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A simple light-driven transmembrane proton pump

KAI SUN AND DAVID MAUZERALL†

The Rockefeller University, 1230 York Avenue, New York, NY 10021

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ABSTRACT **Light-induced lipophilic porphyrin/aqueous acceptor charge separation across a single lipid–water interface can pump protons across the lipid bilayer when the hydrophobic weak acids, carbonylcyanide *m*-chlorophenylhydrazone and its *p*-trifluoromethoxyphenyl analogue, are present. These compounds act as proton carriers across lipid bilayers. In their symmetric presence across the bilayer, the positive currents and voltages produced by the photogeneration of porphyrin cations are replaced by larger negative currents and voltages. The maximum negative current and voltage occur at the pH of maximum dark conductance. The reversed larger current and voltage show a positive ionic charge transport in the same direction as the electron transfer. This transport can form an ion concentration gradient. The movement of protons is verified by an unusual D_2O isotope effect that increases the negative ionic current by 2- to 3-fold. These effects suggest that an interfacial pK shift of the weak acid caused by the local electric field of photoformed porphyrin cations/acceptor anions functions as the driving force. The estimated pumping efficiency is 10–30%. Time-resolved results show that proton pumping across the bilayer occurs on the millisecond time scale, similar to that of biological pumps. This light-driven proteinless pump offers a simple model for a prebiological energy transducer.**

Light-driven proton pumps that sustain proton concentration gradients across cellular membranes by consuming light energy are important in supplying energy in the form of ATP to many cells. The mechanisms of biological ion pumps involve transmembrane electron transport (1) or light-induced isomerization of pigments (2), which drive changes in protein conformations (3). The complexities of the integral membrane proteins constructing these pumps have so far prevented a complete description of the ion pumping mechanisms. A light-driven ion pump composed of simple molecules and a lipid bilayer would serve as a useful model and as a possible analogue of the prebiological steps leading to the actual photobiological ion pumps. A defining characteristic of an ion pump is its ability to move ions across bilayers against a concentration or potential gradient. Since the transmembrane transport of protons requires carriers or channels that have one or more proton binding sites (4), a thermodynamic requirement for proton pumps is to cycle through a change in proton binding constants at the two membrane interfaces. We now describe a lipid bilayer system composed of lipophilic magnesium porphyrin (P) and aqueous electron acceptor (A) as the energy generating component and lipophilic weak acids as proton carriers, which has this desired characteristic.

Previous experiments have shown that a lipid bilayer can be photocharged on the nanosecond time scale by electron transfer from an excited porphyrin in the bilayer to an acceptor in the aqueous phase (5) and that it takes >0.1 s for P^+ to cross the bilayer (6). We have recently demonstrated the pumping of tetraarylborate ions across a lipid bilayer by light-induced electron transfer from porphyrin to acceptor across a single lipid–water interface (7). Carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) can function as mobile proton carriers across lipid bilayers and thus as uncouplers in biological energy transduction (8, 9). We present evidence that, when CCCP or FCCP is added to the lipophilic porphyrin/aqueous acceptor system and the bilayer is illuminated, proton pumping across the bilayer occurs. Our results indicate that the mechanism of this proteinless proton pump involves an interfacial electrostatic pK shift.

EXPERIMENTAL PROCEDURES

The membrane system and the probing methods are schematically shown in Fig. 1*A*. The membrane-forming solution consists of 18 mM 1,2-diphytanoyl-3-*sn*-phosphatidylcholine and 3.6 mM magnesium octaethylporphyrin in *n*-decane. Lipid bilayer membranes are formed in a 1.5-mm diameter hole in a thin Teflon sheet dividing a 4-ml polyethylene cell with glass windows, bathed in 0.1 M NaCl solution with 10 mM Hepes (pH 6–8) or other buffers (phosphate, pH 7–10, or phthalate, pH 4–5). The quality of lipid bilayers is monitored by measuring the capacitance (5–6 nF) and resistance (10^9-10^{10} Ω). For the photogeneration of porphyrin cations, 0.5 mM disodium anthraquinone-2,6-disulfonate is added to the aqueous solution on one side of the membrane as A. CCCP or FCCP, is added symmetrically to the aqueous solutions on both sides of the membrane. Gramicidin A, when used, is added to aqueous phases in the 0.5–20 nM concentration range, which causes a comparable membrane conductivity to that of CCCP or FCCP.

