Golbach, R. W., Borst, P., Bollen-DeVcer, J. E., & Van Bruggen, E. F. (1978) *Biochim. Biophys. Acta 521*, 169-186.
Grunstein, M., & Hogness, D. S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3961-3965.
Horlein, D., McPherson, J., Han Goh, S., & Bornstein, P. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6163-6167.
Kissane, J. M., & Robins, E. (1958) *J. Biol. Chem. 233*, 184-188.
Kream, B., Rowe, D. W., Gworek, S. C., & Raisz, L. G. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5654-5658.
Kushner, S. R. (1978) in *Genetic Engineering* (Boyer, H. B., & Nicosia, S., Eds.) pp 17-23, Elsevier, New York.
Lehrach, H., Diamond, D., Wozney, J. M., & Boedtker, H. (1977) *Biochemistry 16*, 4743-4751.
Lehrach, H., Frischauf, A., Hanahan, D., Wozine, Y. J., Fuller, F., Crkvenjakov, R., & Boedtker, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5417-5421.
Lehrach, H., Frischauf, A., Hanahan, D., Wozine, Y. J., Fuller, F., & Boedtker, H. (1979) *Biochemistry 18*, 3146-3152.
Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499-560.
McDonnell, M. W., Simon, M. N., & Studier, F. W. (1977) *J. Mol. Biol. 110*, 119-146.
Moen, R. E., Rowe, D. W., & Palmiter, R. D. (1979) *J. Biol. Chem. 254*, 3526-3530.
Monson, J. M., & McCarthy, B. J. (1981) *DNA 1*, 59-69.
Monson, J. M., Friedman, J., & McCarthy, B. J. (1982) *Mol. Cell. Biol. 2*, 1362-1371.
Myers, J. C., & Spiegelman, S. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5329-5333.
Myers, J. C., Spiegelman, S., & Kacian, D. L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2840-2843.
Nelson, T., & Brutlag, D. (1979) *Methods Enzymol. 68*, 41-50.
Payvar, F., & Schimke, R. T. (1979) *J. Biol. Chem. 254*, 7636-7642.
Peterkofsky, B., & Diegelmann, R. F. (1971) *Biochemistry 6*, 988-994.
Raisz, L. G., & Kream, B. E. (1983) *N. Engl. J. Med. 309*, 29-35, 83-89.
Rowe, D. W., & Kream, B. (1982) *J. Biol. Chem. 257*, 8009-8015.
Rowe, D. W., Moen, R. E., Davidson, T. H., Byers, P. H., Bornstein, P., & Palmiter, R. D. (1978) *Biochemistry 17*, 1581-1590.
Sandmeyer, S. G., & Bornstein, P. S. (1981) *J. Biol. Chem. 245*, 5022-5028.
Showalter, A. M., Pesciotta, D. M., Eikenberry, E. F., Yamamoto, T., Pastan, I., deCrombrugghe, B., Fietzek, P. P., & Olson, B. R. (1980) *FEBS Lett. 111*, 61-65.
Sobel, M. E., Yamamoto, T., de Crombrugghe, B., & Pastan, I. (1981) *Biochemistry 20*, 2678-2684.
Solomone, H., Hiorna, L., Sheer, D., & Rowe, D. W. (1984) *Ann. Hum. Genet. 48*, 39-42.
Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5201-5205.
Wickens, M. P., Buell, G. N., & Schmike, R. T. (1978) *J. Biol. Chem. 253*, 2483-2495.

The Chromoprotein of Halorhodopsin Is the Light-Driven Electrogenic Chloride Pump in *Halobacterium halobium*†

E. Bamberg,* P. Hegemann, and D. Oesterhelt

ABSTRACT: The chromoprotein of halorhodopsin was isolated from *Halobacterium halobium* strain L-33, a bacteriorhodopsin-deficient mutant, and incorporated into asolectin lipid vesicles. When these vesicles are added to one side of a planar lipid membrane, the membrane system becomes photoelectrically active. The observed photoresponse occurs only in the presence of chloride (and other halides). The action spectrum of the photoresponse is identical with the visible absorption band of the chromoprotein in lipid vesicles. The photoresponse consists of a transient photocurrent, which indicates that the lipid vesicles are adsorbed to the surface of the planar lipid membrane but not integrated into it. The stationary photocurrent is extremely low because the underlying lipid membrane is virtually impermeable to the transported ion. The stationary photocurrent, however, increases drastically upon the addition of the lipophilic anion tetraphenyl borate or of the protonophore tetrachloro-2-(trifluoromethyl)benzimidazole (TTFB, HA) to the system. The TTFB-enhanced stationary photocurrent is caused by the transport of an HA_2^- species. The results obtained demonstrate that the chromoprotein of halorhodopsin is the light-driven Cl^- pump in *H. halobium*.

