The Voltage-Dependent Proton Pumping in Bacteriorhodopsin Is Characterized by Optoelectric Behavior

Sven Geibel,* Thomas Friedrich,* Pal Ormos,† Phillip G. Wood,* Georg Nagel,* and Ernst Bamberg*
*Max Planck Institut für Biophysik, D-60596 Frankfurt am Main, Germany; and †Institute of Biophysics, Biological Research Centre, Hungarian Academy of Sciences, H-6701 Szeged, Hungary

ABSTRACT The light-driven proton pump bacteriorhodopsin (bR) was functionally expressed in *Xenopus laevis* oocytes and in HEK-293 cells. The latter expression system allowed high time resolution of light-induced current signals. A detailed voltage clamp and patch clamp study was performed to investigate the ΔpH versus $\Delta\psi$ dependence of the pump current. The following results were obtained. The current voltage behavior of bR is linear in the measurable range between −160 mV and +60 mV. The pH dependence is less than expected from thermodynamic principles, i.e., one ΔpH unit produces a shift of the apparent reversal potential of 34 mV (and not 58 mV). The M_2–BR decay shows a significant voltage dependence with time constants changing from 20 ms at +60 mV to 80 ms at −160 mV. The linear *I-V* curve can be reconstructed by this behavior. However, the slope of the decay rate shows a weaker voltage dependence than the stationary photocurrent, indicating that an additional process must be involved in the voltage dependence of the pump. A slowly decaying M intermediate (decay time > 100 ms) could already be detected at zero voltage by electrical and spectroscopic means. In effect, bR shows optoelectric behavior. The long-lived M can be transferred into the active photocycle by depolarizing voltage pulses. This is experimentally demonstrated by a distinct charge displacement. From the results we conclude that the transport cycle of bR branches via a long-lived M_1* in a voltage-dependent manner into a nontransporting cycle, where the proton release and uptake occur on the extracellular side.

INTRODUCTION

Proton pumping by the retinal protein bacteriorhodopsin (bR) from *Halobacterium salinarum* has been studied in the past in great detail by various biochemical and biophysical methods. An overwhelming body of data concerning structure and function of this light-driven proton pump has been accumulated. In brief, the following picture about the transport mechanism has been developed. bR undergoes a photocycle which is intimately linked to the transport cycle. After absorption of a photon retinal isomerizes from all-*trans* to 13-*cis*, and via the spectroscopically distinct J, K, L intermediates, the blue-shifted M_{cis} product is formed. During the L to M_{cis} transition the proton of the Schiff base is released to Asp-85 while a proton is released to the extracellular side and the pK of the Schiff base shifts from an original pK > 13 to pK < 4 (Druckmann et al., 1982; Sheves et al., 1986). While still in M_{cis} the pK of the Schiff base increases (pK > 10), proton accessibility changes, and another proton is taken up via Asp-96 from the intracellular side before returning to the bR state again. The transition within the M state involving the change of accessibility for protons from the extracellular side to the cytoplasmic side can be called a molecular switch, whereby the M intermediate can be subdivided into M_1 (with proton accessibility to the extracellular part of proton pathway, EC) and M_2 (with proton accessibility to the cytoplasmic part of the proton pathway, CP). A substantial help for understanding the pumping mechanism was the determination of bR's three-dimensional structure (Henderson et al., 1990; Landau and Rosenbusch, 1996; Essen et al., 1998; Luecke et al., 1999). bR consists of seven α helices (termed A to G) forming a transmembrane channel-like structure. The channel is formed by helices B, D, F and G and is divided into a cytoplasmic (CP) and an extracellular (EC) oriented half-channel by the chromophore retinal, which is bound via a Schiff base to Lys-216 in helix G.

An important aspect of the vectorial transport in bR is the regulation of the pump by the electrochemical gradient. This is essential for the understanding of the pump because under normal, i.e., physiological, conditions the electrical transmembrane potential reaches values of up to −280 mV (Michel and Oesterhelt, 1976). Many attempts have been undertaken to study the influence of an external electrochemical potential on the pump (Bamberg and Fahr, 1980; Braun et al., 1988) but all the studies, including the spectroscopic work, were carried out under undefined membrane potentials or in systems with unsatisfactory orientation of bR in the respective membrane system. The results of these studies have in common that the M decay is remarkably prolonged at potentials that energetically inhibit proton pumping (Quintanilha, 1980; Dancsházy et al., 1983; Westerhoff and Dancsházy, 1984; Groma et al., 1984).

Recently, we succeeded in expressing bR heterologously in *Xenopus laevis* oocytes, which allowed the study of the pump under voltage clamp and patch clamp conditions, i.e., under controlled membrane potential (Nagel et al., 1995, 1998). The outcome of these studies was that a long-lived M

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Address reprint requests to Dr. Ernst Bamberg, Max Planck Institut für Biophysik, Kennedyallee 70, D-60596 Frankfurt am Main, Germany. Tel.: 49-69-6303-300/301; Fax: 49-69-6303-305; E-mail: bamberg@biophys.mpg.de.

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intermediate with a lifetime of up to 300 ms was found (Nagel et al., 1998). From these data it was inferred that in this state, the Schiff base is accessible for protons from the extracellular side. This intermediate is enriched by a negative electrical potential. It was concluded that an applied electrical field regulates the pump current via the $M_1 \rightarrow M_2$ transition, the switch where the accessibility of the Schiff base changes from the extracellular to the intracellular side. By producing the slowly decaying M, negative potential favors a nontransporting parallel cycle, allowing for an efficient regulation of the pump by the membrane potential. However, our previous experiments did not allow us to precisely resolve the voltage dependence of the M decay. Therefore, it was concluded that the "time course of the M decay is virtually unaffected by the electric field" (Nagel et al., 1998). A similar weak voltage dependence of the M decay was also observed by Groma et al. (1984).

In this study we set out to get a deeper understanding of the regulation of the pumping process by $\Delta\Psi$ and ΔpH. For this purpose, bR was heterologously expressed in oocytes and in HEK-293 cells. Especially the latter expression system, together with improvements in our experimental setup (involving the use of a fast shutter device with an opening time of <1 ms and application of light from a pulsed dye laser for blue-light experiments), allowed us to perform a detailed voltage clamp and patch clamp study to find out which steps in the transport cycle are influenced by the electrical potential and/or an applied pH gradient. A distinct voltage dependence of the M_2 decay in the millisecond range was found.

Evidence was obtained that the nontransporting cycle decays via a third M state (which will be called M_1* in the following) to the bR state (see Fig. 1). Furthermore, we show that this M_1* intermediate can be transformed into the bR state by depolarizing voltage pulses, demonstrating an optoelectric behavior of the pump.