Two calomel electrodes with saturated KCl bridges are in the separate bathing solutions. The current is monitored by a fast operational amplifier (Teledyne, Philbrick, MA; model 1021) with a feedback circuit of adjustable gain and time constant. The gain is set to 10^7-10^8 V/A and the time constant is 0.1–1 ms. The voltage is monitored by a low noise voltage amplifier with a 10^9-Ω input impedance and 1-MHz response (Ithaco, Ithaca, NY; model 1201). The resistance × capacitance time of the membrane in the absence of proton carrier is 6 nF × 1000 MΩ = 6s and, in the presence of 10 μM CCCP at pH 7.0, is 6 nF × 10 MΩ = 60 ms. Continuous light (350 mW·cm^{-2}, 400–600 nm) coming from a filtered arc lamp and controlled by a shutter (2 ms half-open time) is used to illuminate the membrane. A 10-ns pulse of light (532 nm, 150 μJ) is from a pulsed Nd:YAG laser (Surelite; Continuum, Santa Clara, CA). Other experimental details can be found elsewhere (10).

RESULTS AND DISCUSSION

Since an active proton pump causes a net ionic charge flow and charge separation across the lipid bilayer, ion pumping can be

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Abbreviations: P^+, lipophilic porphyrin cation; A^-, reduced aqueous electron acceptor; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; D, ^2H; CH, carrier.

†To whom reprint requests should be addressed.

effectively monitored by measuring currents and voltages across membranes (11–13). These electric methods are several orders of magnitude more sensitive than the measurements of ion concentrations in aqueous phases and do not require unbuffered systems. They can directly monitor fast kinetic processes occurring across the bilayer and the interfaces.

Transmembrane Proton Pumping Is Caused by Photoformation of P^+. In Fig. 1*A*, the experimental setup assigns both the photo current and voltage caused by electron transfer from excited porphyrin in the membrane to A a positive (+) sign. On steady-state illumination, the photocurrent rises with the opening-time of the light shutter and decays to zero on the ≈10-ms time scale (Fig. 2*A*, curve a), similar to the rise time of the photovoltage (Fig. 2*B*, curve a). This current transient is the charge displacement current (C dV/dt) and the near-zero steady photocurrent indicates the immobility of P^+. The steady photocurrent is still near zero (5–10 pA) when an electron donor is added to the side trans to the acceptor, verifying this claim. When CCCP is added to the P^+/A^- system, the positive current and voltage are gradually replaced by increasingly negative currents and voltages as the pH of the aqueous phase is decreased from 10 to 6 (Fig. 2). A system using FCCP instead of CCCP shows a similar negative current and voltage. The negative current slowly decays with a half-life of ≈30 s under illumination following an overshoot (Fig. 3). The current is linear with light intensity and concentration of CCCP. The mobile anionic and neutral forms of CCCP and FCCP are not sensitive to the exciting light. Their lack of effect on the positive photo current and voltage at pH>10 proves that the anions of neither carrier reduce the P^+ cation. This is confirmed by the same lifetimes (5 s) of P^+ in the presence and absence of the proton carriers, obtained by measuring the increased transmembrane conductivity of tetraarylborate anions (photogating effect; ref. 14) on this time scale. Both current and voltage are canceled in the presence of electron donor, ascorbate, or ferrocyanide, on the acceptor side. Thus, the inverted signals are not electron transfer reactions and require the presence of P^+.