The retinal-binding protein, halorhodopsin, occurs in the cell membrane of *Halobacterium halobium* in addition to the predominant and well-characterized proton pump bacteriorhodopsin (Matsumo-Yagi & Mukohata, 1977; Lanyi & Oesterhelt, 1982; Mukohata & Kaji, 1980; Wagner et al., 1981). On the basis of transport measurements with cell envelope vesicles from the bacteriorhodopsin-deficient mutant strain L-33, it was proposed that halorhodopsin acts as a light-driven chloride pump (Schobert & Lanyi, 1982). This function was demonstrated with cell membrane fragments on black lipid membranes (Bamberg et al., 1984). Both types of experiments, however, do not determine whether the functional unit of halorhodopsin consists of an individual protein species or whether several different proteins contribute to the function. The retinal binding component of halo-

† From the Max-Planck-Institut für Biophysik, D-6000 Frankfurt am Main, West Germany (E.B.), and the Max-Planck-Institut für Biochemie, Am Klopferspitz, D-8033 Martinsried, West Germany (P.H. and D.O.). *Received March 28, 1984.*

0006-2960/84/0423-6216$01.50/0

rhodopsin was isolated in its native state (Steiner & Oesterhelt, 1983; Taylor et al., 1983). The photochemical and chloride-binding properties of halorhodopsin (Steiner et al., 1984; Falke et al., 1984) were similar to those of halorhodopsin in membrane fragments and cell envelope vesicles (Weber & Bogomolni, 1981; Schobert et al., 1983). Reconstitution of purified halorhodopsin into an artificial membrane system therefore is necessary to order to decide the question whether the chromoprotein is the chloride-transporting unit of halorhodopsin. Passive proton transport can be used as an assay for the light-induced chloride pump activity of halorhodopsin in cell envelope vesicles. This assay, however, appeared not sensitive enough in liposomal systems for the reconstitution of halorhodopsin activity with the purified chromoprotein. Therefore, black lipid membranes were used to show the chloride pump activity of the halorhodopsin chromoprotein in these liposomes.

Materials and Methods

Purification of Halorhodopsin and Preparation of Halorhodopsin-Containing Lipid Vesicles (Proteoliposomes).[1] *H. halobium* strains L-33 and OD 2 were grown and the Tween-washed membrane prepared from the cells as described (Bamberg et al., 1984). Halorhodopsin was purified from Tween-washed membranes by the procedure described (Steiner & Oesterhelt, 1983), except that the hydroxylapatite chromatography was carried out in the presence of 5 mM sodium phosphate, pH 7.

Liposomes were prepared from 80 mg of asolectin (Sigma) in 2 mL of 1 M NaCl by sonication at 30 °C in a sonifying bath (Branson) until the turbidity no longer decreased (about 30 min). The liposomal suspension (50 μL) was mixed with 30 μL of a halorhodopsin-containing solution (80 nmol/mL, 1% octyl glucoside in 1 M NaCl) and 1 M NaCl added to a final volume of 200 μL. After 2-h incubation 2.8 mL of 1 M NaCl was added, and 100 μL (80 pmol of halorhodopsin) of this sample was used for each of the black lipid membrane experiments.

Bacteriorhodopsin (3 nmol) was solubilized in 1% octyl glucoside, pH 5, for 3 days at 32 °C. The protein was incorporated into the lipid vesicle by the same procedure as described for halorhodopsin.

Cell envelope vesicles were prepared from cells as described (Hegemann et al., 1982). Limited proteolysis with chymotrypsin was carried out in 1 M NaCl/0.1 M $MgSO_4$ at 37 °C for 1 h with 0.04 mg of chymotrypsin/mg of vesicle protein. A total of 200 μL of this vesicle suspension (2.5 mg/mL) was used for each experiment.

Preparation of the Planar Lipid Bilayers. Optically black lipid membranes with an area of about 7.5×10^{-2} cm^2 were formed in a Teflon cell filled with an appropriate electrolyte solution (6 mL in each compartment). The membrane-forming solutions contained either 1.5% (w/v) asolectin in decane (negatively charged) or 1.5% (w/v) diphytanoyllecithin (Avanti Biochemicals, Birmingham, AL) and 0.05% (w/v) octadecylamine (Riedel-de Haen, Hannover, F.R.G.) in decane positively charged; Dancshazy & Karvaly, 1976). For adsorption of the negatively charged vesicles to negatively charged membranes, the addition of 1–20 μM Ca^{2+} ions was necessary (Bamberg et al., 1981), whereas the vesicles adsorbed to positively charged membranes without Ca^{2+}. The

