Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 639–643, January 1993
Biophysics

Light-driven proton or chloride pumping by halorhodopsin

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Communicated by Walther Stoeckenius, September 23, 1992 (received for review June 16, 1992)

ABSTRACT **Halorhodopsin from *Halobacterium halobium* was purified and reconstituted with lipids from purple membranes. The resulting protein-containing membrane sheets were adsorbed to a planar lipid membrane and photoelectric properties were analyzed. Depending on light conditions, halorhodopsin acted either as a light-driven chloride pump or as a proton pump: green light caused chloride transport and additional blue light induced proton pumping. In the living cell, both of these vectorial processes would be directed toward the cytoplasm and, compared to ion transport by bacteriorhodopsin, this is an inversed proton flow. Azide, a catalyst for reversible deprotonation of halorhodopsin, enhanced proton transport, and the deprotonated Schiff base in the 13-*cis* configuration (H410) was identified as the key intermediate of this alternative catalytic cycle in halorhodopsin. While chloride transport in halorhodopsin is mediated by a one-photon process, proton transport requires the absorption of two photons: one photon for formation of H410 and release of a proton, and one photon for photoisomerization of H410 and re-formation of H578 with concomitant uptake of a proton by the Schiff base.**

The retinal protein halorhodopsin occurs in the cell membrane of *Halobacterium halobium* in addition to the proton pump bacteriorhodopsin (for review, see ref. 1). Its function was identified as that of an inwardly directed Cl^- pump (2, 3). After photoisomerization of all-*trans*-retinal to the 13-*cis* isomer, halorhodopsin passes a series of distinct intermediates characterized by their respective absorption maxima before returning to the initial state within ≈14 ms (4). No reversible deprotonation of the Schiff base is connected with the Cl^- transport cycle. However, halorhodopsin can produce in a side reaction a species called H410. This product is produced from the photocycle intermediate H520 and contains a deprotonated Schiff base in the 13-*cis* configuration (5) (see Fig. 7). From the H410 state halorhodopsin returns in a thermal reaction in the time range of seconds to the initial state. Thus, continuous green light inhibits Cl^- pumping under concomitant accumulation of H410 (6). Photochemically, the H410 state after isomerization returns much faster to the initial state, and this phenomenon causes the observed blue light regulation of halorhodopsin activity. Thus, irradiance with white light (blue and green light) resulted in an optimal Cl^--dependent stationary photocurrent in black lipid membrane experiments (6).

Azide accelerates accumulation of H410 drastically, and this was seen as an accelerated inhibition of Cl^- transport in green light and a fast reactivation by additional blue light. The effect of azide is catalysis of the deprotonation reaction of the Schiff base (7, 8).

Recent elucidation of the proton transport mechanism in bacteriorhodopsin by structural, genetic, biochemical, and biophysical studies (9, 10) helped considerably in understanding the analogue molecular processes in halorhodopsin. Halorhodopsin lacks the aspartic acid residues corresponding to Asp-85 and -96 in bacteriorhodopsin, which were shown to be crucial for proton translocation in bacteriorhodopsin (11–17). Otherwise, both proteins have 30% amino acid sequence identity and share a similar tertiary structure (18). Both molecules form a transmembrane pore by a circular arrangement of their seven transmembrane helices (9). The Schiff base in the middle separates the cytoplasmic narrow half-channel from the extracellular half-channel. For both molecules, side-specific protonation reactions have been demonstrated. In halorhodopsin, azide mediates proton equilibration between medium and Schiff base through the cytoplasmic channel (8) and photon absorption by the M state in bacteriorhodopsin leads to proton uptake specifically through the extracellular channel (19). This prompted our reinvestigation of halorhodopsin's ion transport properties in white light. If blue light absorption by the intermediate H410 would allow proton uptake through the extracellular channel, a two-photon-driven proton transport would result. This is demonstrated by the results reported here.

MATERIALS AND METHODS

Reconstitution of Halorhodopsin. Lipids from purple membranes were extracted with chloroform/methanol and stored in chloroform solution at −20°C (20). After removal of the solvent, lipids were dissolved in 1 M NaCl/10 mM Tris·HCl, pH 7/1% octyl glucoside at a final concentration of 1 mg/ml. This is the upper limit of solubility under these ionic and detergent conditions.