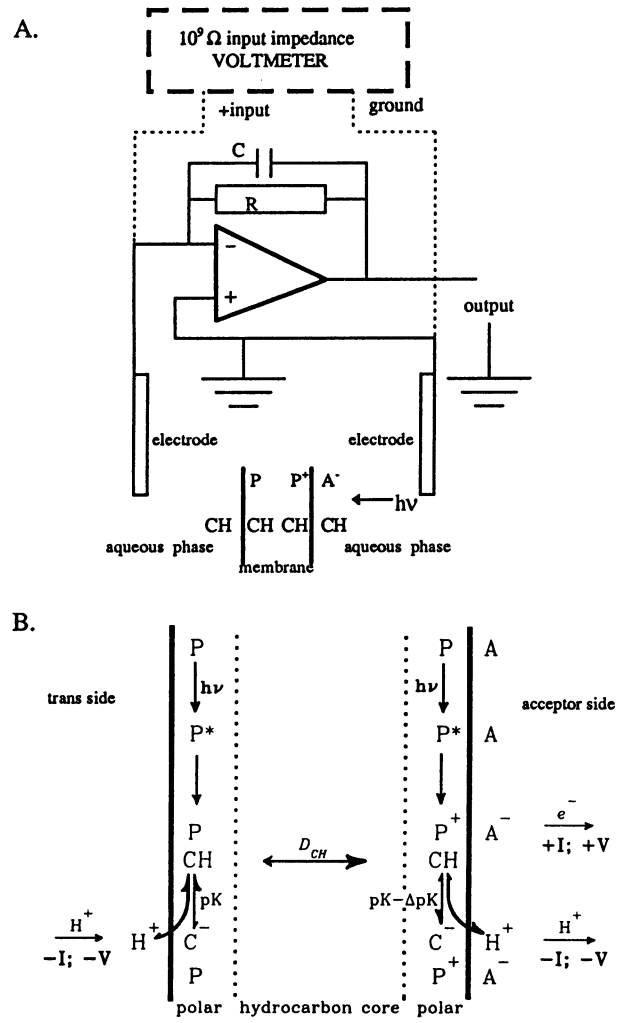

FIG. 1. Schematic diagrams of the membrane systems and the probing methods (*A*) and of the electrostatic pK shift mechanism of proton pumping (*B*). P, P*, and P^+ represent the porphyrin, its excited state, and cation, respectively. A and A^- represent the electron acceptor and its reduced form. CH and C^- represent neutral proton carrier and its anion. In *A*, the circuits with solid and dashed lines represent the conditions for current (short circuit) and voltage (open circuit) measurements, respectively. In *B*, $-\Delta pK$ is the interfacial pK lowering of CH caused by the local electric field of the photoformed P^+ and A^-. This favors dissociation of CH with proton transfer across that interface. D_{CH} is the translocation rate of neutral proton carriers. Thus protons will move across the trans interface to replenish CH. This will continue until the electric field of P^+ is canceled. The transmembrane movements of other species are not shown for clarity. The transmembrane transit time of P^+ is >0.1 s (7), and its field may slow the translocation of the released C^-.

The reversed current and voltage and sensitivity to pH demonstrate that the illumination has transferred protons to the acceptor interface from the trans interface or carrier anions in the opposite direction. There are two parts to our following argument. First, protons are the charge carriers, not the carrier anions; and second, the charge movement is completely transmembrane—i.e., involves the trans (nonacceptor) interface. The claim that the larger reversed signals indicate proton pumping from the opposite to acceptor sides is in accord with the direction of photogenerated local fields: P^+(membrane)/A^-(aqueous). One cannot directly measure the pH change because of the small amount of protons moved, $\approx 10^{-15}$ mol·s^{-1} in the 30-s time range. The proton movement is confirmed by a striking isotope effect on substituting ^2H $(D)_2O$ for H_2O in the system (Figs. 3 and 4). The negative ionic current is increased by 2- to 3-fold on D_2O substitution at pH 7–8 in both the steady-state and pulsed measurements, while the positive photo current and voltage in the absence of the proton carriers are not affected. The mechanism is discussed below. Thus the negative current must be a proton pumping current across the membrane.

The transport of protons completely across the membrane is best argued from the negative photovoltage observed, the amplitude of which exceeds that of the P^+/A^- system alone by >2-fold. The proton movement across the acceptor interface in the presence of P^+ could maximally reduce the positive photovoltage to zero, with a small residual voltage of either sign determined by the orientation of the P^+C^- pairs in the bilayer. It is only if proton movement from the solution to the C^- inside the bilayer occurs at the trans interface that a larger negative voltage will develop, and this movement is in the expected direction of any residual P^+/A^- field. A negative voltage could develop by a large-scale movement of C^- across the bilayer in the opposite direction, but this would have to overcome the electrostatic attraction of the P^+ and is thus unlikely. The apparent contradiction of conservation of charge by the 2-fold increase of the negative voltage over the original positive voltage is in fact a strong argument that ionic charge has moved more deeply or transmembrane. The voltage developed on interfacial charge transfer depends not only on the charge, but on the distance into the bilayer of the hydrophobic charged species. As pointed out earlier (14), the positive photovoltage observed by aqueous electrodes is only the residual polarization of the trans interface by the P^+ at the acceptor interface. In the capacitor analogy, the charge transfer from the excited porphyrin to the acceptor is across the very large polar interface capacitor (≈100 μF·cm^{-2}), while the