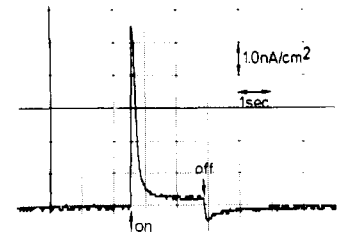

FIGURE 1: Short-circuit photocurrent after addition of 100 μL of chromoprotein-containing asolectin/lipid vesicle suspension. Final protein concentration was 13 nM. Electrolyte was 1 M NaCl/0.1 mM Hepes, pH 6.5; irradiance was 5 mW/cm^2. The arrows indicate light on and off. The membrane forming solution was asolectin in *n*-decane (1.5% w/v).

temperature was kept at 25 °C. The membrane cell was connected to an external measuring circuit via Ag/AgCl electrodes. In order to avoid artificial photoeffects, the electrodes were separated from the aqueous compartments of the Teflon cell by salt bridges. Light from a 250-W halogen tungsten lamp equipped with a heat protection and a cutoff filter ($\lambda > 495$ nm) was focused on the lipid bilayer. The light beam first entered the front compartment; the vesicles were added to the rear compartment.

The action spectrum of the photocurrent was determined by using a series of narrow band interference filters (Balzers B 40, 10-nm half-width). Further details are described in Bamberg et al. (1979).

Results

A solution of purified halorhodopsin[2] ($OD_{580} = 4$) in 1% octyl glucoside and 1 M sodium chloride was added (8 nmol in 100 μL) to one side of the black lipid membrane. The final concentration of the protein in the cuvette filled with 1 M NaCl was 1.3 nmol/ml in 0.16% octyl glycoside. A small transient photocurrent (0.2–0.3 nA/cm^2 peak current) was observed, indicating a preferentially oriented association of the protein with the planar bilayer. Unfortunately, under these conditions the black lipid membrane becomes unstable. Control experiments showed that the destabilization was not caused by the octyl glucoside but by the protein because concentrations of the detergent up to 0.5% did not affect the stability of the bilayer membrane. We therefore added halorhodopsin to the black lipid membrane in a lipid-bound form.

Liposomes were prepared from asolectin and preincubated for 2 h with purified halorhodopsin at a lipid to protein ratio of 1200 (see Materials and Methods). Then these proteoliposomes were added to one side of the negativly charged asolectin bilayer membrane. The aqueous solution contained 1 M NaCl and 20 μM $CaCl_2$. Photosensitivity developed within minutes and continued to increase over several hours.

Figure 1 shows the photocurrent 30 min after the addition of the proteoliposomes. Although halorhodopsin was present only in 1/100 of the concentration used in the experiment with solubilized protein described above, a 20-fold higher peak current, I_0, of 5.5 nA/cm^2 was obtained. As seen from Figure 1, the photocurrent is transient. This is due to an association of the vesicles to the planar bilayer (Herrmann & Rayfield, 1978). Figure 2a shows schematically the tight association that leads to electric coupling between the proteoliposomes and the underlying black lipid membrane. The capacitive decay of the photocurrent can be explained by the equivalent circuit

[1] Abbreviations: TTFB, tetrachloro-2-(trifluoromethyl)benzimidazole; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; TPB^-, tetraphenyl borate; proteoliposomes, halorhodopsin-containing lipid vesicles.

[2] The experiments described in this paper demonstrate that the pure chromoprotein acts as the chloride pump. Therefore, we use from now on the term halorhodopsin instead of chromoprotein of halorhodopsin.

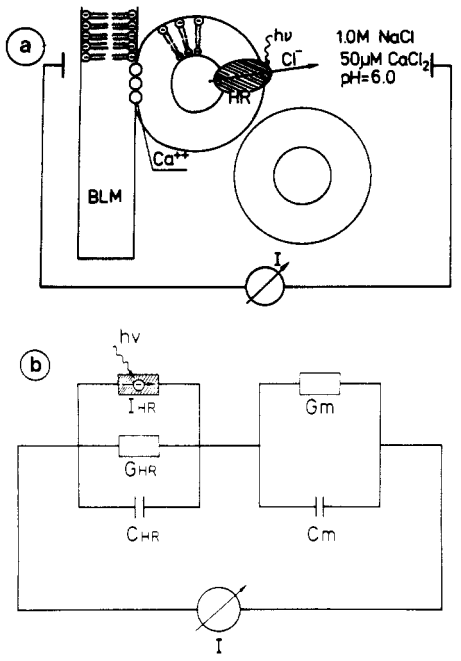

FIGURE 2: Schematic representation of the adsorption of asolectin vesicles on the black lipid membrane. (a) Adsorption of the vesicles to the underlying membrane in the presence of Ca^{2+} ions. (b) Equivalent circuit diagram for the vesicle black lipid membrane system. G_{HR} and C_{HR} are the conductance and the capacitance of halorhodopsin-containing vesicles. G_m and C_m are the corresponding elements for the underlying lipid membrane. I_{HR} is the photocurrent generating element in the liposomal membrane. The RC time of the system is given by $\tau = (G_m + C_{HR})/(G_m + G_{HR} + I_{HR}/V^*)$ where V^* is a constant. Under our experimental conditions (Figure 1), τ is 100 – 200 ms.