Solubilized halorhodopsin in 1% octyl glucoside (21) was mixed with the lipid solution in a molar ratio of 1:10 at 15°C. Excess detergent was removed by dialysis (3 days at 15°C) in a Lipoprep minichamber (Schött, Göttingen, F.R.G.). Reconstitution of membrane sheets was checked by density-gradient centrifugation, and a buoyant density of 1.18 g/ml found with purple membranes as control indicated success (22).

Electrical Measurements. Black lipid membranes with an area of 10^{-2} cm^2 were formed in a Teflon cell filled with the appropriate electrolyte solution (1.5 ml for each compartment). The membrane-forming solution contained 1.5% (wt/vol) diphytanoyllecithin (Avanti Biochemicals, Birmingham, AL) and 0.025% (wt/vol) octadecylamine (Riedel-de-Haen, Hannover, F.R.G.) in *n*-decane, to obtain a positively charged membrane surface (23). Membrane formation was checked by eye and the capacitance of each membrane was determined. Membrane fragments reconstituted by the procedure described above were suspended in distilled water (OD_{570} = 5) and sonicated for 1 min in a sonication bath. Then, aliquots of 30 μl were added with stirring to the rear compartment of the Teflon cell. Photosensitivity of the system developed in time and reached a maximal and constant value after ≈40 min.

The membrane was illuminated by a xenon lamp (100 W). Light reached the membrane after passing through appropriate filters, including a heat protection filter. The intensity of the continuous light source was up to 1 W/cm^2. For white or green light, high-pass filters [$\lambda > 360$ nm or $\lambda > 495$ nm (Schött, Mainz, F.R.G.), respectively] were used. A K40 broadband interference filter (λ_{max} = 405 ± 17 nm; Balzers)

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served for blue light excitation. Light intensity was measured as described (24).

The suspensions on both sides of the black lipid membrane were connected to an external measuring circuit via Ag/AgCl electrodes, which were separated from the Teflon cell by salt bridges. The current was measured with a current amplifier (Keithley; model 427). Stationary currents were obtained after the addition of the Cl^-/OH^- exchanging carrier triphenyltin and the electrogenic protonophore 1799, which together permeabilize the black lipid film for Cl^- and protons. Further details of the system have been described (25). The experimental setup is shown schematically in Fig. 1.

Chemicals. The protonophore 1799 (2,6-dihydroxy)-1, 1,1,7,7,7,-hexafluoro-2,6-bis(trifluoromethyl)heptane-4-one was kindly provided by P. Heydtler (DuPont Nemours). It was used because of its blue/UV light insensitivity. The usual protonophores like carbonylcyanide *p*-trifluoromethoxyphenylhydrazone and Tetrachloro-2-trifluoromethylbenzimidazole show electric artifacts on the planar lipid films caused by blue light absorption (26). All chemicals were analytical grade and were purchased from local distributors.

RESULTS

Capacitive and Photostationary Cl^- Currents. Membrane fragments containing halorhodopsin were added to one side of the planar lipid membrane and a transient capacitive current was observed upon illumination from a continuous green ($\lambda >$ 495 nm) light source and addition of Cl^- (data not shown).