MATERIALS AND METHODS

Preparation of plasmid DNA and cDNA synthesis

bR was expressed in *X. laevis* oocytes using the plasmid pGEM-HE as previously reported (Nagel et al., 1995). A promoter-free *bop* gene was inserted into a modified pGEM-3Z vector containing 3′ and 5′ untranslated regions of the *Xenopus* β-globin sequence to boost expression in oocytes. To further elevate the level of expression in the plasma membrane, the bR pre-peptide was replaced by the first 105 amino acids of the rat gastric H^+/K^+-ATPase β subunit (Shull, 1990). The inserted region provides a transmembrane helix and a loop region containing two potential glycosylation sites. The product is referred to as $_{HK\beta}$bR. The HK region was lifted by PCR (Vent polymerase, NE Biolabs, Beverly, MA) with suitable primers and inserted into the original bR-containing plasmid (pGEM-HE), which was appropriately modified via PCR. The resulting construct coded for the β HK fragment and the mature bR protein. For expression in HEK-293 cells as a GFP fusion protein, the bR and $_{HK\beta}$bR coding sequence was removed via PCR and inserted into a suitably prepared pEGFP-n3 vector (Clontech, Palo Alto, CA). The stop codon of bR was replaced by a *Bam*H1 site, which inserted two additional amino acids into the fusion protein. The product is referred to as bR_{eGFP}-DNA, $_{HK\beta}bR_{eGFP}$-DNA, respectively. All constructs were sequenced using the ABI Prism dRhodamine-terminator cycle sequencing method on an ABI 310 sequencer (Applied Biosystems, Foster City, CA).

Oocyte microinjection and incubation

Oocytes were prepared as described elsewhere (Grygorczyk et al., 1989). In short, ovarian lobes were surgically removed from anesthetized frogs, and individual stage V to VI oocytes were isolated by collagenase digest. cRNA synthesis was carried out using the T7-mMessage mMachine Kit (Ambion, Austin, TX). Usually 25 ng bR cRNA was injected per oocyte. Expression seemed to saturate above 15 ng. The oocytes were incubated 4–6 days at 18°C in the dark in solution ORI (+ 1 mg/ml penicillin + 1 mg/ml streptomycin) with 1 μM retinal in the medium to reconstitute bR from bacterioopsin.

Heterologous expression in HEK-293 cells

HEK-293 cells were transiently transfected with bR_{eGFP}-DNA or $_{HK\beta}bR_{eGFP}$-DNA using calcium phosphate (Chen and Okayama, 1987) or the Effectene transfection reagent (Qiagen, Hilden, Germany). HEK-293 cell culture was performed according to published procedures (American Type Tissue Culture Collection, CRL1573). Cells were used for electrophysiology 24–48 h after transfection.

Electrophysiology

bR-induced currents were recorded in the two-electrode voltage-clamp configuration on *Xenopus* oocytes with either GeneClamp500 (Axon Instruments Inc., Foster City, CA) or Dagan CA-1B (Dagan Corp., Minneapolis, MN) amplifiers. For patch clamp experiments (Hamill et al., 1981) on HEK-293 cells a GeneClamp500 amplifier was used. Data acquisition, shutter triggering, and control of transmembrane potential were performed with pCLAMP 7 software via a Digidata 1200 B interface (Axon Instruments). Current traces were usually recorded at 10 kHz (oocytes) or 20 kHz (HEK-293 cells) after filtering to 5 kHz (oocytes) or 10 kHz (HEK-293 cells) using the amplifier's built-in filtering circuits. Data traces obtained were usually averages of three or five runs. Because the observed pump current signals are quite small (30–100 nA), changes in the electrical parameters of the oocytes (leak conductances, 1-10 μS in the range of 1% can easily induce changes in offset currents of 1–10 nA over the large potential range studied here. Therefore, all currents were calculated as the difference between currents evoked by a specific voltage-pulse protocol first with, then without illumination. Only experiments without recognizable drifts in the background conductances were taken for further analysis. All experiments were carried out at room temperature. The potential values stated throughout the manuscript are given in physiologic convention, meaning that the intracellular space is clamped to a given command potential with the extracellular space set to zero potential as reference.

Voltage clamp solutions

The pipette solutions contained 3 mM KCl, the bath solution contained 100 mM NaCl, 15 mM TEACl, 2 mM $CaCl_2$, and 10 mM TRIS, and pH was adjusted to 8.5 or 7.5. For measurements at pH 6.5 or 5.5 TRIS was replaced by equimolar amount of MES. Typical pipette resistances were 0.5–2 MΩ.

Patch clamp solutions

The recording pipette solution contained 120 mM CsCl, 2 mM $MgCl_2$, 10 mM TEACl, 10 mM EGTA, and 10 mM HEPES, and was adjusted to pH

7.5. The bath solution contained 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM TRIS, and 10 mM glucose, and was adjusted to pH 7.5. Typical pipette resistances were 2–4 MΩ.

Optical equipment

To induce photocurrents, cells were illuminated with light from a mercury arc lamp (Osram HBO 100 W), filtered through an IR-filter and a short-wavelength cutoff filter (Schott GG495 = "green light"), and coupled into an optical light guide. Typical stationary currents at saturating light intensities and 0 mV were 30–100 nA in oocytes, 20 pA/pF in $_{HK\beta}bR_{eGFP}$-transfected and 5 pA/pF in bR-transfected HEK-293 cells. Illumination was controlled by a fast shutter (Uniblitz LS6ZM2, Vincent Associates, Rochester, NY), which provided a response time of <1 ms for high time resolution. For single-flash experiments, light from an XeCl Excimer laser over a pumped dye-dye laser setup (PBBO; 396 nm = "blue light", Rhodamine 6G; 581 nm = "green light"; 10 ns pulse duration) was used.

Absorption kinetic experiments on single oocytes and membrane sheets in solution

To directly follow the photocycle kinetics under the influence of transmembrane voltage, we performed absorption kinetic experiments following flash excitation on single oocytes expressing bR. Two factors make the experiment technically difficult: the small amplitude of the absorption signal and the strong light scattering of the oocyte. A single layer continuously covered by bR has an absorption of OD $\approx 10^{-3}$ (with $\epsilon = 63{,}000$ M^{-1} cm^{-1} and bR molecules arranged as trimers in a hexagonal lattice 63 Å apart). Thus, absorption changes in the order of OD $\approx 10^{-4}$ can be expected. To form an efficient integrating sphere arrangement, the oocytes were placed in a glass capillary of 1.5 mm inside diameter with the outside of the capillary painted matted white. The absorption change of the sample was measured by light carried to or from the cuvette by plastic light fibers of 1 mm diameter. The two fibers reached the capillary at an angle of 90° to each other. The excitation light was also joined into the fiber that led the monitoring light to the cuvette. A xenon lamp (Osram XBO 75W) was used as a source of monitoring light. This lamp shows intensity fluctuations of ~1%. Therefore, the light intensity before the sample was also recorded to allow for compensation of the slow thermal fluctuations. The light was filtered both before and after the sample by 400 nm broadband interference filters. For measuring the signal and reference light intensities, identical photomultipliers were used (EMI, type 97813). The measuring and reference light signals were recorded simultaneously in a two-channel transient digitizer (LeCroy 9310AM).