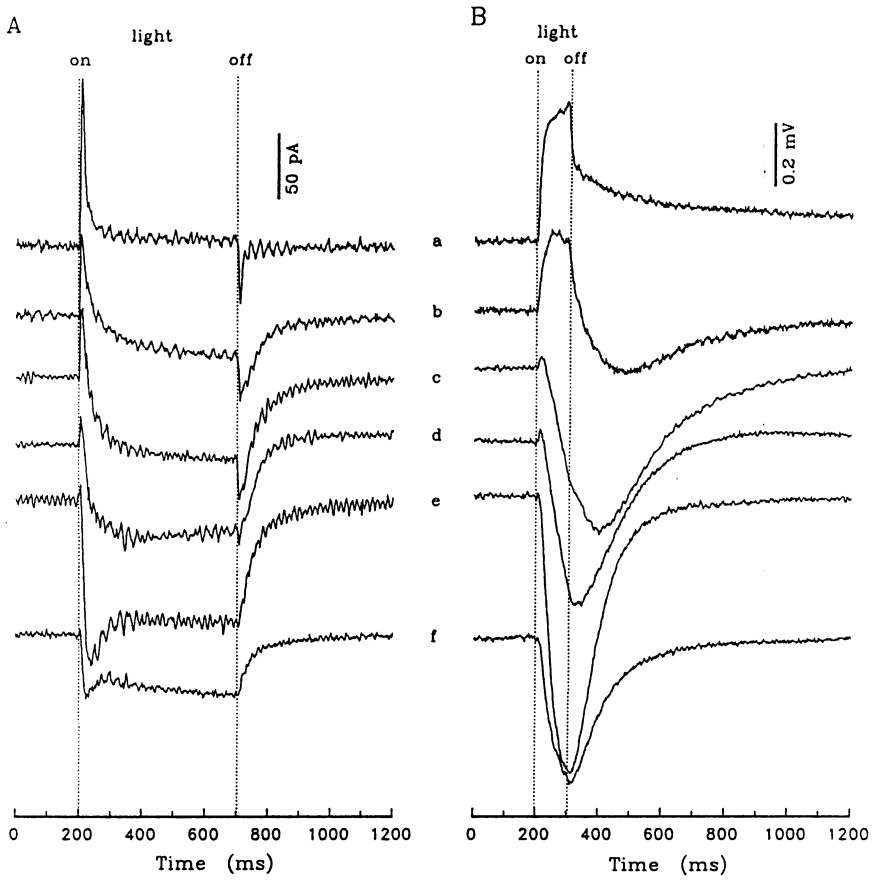

FIG. 2. Currents (*A*) and voltages (*B*) caused by illumination of the system at pH 10–6 are plotted versus time. The half-opening time of the light shutter is 2 ms. The time constant of the current amplifier is 1 ms. A control without porphyrin but with CCCP showed no current (<1 pA). Curve a, no proton carrier (10 ≥ pH ≥ 5). For curves b-f, 10 μM CCCP is present. Curve b, pH 10.0; curve c, pH 9.0; curve d, pH 8.0; curve e, pH 7.0; and curve f, pH 6.0. In *B*, 100-ms illumination is used to show the decay of voltages. Both the positive (no CCCP) and negative voltages reach their maximum values within 300 ms of illumination.