Stationary photocurrents can be obtained by application of the Cl^-/OH^- exchanging carrier triphenyltin together with the protonophore 1799, which permeabilize the underlying lipid membrane for Cl^- ions and protons (data not shown). Because the transient currents depend on the presence of Cl^- and the stationary current also requires triphenyltin as a Cl^- carrier, the photocurrent is exclusively carried by Cl^-. The sign of the photocurrent corresponds to the transport of negative charges in the direction of the protein-containing compartment, meaning that the fragments absorb preferentially with the extracellular side of the protein to the planar lipid membrane. In all experiments, the sign of the current was the same, indicating reproducible orientation of adsorbed membrane sheets. The measured current amplitude in these experiments is ≈50 times larger than previously reported (3). This is due to improved sample preparation: the membrane fragments reconstituted from purified halorhodopsin and halobacterial lipids have a protein content comparable to bacteriorhodopsin in purple membranes and therefore cause much higher currents than the cell membrane fragments used previously. This type of membrane also allows better demonstration of green light inactivation and blue light reactivation of halorhodopsin. Fig. 2 demonstrates this correlation between photocycle and stationary pump currents. The current depends strongly on the color of the incident light. In the absence of blue light, the pump is inactivated by the formation of H410, whereas white light causes a stable current response, indicating that accumulation of H410 is prevented by the additional blue light. The inactivation of the stationary photocurrent in the absence of blue light is accelerated in the presence of 3 mM azide (Fig. 2*B*). This is expected because of azide's property as a catalyst for equilibration of the H520 and H410 species.

Fig. 2*A* (*Inset*) shows that additional blue light induces an oppositely directed current peak preceding reactivation of the stationary current. If this component of the electrical signal represents reprotonation of the Schiff base, then proton uptake must be from the same side as Cl^- uptake—i.e., through the extracellular channel. In the presence of white light, therefore, a proton transport mechanism could exist if release of protons during H410 formation is through the cytoplasmic channel. This has been suggested on the basis of results with cell vesicles (8).

Capacitive Proton Currents in Halorhodopsin. The expected photocurrent corresponding to proton transport should have a sign opposite that during Cl^- transport. When halorhodopsin is excited with green light in the absence of Cl^- ($\lambda >$ 495 nm), a small but distinct transient current response is observed, which corresponds to a charge transport opposite to Cl^- pumping (Fig. 3*A*). Since no ionophore or Cl^- is present in the medium, this charge transport must

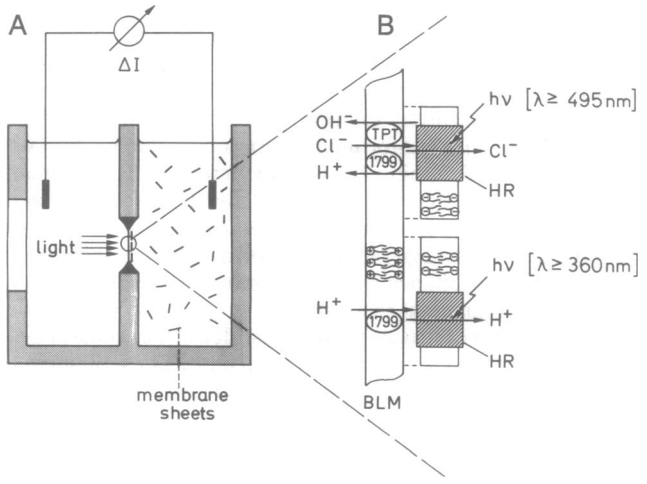

FIG. 1. (*A*) Schematic representation of the bilayer membrane in the Teflon cell. (*B*) Proposed compound membrane of halorhodopsin-containing membranes and the underlying lipid membrane. Ion transport processes causing photostationary currents. Triphenyltin (TPT) is an exchange ionophore for Cl^- and OH^- and 1799 is a protonophore. BLM, black lipid membrane.