The photocycle was initiated with light flashes ($\lambda_{exe} = 575$ nm) from an Excimer laser over a pumped dye-dye laser setup (Lambda Physik, LEXtra 100, Rhodamine 6G, 10 ns pulse duration). The pulse energy at the sample was ~0.1 mJ/mm^2. Five hundred traces with repetition frequency of 0.5 Hz were averaged to achieve a reasonable signal-to-noise ratio. To estimate the number of bR molecules contributing to the signal, the amplitude of the absorption kinetic signal was compared to the signal of a purple membrane suspension of known concentration. It was simply assumed that the signal size is proportional to the number of molecules in a sample of identical volume. During the optical experiments the voltage across the cell membrane was controlled and the current across the membrane was followed.

When bR membrane sheets in solution were used for spectroscopic measurements, the excitation light (from an HBO 100 W lamp, Osram) was filtered by a 495-nm cutoff filter and the measuring light (from a 75 W tungsten lamp, Osram) was filtered by two 403-nm interference filters to minimize scattering light effects. Both beams reached the sample at an angle of 90° to one another. The absorption changes were monitored by a photomultiplier, amplified, averaged (512 traces, 0.2 Hz repetition rate), and recorded by a two-channel transient digitizer (LeCroy 9310AM).

RESULTS

Expression of bR in oocytes and HEK-293 cells

Bacteriorhodopsin was expressed in HEK-293 cells as a GFP fusion protein in two forms: with and without the 105 amino acid fragment of the H^+/K^+-ATPase β subunit serving as a targeting sequence for the insertion of the chimera into the plasma membrane of the cell. Expression was detected under the microscope through the fluorescence of GFP and by whole-cell patch clamp recording of the light-induced pump currents at 0 mV. Bacteriorhodopsin with the HKβ subunit targeting sequence shows a higher functional expression than the wild-type construct (20 pA/pF compared to 5 pA/pF). The sign of the current corresponds to outwardly directed proton pumping by bR. It is important to note that neither photocurrent direction, nor kinetics, nor the current-voltage relationship differ between the two bR constructs used in HEK-293 cells and oocytes. As a result of these changes voltage clamp measurements could be performed over a wide range of voltage and pH, and whole-cell patch clamp experiments could be conducted at higher expression levels with higher time resolution. Photocurrents in giant excised membrane patches from oocytes expressing bR yielded amplitudes of ~10 pA (data not shown), whereas whole-cell currents on HEK-293 cells reached ~100 pA. Because the estimated membrane areas are comparable in both cases a 10-fold higher surface expression was achieved in HEK-293 cells.

Voltage dependence of proton pumping in the presence of pH gradients

Voltage clamp experiments on ion pumps offer the possibility of studying the influence of $\Delta\Psi$ and ΔpH separately, which gives valuable information on the bioenergetic behavior of the pump. From a thermodynamic point of view, the equivalence of concentration gradient and potential difference is given. However, for kinetic reasons the influence of the concentration gradient can differ quite remarkably from that of the applied electrical potential. This was shown in a series of publications by Dimroth and colleagues for the F_oF_1 ATP synthase. They showed that for this protein the electrical potential alone is competent to drive synthase activity (Kaim and Dimroth, 1999).

In the case of bR, the current-voltage behavior was determined under different pH gradients. The pH in the extracellular medium was adjusted simply by adding the appropriate electrolyte, whereas the pH inside the cell was maintained constant by incubating the oocyte between measurements in a solution at pH 7.5. As already shown in a previous publication (Nagel et al., 1998), at neutral pH the current-voltage relationship is linear over the measurable range between −160 and +60 mV. As can be seen from Fig. 2 *C*, the voltage dependence of the pump remains linear

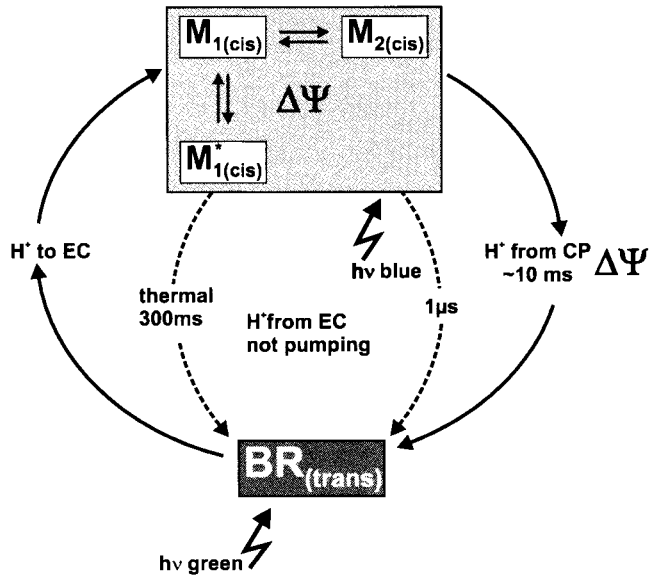

FIGURE 1 Reaction scheme of the photo/transport cycle of bR. Only intermediates relevant for the description of the voltage dependence are shown. The cycle is initiated by green light and the different M states are probed as indicated by blue light.

in the measurable voltage range also under an applied ΔpH (Fig. 2).

From this linearity, it seems justified to determine an apparent reversal potential at which the pump current diminishes to zero by linear extrapolation to the voltage axis. A ΔpH of 1 unit across the membrane (pH 6.5 outside the cell) causes a shift of 34 mV, much smaller than the 58 mV expected from a simple Nernst calculation (data not shown). Inspection of Fig. 2 clearly shows that at a ΔpH of 2 units (pH 5.5 outside the cell) a shift of 68 mV was obtained.

At inwardly directed pH gradients the *I-V* curves are essentially parallel. However, this is not the case at an outwardly directed pH gradient (pH 8.5 extracellular). Here the slope is decreased, i.e., yielding larger currents at negative potential and smaller currents at depolarizing voltages, probably indicating a slight saturation behavior at positive potentials.

To exclude the possibility that a change in external pH effects a change in the intracellular pH, every measurement was preceded and followed by a measurement at pH 7.5. A change in the intracellular pH should then show up as a shift of the *I-V* curve, which was never observed in our experiments.