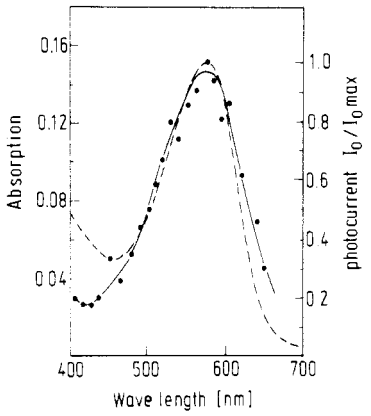

FIGURE 3: Action spectrum of the initial photocurrent I_0 measured with a series of narrow band interference filters (13 nM halorhodopsin). $I_{0,max}$ represents the photocurrent at 578 nm. Correction factors accounting for the emission spectrum of the lamp and transmission of the filters were obtained by calibration with a bolometer. Data points are photocurrent measurements. The dashed line represents the absorption spectrum of the halorhodopsin-containing lipid vesicles. The membrane-forming solution was as in Figure 1.

diagram in Figure 2b. The decay time, τ, which corresponds with the RC time of the system is in the range of 100 ms (Bamberg et al., 1979, 1984; Herrmann & Rayfield, 1978). Similar results were obtained with positively charged octadecylamine-containing black lipid membranes without the addition of Ca^{2+} ions.

The action spectrum of the peak current and the absorption spectrum of the proteoliposomes are compared in Figure 3. The maxima are both at 578 nm, which is very close to the absorption maximum of light-adapted halorhodopsin in solution (Steiner & Oesterhelt, 1983; Taylor et al., 1983). In a

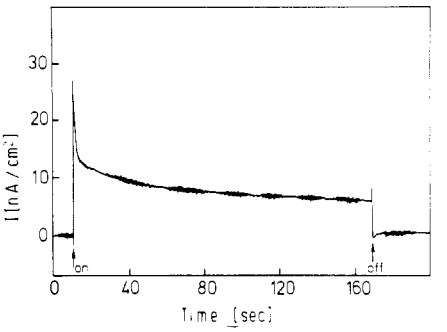

FIGURE 4: Stationary photocurrent induced by halorhodopsin-containing vesicles (13 nM halorhodopsin) in the presence of 1 μM TPB^-, 1 M NaCl, and 0.1 mM Hepes, pH 6.5. Light intensity was 1/mM/cm². The black lipid membrane forming solution was diphytanoyllecitin (1.5% w/v) with octadecylamine (0.05% w/v) in *n*-decane.

previous paper (Bamberg et al., 1984) we have shown that the photoresponse of halorhodopsin-containing membrane fragments depends specifically on the concentration of Cl^- ions (half-saturation at 8 mM) and that a photoresponse is already measurable below 1 mM Cl^-. In contrast to this, the halorhodopsin-containing vesicles give no measurable photocurrent below 50 mM Cl^- even at high ionic strength (1 M Na_2SO_4), preventing the determination of the affinity constant for Cl^-. No dependence of the photocurrent on different cations such as Na^+, K^+, or Mg^{2+} was found.

Since the underlying membrane is nearly impermeable to Cl^-, only transient photocurrents can be observed. The demonstration of transient photocurrents, however, does not prove that halorhodopsin acts as a light-driven ion pump because the transient photocurrent could be a light-induced displacement current, corresponding to a charge movement within the protein. In bilayer systems only the appearance of stationary photocurrents[3] can prove that halorhodopsin acts as an ion pump [see also Bamberg et al. (1984)].

In whole cells and cell envelope vesicles the action of halorhodopsin causes protonophore-enhanced passive proton flow that is abolished upon addition of lipophilic ions (Wagner et al., 1981). The lipophilic anion TPB^- short-circuits by counter transport the current produced by halorhodopsin in a liposome. After association of the liposomes with the planar bilayer, TPB^- is also transported through the planar bilayer in response to the Cl^- transport (Figure 7a).

Figure 4 shows a stationary photocurrent after addition of 0.1–1 μM TPB^- to the aqueous phases of the lipid bilayer system and appropriate preequilibration with the lipophilic anion. After the initial capacitive transient, the photocurrent decays only slowly (in the range of minutes) to a constant value. This indicates the formation of concentration gradients of TPB^- and Cl^- across the vesicle membrane due to the pump action of halorhodopsin. If a continuous layer of proteoliposomes containing only halorhodopsin molecules oriented in one direction would cover the planar film, the total charge corresponding to one Cl^- per halorhodopsin, i.e., a single turnover, would be about 87 nC cm^{-2}. If a current of 5 nA cm^{-2} flows for several minutes (compare Figure 4), the transferred charge is far in excess of 87 nC. This clearly demonstrates the continuous function of the chloride pump in contrast to a displacement current caused by a single photoevent.