observed voltage appears across the far smaller hydrocarbon core capacitor in series (<1 $\mu F \cdot cm^{-2}$). Thus the apparent charge ($C \times V$), measured by outside electrodes, is only a small fraction of the true charge. This has been verified by measurements on the photogating effect (14). As the charge penetrates deeper into the membrane the observed voltage increases, becoming maximal when total transmembrane transfer occurs. Thus the "surplus" negative voltage is a proof that charge has moved more deeply into the membrane and because of electrostatic attraction of P^+ and C^-, it is the proton movement from the trans interface that causes the negative voltage. The negative voltage in Fig. 2*B* continues to rise for ≈100 ms after the light is shuttered. When a 10-ns pulse of light illuminates the membrane, the reversed current and voltage caused by proton pumping occur on the many-millisecond time scale following a positive transient (Fig. 4). These time-resolved results show that the driving force of the proton pumping must be the light-generated interfacial P^+/A^- charge separation, which lives for seconds (10), but in the present system is limited by the 60-ms charge leakage time of the proton carrier doped bilayer.

Note that within the time constant (0.1 or 1 ms) of the negative feedback current amplifier, the transmembrane voltage is reduced to 0 mV by the voltage clamp or "short circuit" in current measurements shown in Fig. 1*A*, so the reverse ionic current occurring on the >1-ms time scale is not driven by the transmembrane photovoltage. Experimental proof for the vanishing transmembrane voltage is obtained by repeating the experiment with low concentrations of the ion channel-forming polypeptide, gramicidin A, replacing the proton carrier with an equal conductance. No negative current is seen, although protons and other monovalent cations can cross the bilayer through the channels (15). The photoformation of P^+ has no observable effects on the properties of the ion channels. The measured positive photovoltage of the gramicidin system simply decays to zero with the shortened resistance × capacitance time of the membrane. Thus, the electrostatic driving force of the transmembrane proton pumping in the CCCP system must be local electric fields.

The Interfacial Electrostatic pK Shift Mechanism. The 2- to 3-fold larger pumping current of the D_2O system (Figs. 3 and 4*A*) suggests a specific proton pumping mechanism. The usual kinetic D_2O effect is a decrease of rate. The membrane with D_2O and proton carriers shows a 20% smaller conductivity than that with H_2O in the dark. D_2O/H_2O isotope effects of ≈3-fold have been observed in multistep proton transfer reactions (16, 17). In the present system, D_2O substitution may cause an increase in proton pumping current by increasing the concentration of neutral proton carriers in the bilayer. A 3- to 6-fold decrease of ionization of weak acids is caused by using D_2O instead of H_2O as solvent (17), which can be partially explained in terms of zero-point energy differences of the O—H and O—D bonds. The $pK_D - pK_H$ difference of weak acids increases as the corresponding pK increases (18). The calculated difference of CCCP or FCCP (both pK_H 6.0–6.2; refs. 8 and 9) on isotope substitution is 0.5. At pH > pK, this causes a larger concentration of deuterated neutral carrier than that of undeuterated neutral CH. Early studies have shown that the partition of the hydrophobic weak acid, picric acid, between organic and aqueous phases can be enhanced

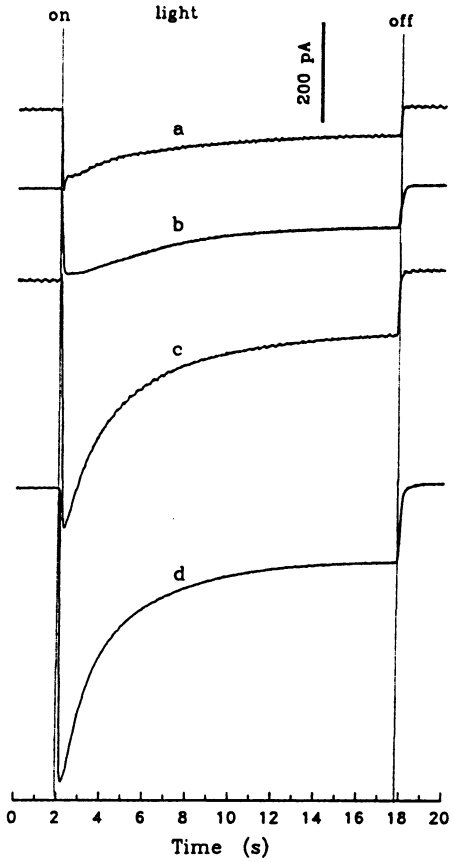

FIG. 3. Proton pumping current caused by continuous light in the H_2O and D_2O systems are plotted versus time. CCCP or FCCP (10 μM) is present in the bathing solutions at pH 7.0 and pD 7.5, which give the maximum negative currents. Curve a, CCCP in H_2O; curve b, FCCP in H_2O; curve c, CCCP in D_2O; and curve d, FCCP in D_2O. Under dark conditions, the D_2O system with the proton carrier has a $\approx$20% smaller conductivity and the same membrane capacitance compared with the H_2O system.