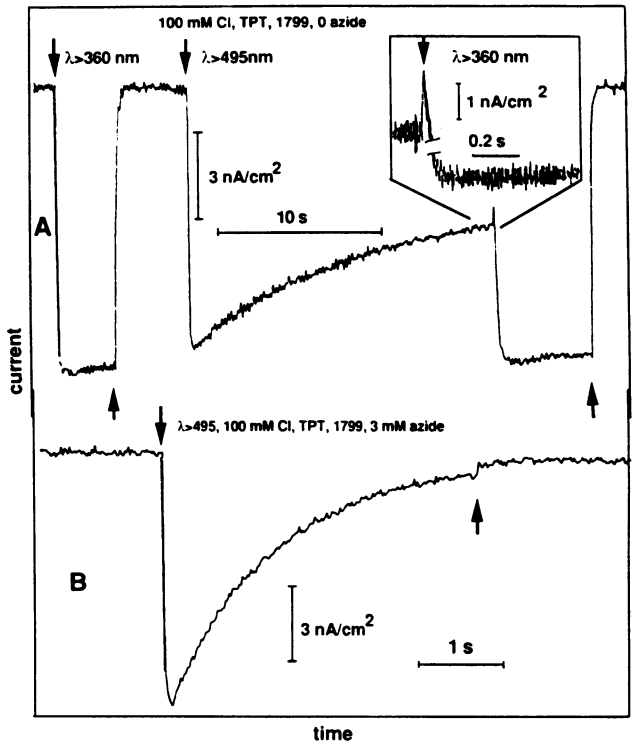

FIG. 2. (*A*) Inactivation of Cl^- pumping activity of halorhodopsin by green light and its reactivation by additional blue light. Additions were 0.1 M NaCl, 1.3 μM 1799, and 10 μM triphenyltin (TPT) in 20 mM Tris·HCl/Hepes, pH 6.35. Irradiances at $\lambda >$ 495 nm and $\lambda >$ 360 nm were 1 and 0.8 W/cm², respectively. (*B*) Same experimental conditions as in *A*, but with additional 3 mM azide.

be caused by a change in halorhodopsin itself. The formation of H410 is responsible for this capacitive current because no accumulation of photoproducts other than H410 has been observed in the absence of Cl^-. Therefore, H410 formation under release of a proton through the cytoplasmic channel is the only acceptable interpretation.

In white light, this current is increased by a factor of 2 (Fig. 3*B*). The difference must be due to proton translocation because in white light the stationary H410 concentration, and therefore stoichiometric proton release, must be smaller than under green light (Fig. 3*A*). This result demonstrates that halorhodopsin photochemically forms H410 by deprotonation through the cytoplasmic channel, but blue light absorption by H410 regenerates the initial state by reprotonation through the extracellular channel. This small net proton transport causes the capacitive current measured.

Addition of 25 mM azide increases the amplitude of this capacitive current by a factor of ≈3 (Fig. 3*C*). At the same time, a pronounced undershoot in current is seen. This is due to discharging of the membrane capacitors during the illumination time because of almost instantaneous formation of H410 in the presence of azide and no contribution of H410 to charge translocation. White light, however, should remove the undershoot because it creates an ion pump action that persists until the capacitors are loaded. This result is actually observed (Fig. 3*D*).

As the final experiment of this series, the sign of the current representing the uptake of the proton by H410 upon blue light absorption was determined. For this, the capacitive currents were measured in the presence of 100 mM Cl^- with no azide present. Halorhodopsin was first illuminated with green light for ≈10 s to form H410. Then, after 2 s of dark phase, blue light was applied (Fig. 4). A transient current was recorded, which shows biphasic behavior, as expected for a single turnover and charge movement. The direction of the blue light-induced current, which occurs only after preexcitation with green light, is in the same direction as was found for the green light-induced current in the absence of Cl^- (formation of H410; see Fig. 3*B*). This means that both electrical events caused by proton release and proton uptake show the same sign, proving the vectorial mode of proton movement.