[3] We use the term stationary for photocurrents that, in contrast to capacitive photocurrents, decay only slower than 1 s. This decay depends on diffusion polarization in the unstirred layers of the planar bilayer and on the formation of concentration gradients.

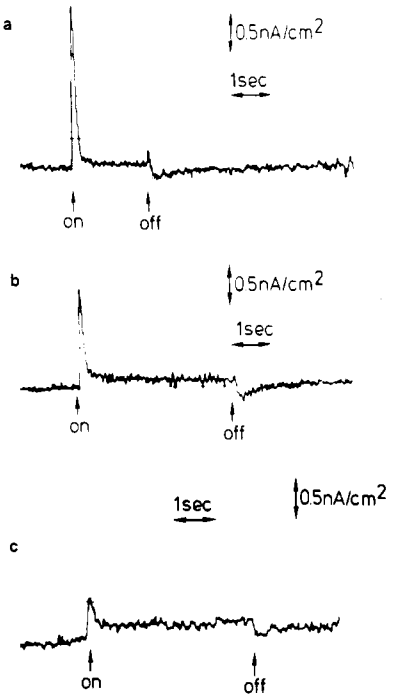

FIGURE 5: Decrease of photocurrent in response to increasing concentrations of FCCP. (a) Without FCCP; (b) 1 μM FCCP; (c) 10 μM FCCP. (a)–(c) contain in addition 0.1 M Hepes, pH 6.0, 1 M NaCl, 50 μM $CaCl_2$, and 13 nM halorhodopsin. Irradiance 5 mW/cm², the black lipid membrane forming solution was asolectin (1.5% w/v) in *n*-decane.

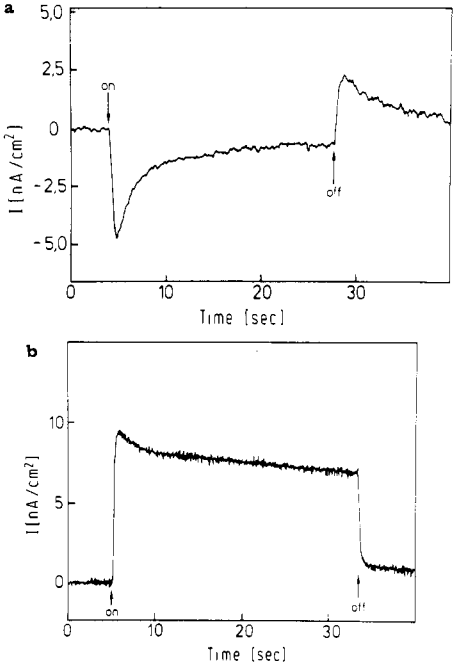

FIGURE 6: (a) Photocurrent induced by cell envelope vesicles (200 μL) in the presence of 1 μM TPB^-, 1 M NaCl, and 0.1 mM Hepes, pH 6.5. The black lipid membrane forming solution was diphytanoyllecitin with octadecylamine. (b) Photocurrent in the presence of halorhodopsin-containing asolectin vesicles (13 nM halorhodopsin). The membrane-forming solutions for (a) and (b) were diphytanoyllecithin (1.5% w/v) with octadecylamine (0.05% w/v) in *n*-decane.

The addition of protonophores should also increase the stationary photocurrent. This was indeed observed after addition of FCCP in the concentration range of 1–10 nM (not shown). Higher concentrations of 0.1–10 μM, however, did not further increase the stationary photocurrent but rather abolished the photocurrent altogether (Figure 5). We interpret this that protons are transported at increasing rates only across the liposomal membrane and that the transport across the lipid bilayer is shunted out. A decreased photoresponse at high concentrations of FCCP is also observed for bacteriorhodopsin-containing liposomes under the same conditions (see Discussion).

The sign of the pump current in the halorhodopsin-containing asolectin vesicles was determined with cell envelope vesicles as the reference. It is known from the pH and light scattering measurements (Schobert & Lanyi, 1982) on these vesicles that chloride is pumped into the interior space as in intact cells. In order to get reasonable electrostatic adsorption, the cell envelope vesicles (1 mL, 10 mg of protein/mL) were slowly brought from 4 to 1 M NaCl by dilution with water. The addition of these vesicles to one compartment of the Teflon cell did not yield any photoresponse. The electrical coupling with the lipid bilayer was presumably hindered by residual cell wall protein on the vesicular surface. Therefore, the vesicles were incubated for 1 h at 37 °C with chymotrypsin (0.4 mg/mg of protein). These pretreated cell envelope vesicles were added to one side of a positivly charged lipid bilayer. The presence of 10 μM TPB^- induced the stationary photoresponse shown in Figure 6a. The stationary current decays within 5 s to a much smaller and constant value (see Discussion). The halorhodopsin-containing asolectin vesicles added under exactly the same conditions to a positivly charged lipid bilayer showed the opposite sign of the photocurrent (Figure 6b). Therefore, Cl^- transport must occur in these liposomes from the inside to the outside. The different decay times of the photocurrents in the two different preparations will be discussed below.