Voltage-dependent kinetics

Information about the influence of the applied electric field and/or pH on the kinetic behavior of the pump currents can be obtained in different ways: 1) by analysis of the on and off response of the stationary currents or laser flash-induced time-resolved currents; 2) by probing the formation or disappearance of the M intermediate with blue light. Blue light induces the *cis-trans* isomerization of the retinal, followed by uptake of a proton from the extracellular side completing a nonpumping two-photon reaction cycle (Oesterhelt and Hess, 1973; Ormos et al., 1978; Nagel et al., 1998); and 3) by fast voltage jumps in the patch clamp or voltage clamp configuration.

The upper part of Fig. 2 shows the photocurrent measured on oocytes at two different voltages at an extracellular pH of 7.5 (Fig. 2 *A*) and at an extracellular pH of 5.5 (Fig. 2 *B*, corresponding to $\Delta pH = 2$ against the pump direction). The peak current reflects the deprotonation of the Schiff base whereby M_1 is formed. It displays only weak voltage dependence, in accordance with $M_2 \rightarrow bR$ decay being the main electrogenic and rate-limiting step. Therefore, the stationary current is primarily influenced by the externally applied electric field. An additional pH gradient induces a larger effect on the peak current. The ratio of the amplitudes of the peak currents at +40 mV and −120 mV (indicated by the arrows in Fig. 2, *A* and *B*) in the presence of a pH gradient is 1.7, and in the absence of a pH gradient is 1.3.

The off response represents the time dependence of the $M_1 \rightarrow M_2 \rightarrow bR$ decay. The data traces in Fig. 2 clearly show that the decay after switching off the light consists of at least two processes (in agreement with Nagel et al., 1998). The fast one (τ_1) is almost voltage independent within the time resolution of our measuring system. It can be fitted with a time constant of 1–3 ms, and is mainly influenced by the RC constant of the voltage clamp and the switching time of the shutter. Similar experiments in HEK-293 cells resolved the fast decay with a better system time constant, although still limited by the switching time of the shutter. Even in single-flash experiments the time constant (τ_1) was voltage independent between +40 mV and −120 mV, and remained in the same order of magnitude (~0.8 ms, data not shown).

The second, slower process (described by the relaxation time τ_2) is voltage dependent and varies from 20 ms at +60 mV to 80 ms at −160 mV. As can be seen in Fig. 2 *D*, the slope of the rate $1/\tau_2$ versus *V* is almost similar to the slope of the current-voltage curve in Fig. 2 *C*, so that the linear behavior of the *I-V* relation can be explained by the voltage dependence of the M_2 decay. It is remarkable that $1/\tau_2$ depends on ΔpH similarly to the stationary currents at different potentials, showing that the *I-V* curve in the measured range is mainly governed by this process.

Over a range of 200 mV the *I-V* curve shows a variation of current by a factor of 5, whereas the $1/\tau_2$ changes by a factor of 3, i.e., the *I-V* curves are somewhat steeper than the $1/\tau_2$ versus *V* relationship. This comparison strongly suggests that a second voltage-dependent regulation step might be important for the decrease of the pump current at negative potentials (see Discussion). As demonstrated in our earlier publication, at negative potentials an M intermediate appeared that decayed in a dark reaction with a lifetime of

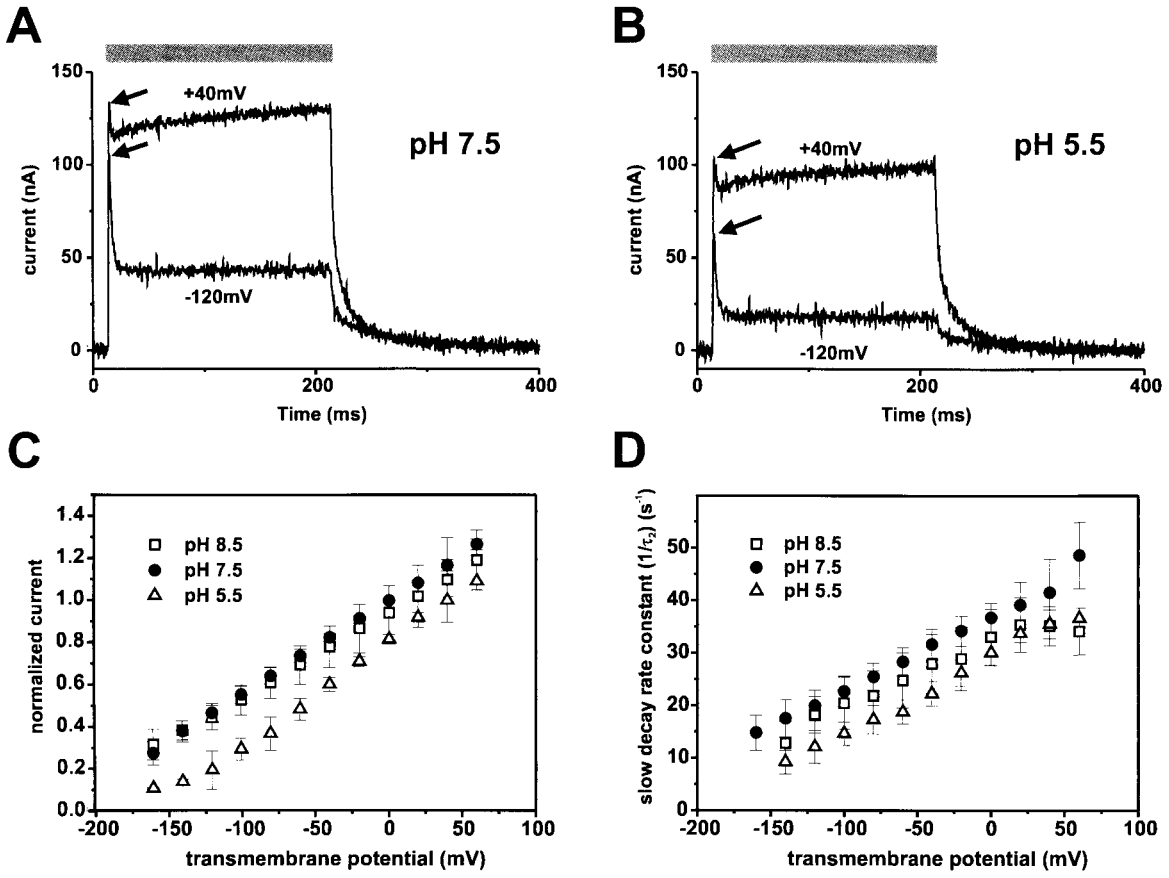

FIGURE 2 ΔpH and voltage dependence of the photocurrent of bR expressed in oocytes from *X. laevis*. (*A*) Photocurrents at two different potentials at an extracellular pH of 7.5 and (*B*) at an extracellular pH 5.5 (presence of a ΔpH of 2 units). The gray bar above the current traces indicates the period of illumination with green light. Peak current amplitudes are indicated by arrows. (*C*) Evaluation of the current-voltage dependence under different pH gradients (currents normalized to the mean current at 0 mV, pH 7.5) and (*D*) of the off response of the voltage-dependent photocurrent (slow component), as shown in *A* and *B*. Each point represents the data from 5 (pH 5.5, 8.5) or 15 (pH 7.5) oocytes. Data points in *C* and *D* are given as mean ± SE.

hundreds of milliseconds (Nagel et al., 1998). Therefore, it was suggested that the photocycle splits into a transporting and a nontransporting cycle.