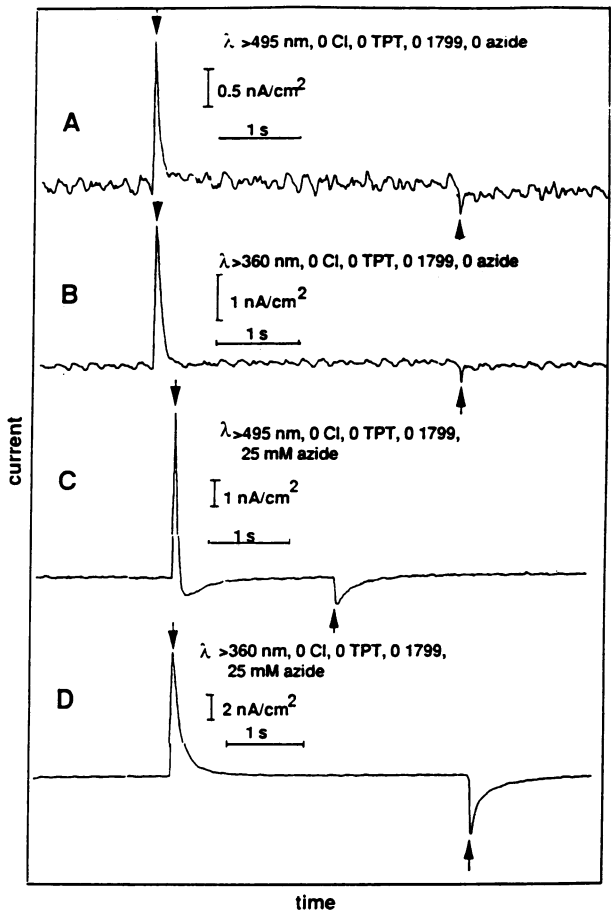

FIG. 3. Capacitive currents in the absence of Cl^-. Sodium gluconate (0.1 M) was added in 20 mM Tris·HCl/Hepes, pH 6.35. Irradiance was as in Fig. 2. Azide (25 mM) was added in *C* and *D*. Green light (λ > 495 cm) was applied in *A* and *C*; white light (λ > 360 nm) was applied in *B* and *D*. TPT, triphenyltin.

Continuous Proton Pumping by Halorhodopsin. To demonstrate that halorhodopsin can pump protons continuously, the bilayer membrane was made ion selective for protons by the protonophore 1799 (Fig. 5*A*). In the presence of 50 mM azide and under green light conditions, a transient current is observed, indicating H410 formation. Additional blue light yields a large photostationary current. This must be a proton current because the anions of the buffer and gluconate are not permeable for the lipid membrane and therefore cannot contribute to the stationary current component. Without the protonophore 1799, no stationary current was observed. In addition, in experiments with several cations—i.e., K^+ or Mg^{2+}—no difference in the photoresponse was observed. This unequivocally proves that the proton is the only ion species that can be continuously transported in halorhodopsin under these experimental conditions.

It is of interest to demonstrate proton transport in the presence of Cl^-. This is shown in Fig. 5*B*. After permeabilization of the lipid membrane for Cl^- with triphenyltin and the protonophore 1799 under green light conditions, only a capacitive current is detected because of release of protons during H410 formation. Additional blue light induces a stationary current of the same sign with a large overshoot, reflecting establishment of a new steady state between H410 and H578. The opposite sign of the stationary current compared to Cl^- currents in the absence of azide (compare Fig. 2) proves that proton pumping in halorhodopsin has the same direction as Cl^- translocation.

The proton transport demonstrated in Fig. 5 is obtained in the presence of 50 mM azide. Proton pumping can also occur in the absence of azide if pump currents were measured in the absence of Cl^- at higher sensitivity (data not shown). The competition of protons and Cl^- for translocation was tested by the experiment shown in Fig. 6. For this, increasing amounts of azide were added to the bathing solution and stationary photocurrents in the presence of triphenyltin and protonophore were compared for green and white light. For both conditions, increasing azide concentrations abolish Cl^- translocation, but only white light changes the sign of the

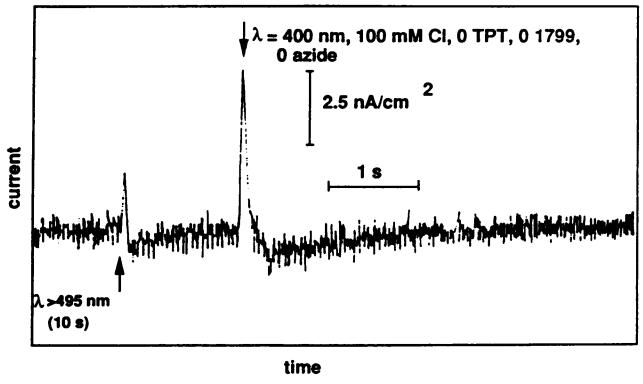

FIG. 4. Electric events during reprotonation of the Schiff base in halorhodopsin. Green light (10 s) was followed by a dark period and by blue light. Additions were 0.1 M NaCl and 20 mM Tris·HCl/Hepes, pH 6.35. Irradiances were as in Fig. 2. TPT, triphenyltin.