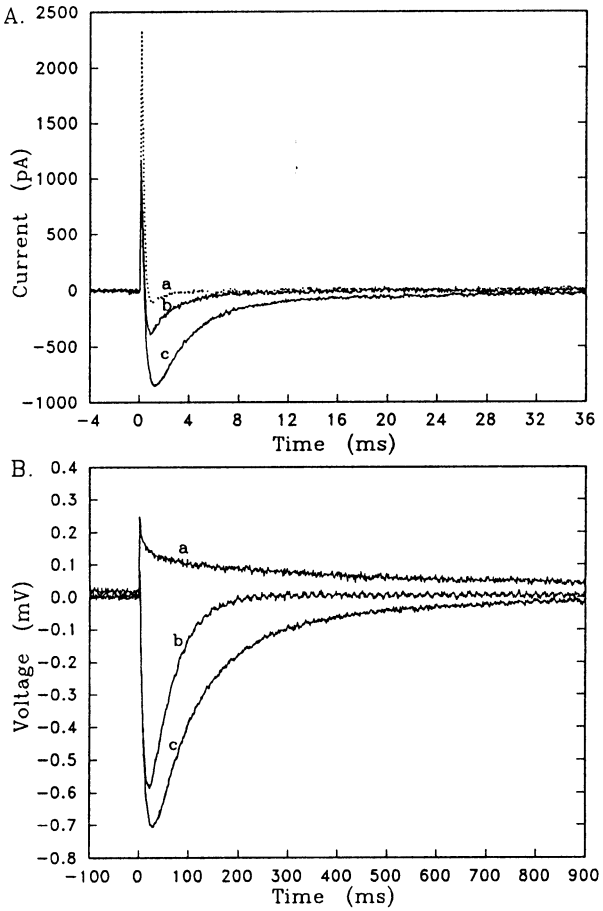

FIG. 4. Currents (A) and voltages (B) induced by a 10-ns pulse of light for the D_2O and H_2O systems are plotted versus time. The time constant of the current amplifier in A is 0.1 ms. The bathing solutions have a pH of 7.0 or a pD of 7.5. Curve a, no proton carrier in D_2O or H_2O. In A, this system shows a displacement current arising from the forward and the rapid component of the reverse interfacial electron transfer. The integral of the total current is zero. Curve b, 10 μM FCCP in H_2O. Curve c, 10 μM FCCP in D_2O.

4-fold by D_2O replacing H_2O (19). The UV-absorption measurements of CCCP and FCCP show that their partition coefficients between benzene and water phases are enhanced 2- to 4-fold by replacement of H_2O with D_2O (unpublished data). The partition coefficients between the lipid bilayer and the water phase may be similarly increased by D_2O substitution. The concentration of deuterated neutral carrier in the bilayer may be larger than that of CH even at pH < pK. Thus, the increased concentration of neutral proton carriers can explain the observed increase of the negative current by D_2O over the complete pH range, not only at pH > pK.