Under the improved experimental conditions, the kinetics of the long-lived M was investigated in more detail with spectroscopic and electrical methods. Surprisingly, the long-lived intermediate was found (though with a relatively small amplitude) already at zero voltage in HEK-293 cells with a lifetime, τ_3, of ~200 ms. The effect is drastically increased at negative potentials (see Fig. 3). The experiment was carried out as follows.

Under whole-cell patch clamp conditions a HEK-293 cell was illuminated with green light for 300 ms at five different voltages between +40 mV and −120 mV to evoke a stationary photocurrent. For clarity, only the current traces at the upper and lower end of the voltage scale are represented in Fig. 3, *B* and *C*. For adequate display of the fast blue light-induced signals, the filter was set to 10 kHz and the current scale had to be expanded. Therefore, the stationary currents appear small and noisy in this representation.

To measure the relative amount of the M intermediates, blue laser flashes (396 nm, 10 ns duration) were applied to evoke the well-known quenching of the photocurrent due to the reprotonation of the Schiff base from the extracellular side upon blue light-induced reisomerization. During illumination with green light the amplitude of these transient currents varied with the transmembrane potential. The insets of Fig. 3, *B* and *C*, show the blue light-induced currents on an expanded time scale. Note that for the evaluation of the amount of M, the integral (i.e., the charge) instead of the amplitude was taken. At +40 mV the stationary current is larger and the blue light-induced charge displacement smaller, whereas at −120 mV the stationary current is smaller and the blue light-induced charge movement is ~1.6 times larger. This result is in agreement with the interpretation that under continuous illumination the concentration of M is larger at negative potential than at positive potential. To show that other mechanisms are also involved in the voltage dependence, the lifetime of M was

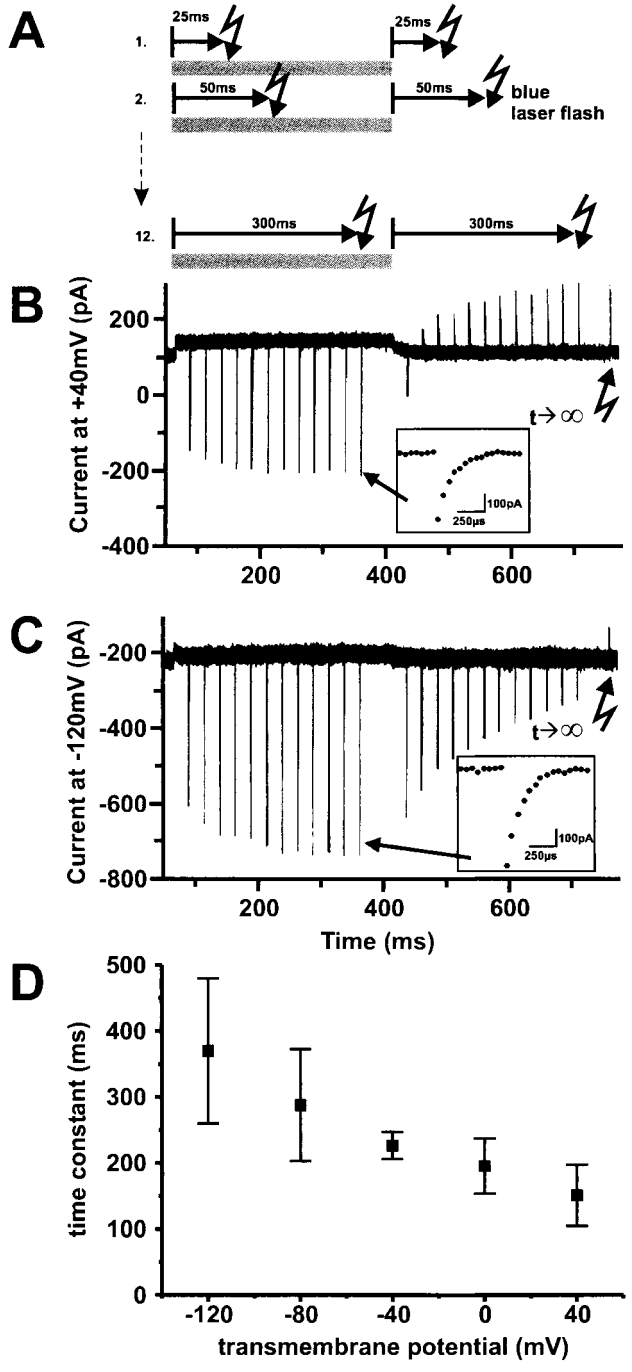

FIGURE 3 Blue light-induced quenching of the photocurrent by bR expressed in HEK-293 cells in whole-cell patch clamp recordings at pH 7.5 on both sides of the membrane. (*A*) Scheme of the illumination protocol. The gray bars indicate the period of illumination with green light, the flash arrows denote the application of the blue laser flash (396 nm). (*B*) Quenching of the stationary photocurrent at +40 mV. The inset shows the time-resolved blue light-induced current indicated by the arrow. (*C*) Same protocol as in *B*, at a potential of −120 mV. The flash arrows in *B* and *C* indicate current signals evoked by a blue laser flash without previous illumination with green light (therefore denoted $t \rightarrow \infty$). (*D*) Voltage dependence of the decay time constant of M_1*. Each point represents the data from four HEK-293 cells (data points are given as mean ± SE).

determined by test flashes after switching off the stationary light source.

In our previous publication we were able to show that at negative potentials an extremely slow process for the M decay occurs (~300 ms at neutral pH), which led us to the interpretation that M_1 probably decays at these potentials via the reprotonation of the Schiff base (pK = 3.0) from the extracellular side to the bR state. This was concluded from the pH dependence of this process, i.e., the lower the pH the smaller τ_3. A reduction from 700 ms at pH 8.0 to 40 ms at pH 6.0 was found.