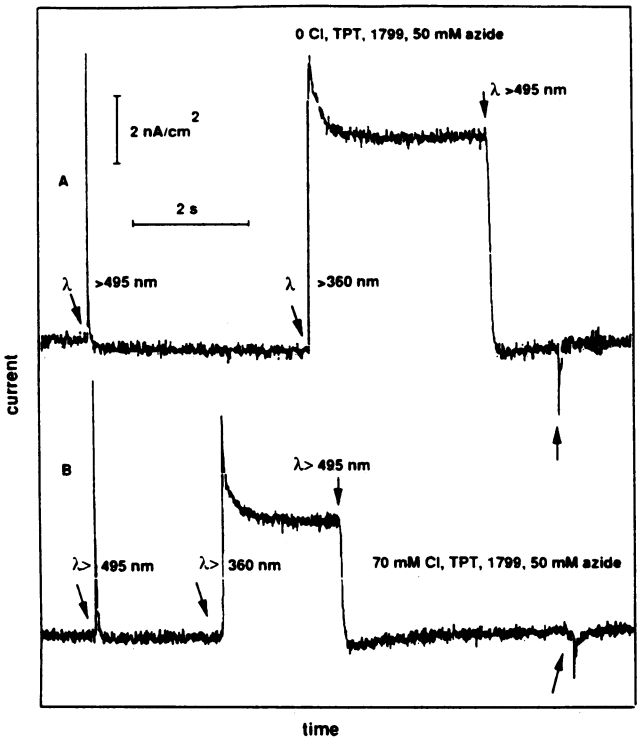

FIG. 5. (*A*) Proton pumping by halorhodopsin. Pump currents were measured in the absence of Cl^-, but in the presence of 50 mM azide, 1.3 μM 1799, 10 μM triphenyltin (TPT), and 0.1 M sodium gluconate in 20 mM Tris·HCl/Hepes, pH 6.35. (*B*) Proton pumping in the presence of Cl^-. Additions were 0.1 M NaCl, 50 mM azide, and 20 mM Tris·HCl/Hepes, pH 6.4. Irradiances were as in Fig. 2.

current at ≈70 mM azide, indicating that proton translocation now becomes more effective than Cl^- transport. This experiment clearly demonstrates alternative ion pumping by halorhodopsin.

DISCUSSION

The results presented here prove that the light-driven Cl^- pump halorhodopsin under certain conditions can also pump protons. The discussion will mainly cover two aspects: (*i*) directionality of proton movements in the molecule and (*ii*) a mechanistic interpretation of the observed proton translocation.

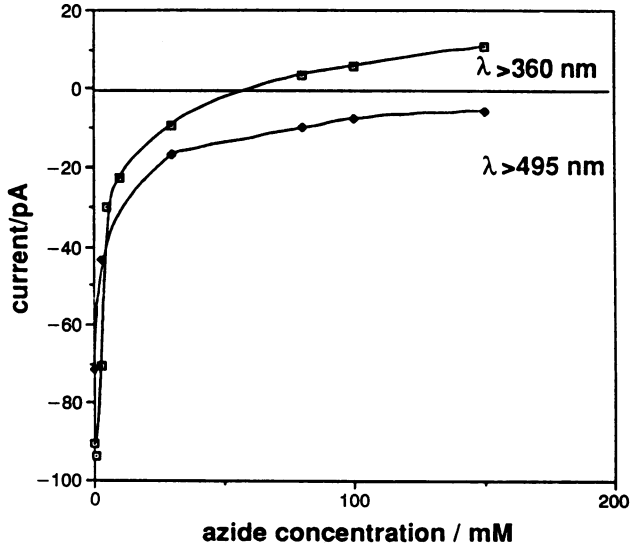

FIG. 6. Stationary photocurrents in white light (λ > 360 nm) and green light (λ > 495 nm) at increasing azide concentrations. Additions were 0.1 M NaCl, 10 μM triphenyltin, and 2 μM 1799 in 10 mM Tris·HCl/Hepes, pH 6.3. Irradiances were as in Fig. 2.