Discussion

In a previous paper we have shown that halorhodopsin-containing membrane fragments, adsorbed to black lipid membranes, exhibit chloride pump activity (Bamberg et al., 1984). Since these membrane fragments also contain many other proteins, the possibility existed that the functional unit consists of the chromoprotein together with another protein. The isolation of pure halorhodopsin provided the opportunity to decide between these two alternatives. The chloride pump activity was first measured by passive proton flux as in the standard assay of cells or cell envelope vesicles. Purified halorhodopsin was incorporated into artificial asolectin vesicles, and upon illumination, only a small acidification of the liposomal suspension was observed that could not be enhanced by the addition of protonophores and corresponded to not more than one proton per halorhodopsin. This can be explained in several ways. Either the vesicles are rather permeable to chloride ions and protons or the protons dissociate stoichiometrically from the protein. A third possibility is an almost complete random orientation of the protein molecules. We therefore preferred electrical measurements for the direct demonstration of the pump activity of purified halorhodopsin.

The most direct approach, i.e., the addition of the solubilized halorhodopsin to one side of the lipid membrane, resulted in a small transient photocurrent, demonstrating an electric coupling of halorhodopsin with the planar bilayer. If single molecules associate with the bilayer, the small current can be due to a small degree of preferential association of the molecules with one of their surfaces to the planar lipid membrane. Alternatively, aggregates of protein resulting from dilution of the detergent could associate with the bilayer. Again the small current is due to only a small excess of pump molecules electrically coupled to the bilayer in one direction. The third possibility, i.e., insertion of halorhodopsin molecules into the lipid bilayer, is excluded by the lack of stationary photocur-

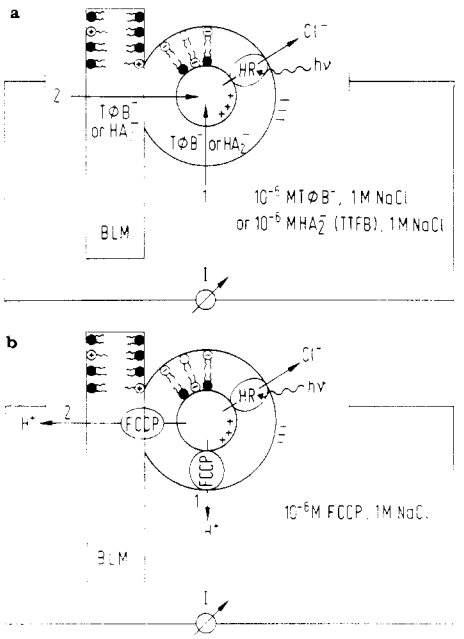

FIGURE 7: Schematic representation of the action of the different lipophilic transport systems on the black lipid/liposomal system. (a) Action of TPB^- and TTFB (HA_2^-); (b) Action of FCCP.

rents. The destabilization of the bilayer indicates a strong interaction of the protein with the planar lipid bilayer. The small current and instability induced by the extremely hydrophobic nature of the protein (results from amino acid analysis) on the one hand and low measurable pump activity after incorporation into proteoliposomes on the other hand (pH change) can be overcome by association of proteoliposomes with the planar lipid bilayer and led finally to the successful demonstration of large stationary photocurrents.

These photocurrents were obtained with only 1/100 of the amount of halorhodopsin necessary without liposomes. Five times higher halorhodopsin concentrations in the proteoliposomal preparations did not increase the photocurrents but affected the planar bilayer to become unstable again. This is probably caused by an excess of free halorhodopsin not incorporated into the lipsomes by the dilution method we used for the preparation of the proteoliposomes.

The appearance of transient photocurrents as shown in Figure 1 can be interpreted by an absorption of the proteoliposomes to the lipid bilayer, so that only capacitive photocurrents can be observed (see also the equivalent circuit diagram in Figure 2b). Such a displacement current gives no evidence that the protein acts as an ion pump but could also be expected by a light-induced charge movement within the protein.

In the case of the light-driven proton pump, bacteriorhodopsin, proteoliposomes show continuous photocurrents on black lipid membranes after the underlying lipid bilayer has been made permeable for protons with lipophilic uncouplers like FCCP (Herrmann & Rayfield, 1978).