Inspection of Fig. 3 shows that under the improved experimental conditions (i.e., better time resolution, higher expression levels in HEK-293 cells) already at +40 mV, where the linear photocycle lasts for ~20 ms, such a long-lived intermediate was detected with a characteristic time constant of ~150 ms. As can be seen from Fig. 3 *B*, after switching off the stationary light source a peak current can be observed following the next illumination at $\Delta t = 25$ ms. Already after $\Delta t = 50$ ms the blue laser pulse induces a positive current, showing that the concentration of the bR state is increased, i.e., blue light is also absorbed by the bR state, inducing a transient pump current in the normal pump direction. The positive peak current reaches a saturation value in the dark with a characteristic time constant of τ = 150 ms, which implies that already upon illumination at +40 mV a long-lived M state must exist. The situation at −120 mV shows the existence of the long-lived M state much more clearly. As mentioned above, the blue laser pulse induces a larger negative current, indicating an increased amount of M. At $\Delta t = 25$ ms almost all of the M still exists in the dark. Following the same double flash pulse sequence as at +40 mV, it is obvious that a large amount of M is arrested by the negative potential (−120 mV). Here the decay time for M is ~380 ms. The results support the interpretation that the proton-transport cycle branches via an intermediate M_1*. Otherwise, the M state would accumulate under stationary light conditions with the consequence of a very slow, thus ineffective, pump cycle.

Attempts were also made to spectroscopically measure the M decay in oocytes (Fig. 4 *B*). In voltage clamped oocytes the M decay as determined from absorption spectroscopic measurements has an exponential component with a τ of 160 ms (Fig. 4 *B*), much slower than the values usually observed on suspensions of purple membranes. The value of the time constant varied from oocyte to oocyte, but it was always in the range of 100–300 ms. However, the decay of M could not significantly be influenced by an externally applied electric field. We believe the reason for this is that the bR molecules contributing to the absorbance signal are not only located in the plasma membrane, but also in the membranes of intracellular organelles. These most probably contain a major fraction of bR protein within the cells and cannot be influenced by the external voltage clamp (see Discussion for details).

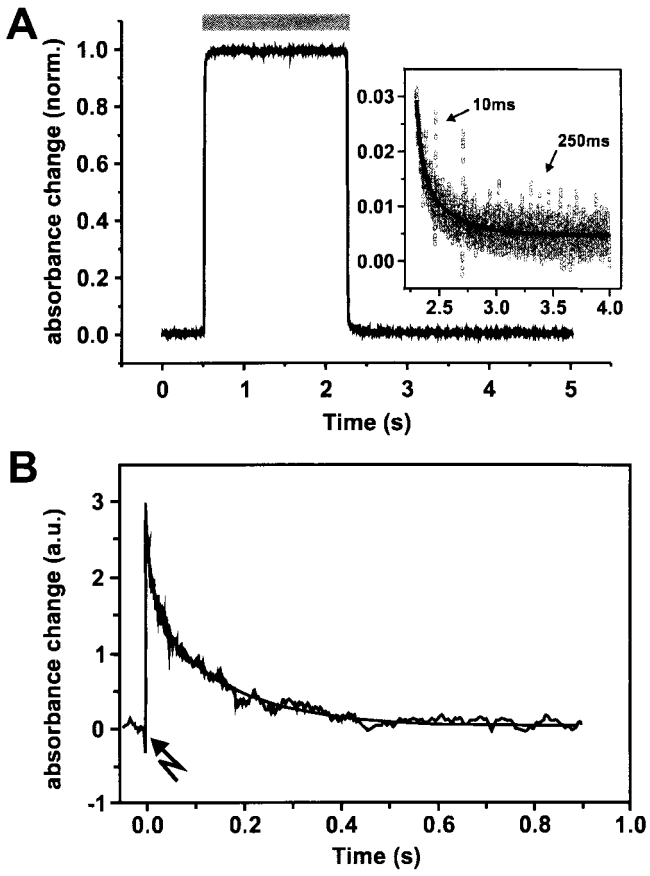

FIGURE 4 (*A*) M decay of bR in purple membrane suspensions with time constants $\tau_1 \approx 10$ ms and $\tau_2 \approx 250$ ms. The inset demonstrates the slow component (with a fractional amplitude of 1% of the total signal) on an expanded scale. The gray bar above indicates the period of illumination with green light. (*B*) Spectroscopic detection of the M decay of bR expressed in oocytes (for details see Material and Methods). Wavelength of the monitoring light $\lambda_{meas} = 400$ nm, wavelength of the excitation flash $\lambda_{exe} = 581$ nm (*arrow*). Average of 500 traces, repetition rate 0.5 s^{-1}. The trace can be fitted with two exponentials: $A_1 = 1$, $\tau_1 = 17$ ms; $A_2 = 1$ 1.6, $\tau_2 = 166$ ms.

The electrophysiological experiments demonstrated that the slow decay process is also present at 0 mV. Therefore, it should also be possible to detect it spectroscopically on purple membrane suspensions. A simple determination of the M decay after steady illumination was carried out ($\lambda >$ 495 nm excitation, 403 nm detection beam). As can be seen in Fig. 4 *A*, the usual M decay in the millisecond range can be observed. However, careful analysis of the off response shows a small but distinct process with ~1% of the total amplitude with a decay time of ~250 ms. Unfortunately, spectroscopic investigation of purple membrane preparations under defined voltage conditions is not possible. However, on purple membranes attached to black lipid membranes (BLMs) the slow M decay electrically was easily detected (Nagel et al., 1998). Equivalent absorption kinetic experiments were also performed on purple membranes attached to BLMs. Here the negative voltage build-up due to proton pumping also results in a slow M decay component with a several hundred millisecond time constant and a significant (several tens percent) relative amplitude (data not shown). This proves that the kinetic behavior of the M decay is not dramatically different between bR expressed in oocytes and the trimeric form, which is prevalent in the purple membrane.

Voltage pulse experiments

From our earlier publication and from the results in this study it is apparent that M is accumulated at hyperpolarizing voltages. The accumulation was found to be extremely voltage dependent ranging from nearly zero at +80 mV to 90% at −160 mV (Nagel et al., 1998). The result was obtained by quenching the photocurrent with steady blue light. Because M accumulates to a large extent at negative potentials, one would expect that its formation and disappearance is accompanied by a voltage-dependent charge displacement. To prove this hypothesis, the following experiment was carried out: bR-expressing oocytes were hyperpolarized to a potential of −120 mV. Then depolarizing voltage pulses to +40 mV were applied before, during, and after illumination of oocytes with green light. The same experiment was carried out without illumination so that voltage-induced leak currents could be deducted. As can be seen in Fig. 5, the first depolarizing voltage pulse in the dark has no effect. After turning on the light the typical photocurrent with the preceding transient current appears. Depolarization of the cell during illumination yields a drastically increased stationary current without a preceding peak current. After repolarization to −120 mV the same stationary photocurrent as initially seen is observed. The most interesting phase of this experiment is the last part, when the light is switched off again. In contrast to a depolarizing voltage pulse before illumination, the same depolarizing pulse given 150 ms after illumination induces a positive current transient, most probably demonstrating the charge displacement connected with the disappearance of M. This interpretation was confirmed by an experiment in which a blue laser pulse (which transfers M into bR) abolishes the voltage jump-induced charge displacement when given before the depolarizing voltage pulse (data not shown).