Several procedures for isolation of halorhodopsin have been reported over the years and the simplest turned out to be a procedure in which cholate was used as detergent in 1 mM NaCl with subsequent exchange for octyl glucoside (21). Black lipid membrane experiments, however, require halorhodopsin in a lipid phase. Therefore, previous work has used the so-called Tween-washed membranes, which are cell membranes from which part of the protein is removed and halorhodopsin is therefore enriched. Although these membrane fragments allowed the description of halorhodopsin's photoelectric properties, only the reconstitution of halorhodopsin and halobacterial lipids into planar membrane sheets reported here made photoelectric experiments possible with a sensitivity high enough for detailed analysis of ion currents. An additional advantage is found in the preferential adsorption of the membrane sheets with one side to the positively doped black lipid membrane. Electric signals can be defined as charge movements with respect to the sidedness of the molecule. Since it is known that Cl^- currents through halorhodopsin are directed from the outside to the inside of the cell, the observed proton currents that have the opposite sign must also be proton movements from the outside to the inside, a direction opposite proton flow in bacteriorhodopsin (Fig. 5*B*).

Stationary currents are taken to prove continuous ion translocation driven by light. Capacitive currents, however, can be very useful in analyzing the direction of individual electrogenic steps of a catalytic ion translocation cycle. It is known that the green part of the light will cause formation of H410 under release of a proton, a reaction that is accelerated by azide. The side from which azide acts as a catalyst for this deprotonation reaction was suggested to be the cytoplasmic channel of halorhodopsin (8). Direct support is the fact that azide increases the stationary proton current by a factor of 10. Additional evidence may be derived from similar properties of bacteriorhodopsin. Azide acts as a protonophore exclusively in the cytoplasmic channel of mutants lacking Asp-96 (27) and cannot induce M formation by deprotonation of the Schiff base in mutants lacking Asp-85 in the extracellular channel (unpublished observation).

The blue portion of light will cause re-formation of the initial state of halorhodopsin from H410. This reaction is similar to the photochemical conversion of the M intermediate of the bacteriorhodopsin cycle to the initial state. Photoelectric experiments showed that blue light will decrease the green light-sustained proton current (19). Since during M formation in bacteriorhodopsin the proton is ejected through the extracellular channel via Asp-85, blue light must cause proton reuptake through the same half-channel to explain a decrease in net proton pumping. For the H410 state in halorhodopsin blue light apparently caused a capacitive current in black lipid membrane experiments (6), which had to be interpreted as proton uptake through the cytoplasmic channel during the H410 → H575 transition. This would exclude a net proton translocation by white light because the proton would be released through the cytoplasmic channel and would be taken up again through the same side. Fig. 2*A* (*Inset*) shows that reactivation of the Cl^- current by additional blue light is accompanied by a transient capacitive current in the opposite direction of the stationary (Cl^-) current in green light. This shows that proton uptake accompanied by H578 formation from the H410 state is from the extracellular side. More directly, a 50-fold higher amplitude of the capacitive currents in the experiments presented here allowed a much higher time resolution of <1 ms. This resolves the capacitive current in two phases. Fig. 4, which shows an experiment reproducing the conditions of our report in 1985 (6), clearly shows that these two phases have

opposite signs, with the fast signal indicating H410 decay followed by an undershoot. If the time resolution is decreased to the level of previous experiments the fast signal disappears and only the undershoot, now indicating proton movement of opposite sign, is left (data not shown). With the information provided by the experiments of this contribution, we must conclude that blue light causes a photochemical reprotonation of the Schiff base in halorhodopsin through the extracellular channel. Taking these results together with the photostationary current in the presence of only a protonophore, the conclusion unambiguously is reached that protons are pumped through halorhodopsin from the outside to the inside. A mechanistic interpretation of this ion translocation has been presented in a recent review (18).