Experiments have demonstrated that an electric field can enhance the dissociation of neutral weak acids (20). A recent calculation has shown that electrostatic pK shifts of residues caused by photoformed negatively charged quinone in the photosynthetic reaction center can explain proton transfer through the quinone cluster (21). The striking D_2O effect suggests that the proton pumping involves interfacial deprotonation of CH at the acceptor side. The electric field of the P^+/A^- charge separation or the direct effect of P^+ by binding C^- in an ion pair favors deprotonation of CH from the membrane interface to the aqueous phase—i.e., the interfacial pK of CH is lowered ($-\Delta$pK). A ΔpK of 0.1 represents a free energy difference (ΔG) equivalent to 6 mV, a $\approx$10-fold stronger driving force than the transmembrane photovoltage observed in open circuit measurements. This free energy difference can cause a pumping current of magnitude shown by Fig. 2. Thus, the interfacial local fields and their effect on pK are the driving force of the proton pumping (Fig. 1B). The interfacial proton flow should be proportional to both the concentration of CH and the electrostatic driving force. Previous studies (8, 9) suggest that free $CCCP^-$ and $FCCP^-$ anions cross the hydrocarbon core of a lipid bilayer on the millisecond time scale, so the transmembrane current is carried by the movement of hydrophobic anions in the hydrocarbon region, but it is carried by protons across the interfaces. Our results are consistent with this model. When illumination forms P^+ and causes CH deprotonation at the acceptor interface, this neutral molecule can be rapidly (<1 ms) replaced by CH from the trans side, which induces H^+ transfer into the bilayer at the trans interface (Fig. 1B). The movement of C^- from the acceptor interface may be slowed by electrostatic interaction with P^+.

This pK shift mechanism (Fig. 1B) can explain the sensitivity of the pumping current to pH changes; proton pumping is immeasurable with pH > 10 or pH < 5. At pH > (pK $-$ ΔpK), the concentration of active CH at the acceptor interface decreases, while at pH < (pK $-$ ΔpK), the concentration of C^- at the trans interface becomes limiting. The fact that the largest current occurs at a higher pH ($\approx$7) than the aqueous pK ($\approx$6) of CCCP and FCCP suggests that they have a higher pK at the membrane interface than in the aqueous phase. The bilayer–CH system also has a maximum conductivity at pH 7 (8, 9).

The Efficiency of This Proton Pump and Comparison to Biological Pumps. This electrostatic ion pump cannot operate

in a true steady state, for then the net current across the membrane is zero, the local electric fields being canceled. In the present system, this saturation is light intensity-dependent and occurs on the time scale of 30 s. Since these slow times are unaffected by stirring, they represent ion gradient formation in the interfacial unstirred layer and within the bilayer. These ion gradients cancel the electrostatic fields producing the enhanced deprotonation of neutral proton carrier and thus attenuate the proton pumping current. The total charge transferred ($I_{peak} \times t_{½}$) is ≈3 nC (Fig. 3, curve a or b). This is ≈100 times that measured in the positive photoinduced charge displacement (Fig. 2*A*, curve a) but, as discussed above, is close to our estimate of the total P^+ that can be formed in the bilayer (14). This saturation is analogous to that in biological ion pumps which, although they cycle more rapidly than the present simple pump when fed photons or ATP, must also reach the thermodynamic limit where the back pressure of the proton gradient equals the pumping force.

Since the asymmetric photoformation of P^+ produces the driving force for the proton pumping current, the efficiency of this pump can be expressed as the ratio of pumped protons to photoformed P^+ cations. To determine the latter we make use of the photogating effect (14) wherein tetrakis(*p*-chlorophenyl)borate anions increase the mobility of P^+ by 100-fold (10), allowing rapid reaction with an aqueous electron donor at the trans interface. The steady maximum electron transfer current is 1200 pA under the same illumination as that of the proton pumping. By ratio with the maximum pumping current with FCCP (≈200 pA; Fig. 3, curve b), the efficiency is ≈15%. The D_2O system has a value of ≈30%. This proton pump is inherently much more "leaky" than biological pumps because it uses a mobile proton carrier.

The simplest biological ion pump is the light-driven proton pump in bacteriorhodopsin. It has been shown to function by flip-flopping between different protein conformations caused by light-induced isomerization of the retinal chromophore (2, 11). The pumping cycle consists of two half-cycles: the first half for proton release from the protonated Schiff base to the extracellular side via Asp-85, and the second half for reprotonation of the deprotonated Schiff base from the cytoplasmic side via Asp-96. How the pigment isomerization affects the polypeptide chain and causes pK shifts of the active sites is still poorly understood (11, 22). When compared with the light-driven proton pump, bacteriorhodopsin, and other biological proton pumps (3, 13), the present pump similarly involves pK shifts of the crucial proton carriers. The present light-driven system also pumps protons across the membrane on the millisecond time scale (Fig. 4), via an electrostatic pK shift. However, it requires no complex proteins. As such it is a simple model for a prebiological energy transducer (23).

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