After switching off the light, the cell was depolarized at different Δt. The current-time curve was integrated and the resulting charge displacement was plotted against the time (see Fig. 5, *inset*). Two relaxation times (30 and 460 ms) could be detected. It is important to note that the evaluation of the charge displacement shows similar kinetics as those obtained with the double-flash experiments (Fig. 3).

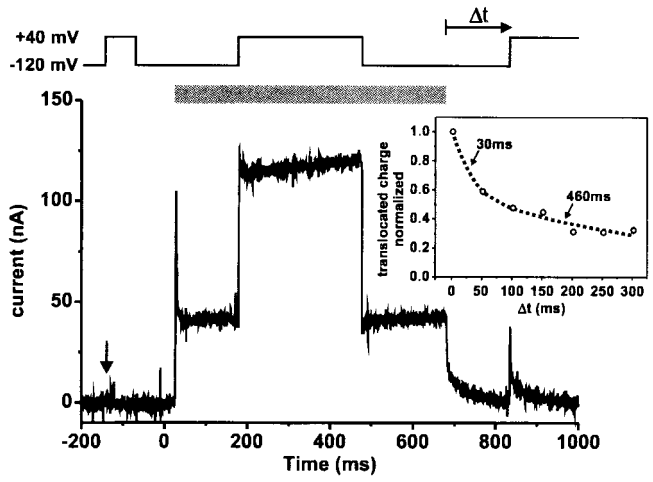

FIGURE 5 Optoelectric behavior of bR expressed in oocytes (extracellular pH = 7.5). The upper panel shows the applied voltage protocol, the gray bar indicates the period of illumination with green light. No extra charge displacement upon a voltage jump from −120 mV to + 40 mV is visible before illumination (*arrow*). The same depolarizing voltage jump during illumination simply evokes an immediate increase of the stationary current, as expected from its voltage dependence (see Fig. 2). The depolarization of the cell at a time Δt after illumination, however, shows a transient current. The *inset* represents the charge displacement at different time intervals Δt after turning off the green light. An analogous behavior is found for blue light-induced charge displacement at different time intervals (as demonstrated in Fig. 3). The data trace represents the average of five runs of the indicated illumination/voltage protocol.

DISCUSSION

The aim of this publication is to get a deeper understanding of the regulation of the pumping process in bR by the electrochemical gradient. For this, bR was heterologously expressed in the plasma membrane of *X. laevis* oocytes or in HEK-293 cells. Both cell types are ideally accessible for electrophysiological techniques, so that the potential and the ΔpH dependence could be studied either by the voltage clamp or the patch clamp technique. It was necessary to demonstrate that bR (and the fusion protein constructs thereof used in this study) retains its characteristic properties in both heterologous expression systems. As discussed below, the formation of the M intermediate and its decay are essentially similar to those obtained in purple membranes. In addition, both GFP fusion protein constructs, one with and the other without the 105 amino acid fragment of the H^+/K^+-ATPase β subunit for better expression in HEK-293 cells, show results indistinguishable from those obtained on oocytes expressing bR alone and are comparable to the purple membrane/BLM system (Nagel et al., 1998).

Both HEK-293 cells and the oocytes allow the study of light-induced pump currents under controlled conditions over a voltage range between −160 mV and +60 mV and a pH range between 5.5 and 8.5 outside the cell. The accessible voltage range is still somewhat limited, since voltages between −250 mV and +150 mV would be desirable to directly obtain the crucial values that determine the complete voltage dependence of the pump. However, interesting results have been obtained on the influence of ΔpH and on the *I-V* behavior. Inspection of Fig. 2 clearly shows the influence of voltage and ΔpH. The peak currents reflect the charge movement due to the deprotonation of the Schiff base, i.e., the formation of M. Because this process contributes only a small fraction of the total translocated charge during the pump cycle it shows only a weak voltage dependence, whereas the dependence on extracellular pH is more pronounced (see arrows in Fig. 2, *A* and *B*). The stationary current changes drastically with both ΔpH and ΔV, which must be associated with the decay of M.

The current-voltage behavior is linear over the measurable voltage range also in the presence of an applied proton gradient. This justifies the linear extrapolation to zero current to determine an apparent reversal potential. As a result of this evaluation a stronger effect of the electric field is obvious, because at a ΔpH of two units a shift of the apparent reversal potential of only 68 mV can be detected. The reason for this behavior is probably the charge displacement within the protein, which is kinetically preferred, because the voltage dependence of the M decay is rate-limiting. The *I-V* curves are essentially parallel at ΔpH directed against the pump direction. However, at the external pH of 8.5 (a ΔpH in the direction of the pumping process) the slope of the current-voltage curve is significantly less steep. Inspection of this specific *I-V* curve indicates saturation already at +40 mV. Under these conditions the pump is accelerated to such an extent that light absorption probably becomes rate-limiting and, therefore, voltage independent. The linearly extrapolated value to the voltage axis shows the expected direction toward more negative values.

To summarize the influence of ΔpH, the shift of the current voltage curve shows the expected behavior. However, the effect is quantitatively smaller than expected from basic thermodynamics.

The *I-V* curves can be explained by the voltage dependence of the M_2 decay, as demonstrated in Fig. 2 *D*. The variation of the slow component τ_2 of the off response with *V* in Fig. 2 is sufficient for the reconstruction also under applied pH gradients. Also, here a tendency of saturation of $1/\tau_2$ at positive potentials and pH 8.5 is visible, yet the slope of the *I-V* curve is steeper by a factor of significantly larger than 1 compared to the value for $1/\tau_2$ versus *V*. Therefore, another process should additionally be involved in the regulation of the pump.

In our earlier publication we described an M with extremely long decay times, up to 700 ms. Because this decay is pH dependent it was argued that the photocycle was simply arrested in the M_1 state, where the pK of the Schiff base is around 3 and the reprotonation via Asp-85 is rather improbable, and therefore slow. That means, the more neg-

ative the potential, the more M_1 is arrested. As a consequence, M_1 decays back to bR in a futile pump cycle, where deprotonation and reprotonation occur on the extracellular side.