A simplified scheme for Cl^- translocation is shown in Fig. 7. The cycle represents the thermoreversible photochemical trans to cis isomerization also known for bacteriorhodopsin and specifically states that H410 is formed from intermediate H520, not H640 (28). Previous work was unable to decide between these two possibilities and only our recent unpublished experiments demonstrate H520 as the source of H410 (J.T. and D.O., unpublished data). In the context of our results, however, this fact is not significant.

The most intriguing aspect of ion translocation in halorhodopsin is the fact that apparently protons and Cl^- ions can be transported alternatively, which supports the model of a branched catalytic cycle under white light conditions. After green light absorption, the molecule turns into the cis state and H520 (see below) has either the possibility to release a Cl^- ion or a proton from the Schiff base. In the absence of blue light, the latter would result in a decrease of active pump molecules because H410 under these conditions forms a dead end and only molecules cycling through the photochemical cycle connected to Cl^- transport would be operative. Thus, proton translocation has a strict blue light requirement and the azide concentration regulates the distribution between Cl^- and proton translocation rates. This is experimentally demonstrated by the result of Fig. 6, where the continuous change from an electrogenic Cl^- via an apparent electroneutral HCl pump to an electrogenic proton pump is shown. The same result is expected without azide for variation of the blue/green light intensity ratio, but at a lower level of activity. Blue light regulation of halorhodopsin has been reported (6), but there was no indication that the regulatory process is 2-fold. Inactive states are regenerated for Cl^- pumping but the same process is linked to proton translocation, decreasing the membrane potential and short-circuiting the proton pump bacteriorhodopsin. To what extent the proton translocation activity of halorhodopsin has a physiological function in intact cells and without azide requires further experimentation.

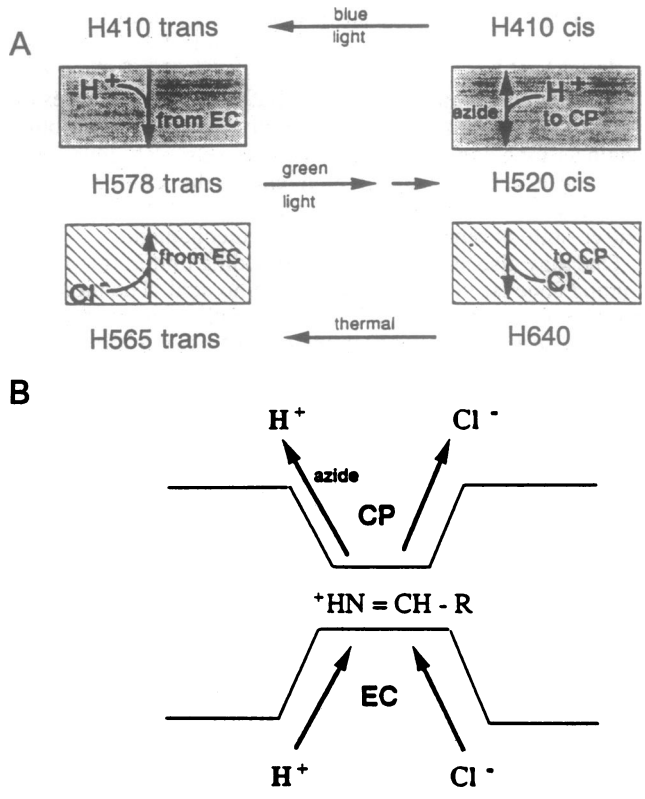

FIG. 7. (*A*) Mechanistic model of proton and Cl^- translocating catalytic cycles in halorhodopsin. Reactions defining the vectoriality of ion transport are boxed. (*B*) Scheme of ion translocation. CP, cytoplasmic; EC, extracellular.

In summary, the results presented here not only add more evidence for a common mechanistic principle in retinal proteins (18) but also allow a detailed molecular interpretation of alternative movement of cations and anions by the same biological ion pump.

The authors thank W. Stoeckenius for valuable comments and critical reading of the manuscript and Luise Eckhardt-Strelau, Afra Hüby, and Susanne von Grunau for excellent technical assistance. This work was partially supported by the Deutsche Forschungsgemeinschaft SFB 169.

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