For the analogous experiments with halorhodopsin, no appropriate Cl^- carrier is available and thus the lipophilic anion tetraphenyl borate was used to obtain stationary photocurrents (Figures 4 and 6). A schematic representation of the ion movement is given in Figure 7a. The underlying lipid bilayer is virtually impermeable to chloride. TPB^- can diffuse in response to the potential difference created by halorhodopsin into the inner space of the liposome either from the cis side (pathway 1) or from the trans side (pathway 2) of the planar bilayer. Pathway 1 involves larger areas of the liposomes and only the crossing of one bilayer but is electrically silent. Pathway 2 involves small areas and two bilayers but leads to stationary photocurrent. Its decrease, in the range of minutes, must be due to the creation of concentration gradients of TPB^- and the pumped chloride ions across the liposomal membranes. Because TPB^- is present in μM concentrations, the gradient that finally limits the the net current is that of the TPB^- ions.

The photocurrent induced by cell envelope vesicles decreases faster than that in the proteoliposomes under the same conditions. Three parameters can be responsible for this effect. First, differences in inner volume, second, the differences in the direction of chloride translocation (Figure 6), and third, differences in permeability. The membrane potential created by the pump can reach values of at least 180 mV (Mukohata & Kaji, 1980) corresponding to gradients of TPB^- with concentration ratios of 1000. With 1 μM TPB^- in the chamber, proteoliposomes will therefore accumulate TPB^- to 1 mM until the stationary photocurrent decreases. Cell envelope vesicles will deplete their inner volume by nearly 1 μM TPB^-, which was present in the inner space after preequilibration. From this, one would expect that the total number of charges translocated during the stationary current would lead, in envelope vesicles, to only about 1/1000 of that on proteoliposomes and the current would decay at a much faster rate. This effect, however, is counterbalanced to some extent by the much larger volume of the envelope vesicles and may be further influenced by differences in permeability of the proteoliposomes and the envelope vesicles.

Furthermore, it is interesting to note that the calculated number of TPB^- molecules in the envelope vesicles (diameter 500 nm; inner volume 6.5×10^{-17} L) is only 40 TPB^- molecules. These would be removed within 1 s, assuming a turnover number of 100/s (Weber & Bogomolni, 1981) if we neglect a buffering effect due to the distribution of TPB^- between the aqueous phase and the liposomal membrane [the distribution coefficient β is ca. 10^{-2} cm (Ketterer et al., 1971)].

Surprisingly, the presence of the protonophore FCCP at concentrations from 0.1 to 10 μM decreases the photoactivity of cell envelope vesicles as well as that of the proteoliposomes via pathway 2 (Figure 7b) to an almost undetectable level. Pathway 1 (electrically silent in our measurement) and pathway 2 in Figure 7b abolish the membrane potential enhanced by the chloride pump in a competitive way. Increasing amounts of FCCP must shunt out pathway 2 by a high transport rate across the liposomal membrane (pathway 1). Without attempts to interprete the molecular basis for such an effect, this assumption explains the lack of a stationary photocurrent at high FCCP concentrations. In addition, in the presence of 10 μM FCCP the chloride-mediated membrane potential is immediately abolished in the vesicles because the proton translocation is about 100–1000 times faster than the chloride transport by halorhodopsin (Benz & McLaughlin, 1983; Weber & Bogomolni, 1981). Because the time constant for the proton translocation in the presence of FCCP is within the range of the RC time of the system also, the capacitive photocurrent is reduced (Figure 5).

To support this explanation, an analogous experiment with bacteriorhodopsin was carried out. Under the same conditions, i.e., in the presence of bacteriorhodopsin-containing liposomes (13 nM bacteriorhodopsin), the photocurrent increased by a factor of 5 when FCCP was added in concentrations up to 0.1 μM, whereas 5 μM abolished the photocurrent (0.7 $\mu A/cm^2$) completely. This effect occurs only at these low concentrations of bacteriorhodopsin, where the total pump capacity per vesicle is small compared to experiments described earlier with at least

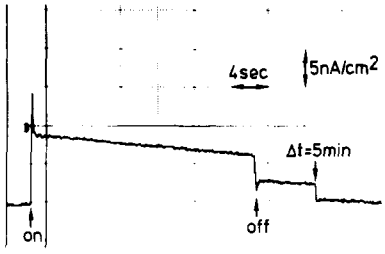

FIGURE 8: Photocurrents induced by chromoprotein-containing vesicles (13 nM halorhodopsin) in the presence of 1 μM TTFB, 0.1 mM citrate, pH 5.8, and 1 M NaCl. The black lipid membrane forming solution was asolectin (1.5% w/v) in *n*-decane.

100 times higher bacteriorhodopsin concentrations (Herrmann & Rayfield, 1978).