By careful analysis of the results under the improved experimental conditions, the slow component (~380 ms, −120 mV) is already found at zero (~200 ms) or at positive voltage (~150 ms, +40 mV; Fig. 3, *B* and *C*), however with a relatively small amplitude. Combined with the finding that the slope of the *I-V* curve and the $1/\tau_2$ versus *V* curve are significantly different, this implies that the photocycle has to be branched into a third M (M_1*), because in a strictly sequential proton pump cycle an accumulation of M1 would occur. This would lead to a much less efficient pump cycle, with a rate-limiting step of several hundred milliseconds. In our previous publication (Nagel et al., 1998) we reported that all three time constants were voltage independent, but with the improved experimental conditions we were able to improve resolution and minimize error bars. As a result we can now see significant changes in the two slower time constants, while the faster one is still dependent on experimental limitations and, therefore, appears not to be voltage dependent.

To investigate the slow component, the M kinetics were measured spectroscopically on membrane sheets in suspension, where no voltage drop across the membrane occurs. Surprisingly, after detailed inspection of the absorption change, a 250-ms component was found (with a relative amplitude of 1%), thus confirming the electrical data.

In spectroscopic measurements on bR expressed in oocytes a long-living M intermediate was found, too. From the absorption experiments the number of bR molecules within an oocyte can be calculated (see Results), whereas the number of molecules within the outer membrane can be estimated from the photocurrent across the membrane. This value can be determined simply by dividing the stationary pump current by the turnover of the pump (Nagel et al., 1995). The estimate yields that an oocyte contains several times 10^{10} bR molecules, and 10% of these are in the surrounding plasma membrane. It was also concluded that ~1% of the outer membrane is covered by bR (Nagel et al., 1995). Although the externally applied electric field reaches only a small fraction of the bR molecules, the excitation light drives all bR molecules in the oocyte, which leads to the build-up of an electrochemical gradient across the membranes of the intracellular organelles. Under the assumption that bR is similarly oriented as in the plasma membrane (with the lumen of the organelles being topologically equivalent to the exterior of the cell), the potential against the pumping direction of bR across the organelle membrane would lead also to a slowdown of the M decay. Because only a minor fraction (10%) of expressed bR is present in the plasma membrane and can thus be influenced by the external voltage clamp, we could not find a significant voltage dependence with these spectroscopic measurements.

Under the assumption that bR creates an electrochemical potential of ~150 mV across the organelle membranes, the relative amplitude of the slow component of ~60% in absorption kinetic measurements is in good agreement with the voltage dependence of the stationary pump currents at the same potential difference (Fig. 2, *A* and *C*).

Comparison of Fig. 3, *B* and *C* shows a drastic increase of the M_1* product at negative potential (−120 mV) compared to positive potentials. The argument that most of the bR is in the M_1* state can be derived from the fact that after a Δt of 100 ms the blue laser pulse induces half of the current amplitude. This value can be considered as a rather lower limit because at this point blue light already activates the bR state, which stems from the M_2 decay in the normal pumping cycle.

In addition, by comparison of the blue light-induced charge displacement at +40 mV to that at −120 mV, it is obvious that at −120 mV a much larger amount of charge is displaced (Fig. 3, *B* and *C*, *insets*).

The branching of the photocycle at the negative potential to an almost inactive intermediate (M_1*) might be the alternative solution for the regulation of the pump by $\Delta\psi$ at very negative potentials. In this model the rate-limiting voltage-dependent step in the photocycle does not have to be slowed down so much to diminish the pump current.

The M_1* state, which is largely depleted at zero or positive potentials, might serve as a reservoir for the pumping cycle when the membrane is hyperpolarized. Under these circumstances it may be a physiological advantage for halobacteria to store bR in an energized M_1* state. Under conditions that lead to depolarization (i.e., insufficient illumination) this reservoir can reenter the normal pump cycle and contribute to repolarization. This mechanism can also serve as a process of adaptation to high light intensities.

As only 1% of the absorption signal in purple membranes shows the slow decay (Fig. 4), the increase of the photocurrent around 0 mV largely arises from the acceleration of the M_2 decay. The apparently increased amount of M_1 found on heterologously expressed bR compared to bR in purple membranes may be caused by the intrinsic membrane potential induced by the asymmetric charge distribution of the membranes.

Optoelectric properties of bR

As demonstrated in Fig. 5, bR can be either switched by light or—and this is an entirely new observation—switched by a voltage jump from the M state into the bR state. An analysis of the result of the optoelectric measurements shows the M_2 decay with 30 ms and the M_1* decay with 460 ms (upon a voltage pulse from −120 mV to +40 mV, pH 7.5). This behavior is quite interesting because a charge displacement in the absence of light correlates with the

formation of bR. In other words, the absorption maximum at 410 nm can be shifted by the applied electric field to a value of 570 nm. Under steady-state illumination and negative potential bR can be arrested in the M_1* state for several hundreds of milliseconds. Simply by depolarization of the cell to zero and positive potential the molecule returns to bR with the demonstrated charge displacement. Here we cannot decide whether the voltage-induced transient current is due to the M_1* to M_1 transition, or (if the M_1* to M_1 is faster than the time resolution of the setup) due simply to the $M_1 \rightarrow M_2 \rightarrow$ bR decay.

In contrast to the blue light-induced reprotonation of the Schiff base from the extracellular side, the charge displacement reflects (at least in part) the reprotonation from the intracellular side, because the sign of the current upon depolarizing voltage pulses corresponds to the direction of proton pumping, i.e., upon depolarizing voltage pulses M_1* is depleted in a voltage-dependent manner to form M_1, which decays via M_2 in the usual way to bR. This implies that depolarizing voltage pulses activate the switch, which regulates the accessibility for protons to the Schiff base.

In conclusion, the results reported in this publication can be described by the reaction scheme in Fig. 1. The pumping cycle shows a voltage dependence governed by the $M_2 \rightarrow$ bR decay, with time constants varying from 20 ms (+60 mV) to 80 ms (−160 mV). In addition, we assume a branching of the transport cycle into a nontransporting cycle forming M_1*. The decay time constant of M_1* varies between 150 ms (+40 mV) and 380 ms (−120 mV) with an apparently linear voltage dependence. In the M_1* state, the binding site for protons to the Schiff base is extracellular and the pK of the Schiff base must be similar to that in M_1, as we showed in our earlier publication that the reprotonation of M_1 occurs via Asp-85 and is strongly dependent on the extracellular pH (Nagel et al., 1998).

As M_1* is formed in a voltage-dependent manner under light conditions, it can be transformed to bR by depolarizing potentials in the absence of light (via M_1 and M_2), showing, in principle, optoelectric properties of bR.

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