A difference between the bacteriorhodopsin- and the halorhodopsin-containing systems is a constant stationary photocurrent for bacteriorhodopsin and a stationary photocurrent for halorhodopsin that decrease in the minute range. This is explained by the fact that the proton pump, bacteriorhodopsin, and a protonophore acting in series never deplete the inner space of the liposome from protons, whereas a single molecule of the chloride pump will deplete a proteoliposome of 100-nm diameter containing 1 M NaCl in about 5 min.

The uncoupler TTFB, which acts within a pH range of 6–7 predominantly as a HA_2^- anion (Neumcke & Bamberg, 1975), also enhanced stationary photocurrents (Figure 8), which decrease in the range of minutes to a constant level. If the light is turned off before this happens, the dark current persists and decays within 5 min to the basic value. This indicates a diffusion potential of the uncoupler ions that were accumulated before in the light. The ion gradient of chloride is negligible because chloride is present at a concentration of 1 M. A similar observation was also made in the presence of TPB^-.

The addition of the lipophilic agents TPB^- or TTFB also increases up to 5 times the peak currents [compare Figure 1 and Wagner et al. (1981)]. We attribute this to the distribution of tetraphenyl borate within the lipid bilayer as a response to light-induced Cl^- transport.

Summarizing our results, we have shown by the demonstration of stationary, chloride-dependent photocurrents on the black lipid membrane system that the purified chromoprotein of halorhodopsin is the light-driven chloride pump of *H. halobium*. Therefore, the chromoprotein is identical with halorhodopsin. Because under exactly the same experimental conditions the amplitude of the photocurrents induced by bacteriorhodopsin is comparable with those induced by halorhodopsin, one can conclude that halorhodopsin pumps chloride with a similar efficiency as bacteriorhodopsin pumps protons. This was anticipated by the similar half-times of their photocycles and a similar degree of orientation.

Acknowledgments

We thank Afra Hüby and Utah Hegemann for excellent technical assistance and Tracy Topping and Walter Stoeckenius for critical reading of the manuscript.

Registry No. TTFB, 2338-29-6; FCCP, 370-86-5; TPB^-, 4358-26-3; diphytanoyllecithin, 32448-32-1; octadecylamine, 124-30-1; chloride, 16887-00-6.

References

Bamberg, E., Apell, H.-J., Dencher, N. A., Sperling, W., Stieve, H., & Läuger, P. (1979) *Biophys. Struct. Mech. 5*, 277–292.

Bamberg, E., Dencher, N. A., Fahr, A., & Heyn, M. P. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7502–7506.

Bamberg, E., Hegemann, P., & Oesterhelt, D. (1984) *Biochim. Biophys. Acta 773*, 53–60.

Benz, R., & McLaughlin, S. (1983) *Biophys. J. 41*, 381–398.

Dancshazy, Z., & Karvaly, B. (1976) *FEBS Lett. 72*, 136–138.

Falke, J. J., Chan, S. I., Steiner, M., Oesterhelt, D., Towner, P., & Lanyi, K. (1984) *J. Biol. Chem. 259*, 2785–2789.

Hegemann, P., Steiner, M., & Oesterhelt, D. (1982) *EMBO J. 1*, 1177–1183.

Herrmann, T. R., & Rayfield, G. W. (1978) *Biophys. J. 21*, 111–125.

Ketterer, B., Neumcke, B., & Läuger, P. (1971) *J. Membr. Biol. 5*, 225–245.

Lanyi, J. K., & Oesterhelt, D. (1982) *J. Biochem. (Tokyo) 255*, 243–250.

Matsumo-Yagi, A., & Mukohata, Y. (1977) *Arch. Biochem. Biophys. 199*, 297–303.

Mukohata, Y., & Kaji, Y. (1980) *Arch. Biochem. Biophys. 206*, 72–76.

Neumcke, B., & Bamberg, E. (1975) in *Membranes. 3. Lipid bilayers and biological membranes: dynamic properties* (Eisenman, G., Ed.) pp 215–253, Marcel Dekker, New York.

Schobert, B., & Lanyi, J. K. (1982) *J. Biol. Chem. 257*, 10306–10313.

Schobert, B., Lanyi, J. K., & Cragoe, E. J. (1983) *J. Biol. Chem. 258*, 15158–15164.

Steiner, M., & Oesterhelt, D. (1983) *EMBO J. 2*, 1379–1385.

Steiner, M., Oesterhelt, D., Ariki, M., & Lanyi, J. K. (1983) *J. Biol. Chem. 259*, 2179–2184.

Taylor, M. E., Bogomolni, R. A., & Weber, H. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6172–6176.

Wagner, G., Oesterhelt, D., Krippahl, G., & Lanyi, J. K. (1981) *FEBS Lett. 131*, 341–345.

Weber, H. J., & Bogomolni, R. A. (1981) *Photochem. Photobiol. 33*, 601–608.