

## Temperature Dependence of Light-Induced Proton Movement in Reconstituted Purple Membrane

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Received June 20, 1983, and in revised form October 3, 1983

Bacteriorhodopsin (BR) was incorporated into phosphatidylcholine (PC) vesicles containing different amounts of other lipids. Under the conditions of nullified membrane potential, light-induced proton movement seemed to follow a kinetic scheme which assumed the existence of a proton-pumping inhibition process characterized by a rate constant,  $k_I$ . The temperature dependence of both  $k_I$  and the membrane proton leak rate constant ( $k_D$ ) obeyed a simple Arrhenius equation. The presence of cholesterol in the membrane significantly increased the activation energy ( $E_a$ ) of both the inhibition and leak process. However, further addition of phosphatidic acid (PA) suppressed the increase of  $E_a$  associated with  $k_I$ . The initial proton pumping rate ( $R_0$ ) of vesicles reconstituted with PC showed a bell-shaped temperature dependence with a maximum at  $\sim 20^\circ\text{C}$ . The addition of cholesterol abolished this dependence. These results suggest that the molecular origin of the inhibition process characterized by  $k_I$  is different from that of  $R_0$  or  $k_D$ . The temperature dependence of the steady-state fluorescence polarization of dansylated bacteriorhodopsin in vesicles was also investigated. The polarization of the labels in the vesicles without cholesterol showed a bell-shaped temperature dependence with a maximum at  $\sim 20^\circ\text{C}$ . However, in the presence of cholesterol, the polarization increased linearly as temperature decreased. A comparison of these results with the observed proton movement in similarly reconstituted systems with unmodified protein indicates that membranes with a low fluidity and negatively charged surfaces enhance proton pumping efficiency of bacteriorhodopsin.

Bacteriorhodopsin in the purple membrane of *Halobacterium halobium* functions as a light-driven proton pump which transports protons from the cytoplasmic to the exterior medium of the cell membrane (1). This movement generates a transmembranous proton electrochemical potential ( $\Delta\mu_{\text{H}^+}$ ). It is generally accepted that the proton-pumping process is initiated by the photochemical events of the retinal group in the protein (2). However, the exact coupling mechanism between the photochemical cycle and proton translocation is still unresolved (3).

Bacteriorhodopsin has been incorporated into phospholipid vesicles (4, 5). The reconstituted systems exhibit a light-induced proton pumping. This activity is influenced by the nature of the phospholipid used (6, 7), the method employed in reconstitution (8), the pH of the medium (9, 10), and the metal ion composition of the assaying solution (11). However, only semiquantitative effects were discussed in these reports.

A quantitative procedure based on mosaic nonequilibrium thermodynamics has been developed to account for the proton flux of reconstituted bacteriorhodopsin (12, 13). In this model, the active proton pumping, the membrane proton leakage, and the leakage of other ions are thought to be independent of each other, except for their

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mutual influence exerted through the membrane potential and ion gradients. Thus, the active proton pumping may be decreased by the generated electrochemical potential through a back-pressure effect. This decrease, however, may also be accounted for by pump slipping which represents a certain number of failures of molecular events that result in no transmembranous proton movement in a loosely coupled system (14, 15).

Recently, we have described the effects of a number of factors affecting the kinetics of proton translocation in reconstituted systems (16-19). The kinetic scheme employed for analysis was originally developed to account for the proton movements in broken chloroplast systems (20), and, more recently, for mitochondrial particles (21). In this procedure, an additional leak, presumably through an ATPase complex in chloroplast or mitochondrial membranes, induced by energization processes, was assumed to account for the observed decrease of the apparent rates of proton uptake or extrusion. Empirically, this kinetic model also provided an excellent fit to the experimental data obtained in our reconstituted bacteriorhodopsin systems.

In the present investigation, we have studied the temperature dependence of the kinetics of proton pumping and the rotational freedom of bacteriorhodopsin in vesicles with different lipid compositions. The results support our previous claim (19) that the coupling between proton pumping and its associated inhibition process is indirect in nature. The data also indicate that membranes with low fluidity and negatively charged surfaces enhance proton pumping efficiency of bacteriorhodopsin in reconstituted systems.

#### MATERIALS AND METHODS

*Isolation and reconstitution.* Purple membrane containing bacteriorhodopsin was isolated from laboratory cultured *Halobacterium halobium* R<sub>1</sub> according to a published procedure (22). The amount and purity of the protein were established as previously mentioned (16).

Bacteriorhodopsin was incorporated into sonicated phospholipid vesicles by the octylglucoside dilution method of Racker *et al.* (5). To a 4-ml solution (75

mM KCl, 1 mM Hepes,<sup>3</sup> pH 8.0) containing 2 mg of bacteriorhodopsin and 100 mg of octylglucoside, an equal volume of sonicated vesicles (120 mg lipids, turbidity = 0.1 absorbance at 600 nm) in the same solution was added slowly with constant stirring. The mixture was incubated overnight at 0°C in the dark, and was then diluted 20 times in volume with 150 mM KCl. The reconstituted bacteriorhodopsin vesicles were collected by centrifugation at 100,000*g* for 1 h at 0°C. The concentration of the protein in reconstituted vesicles was determined by absorbance measurements in the presence of 0.1% Triton X-100 (to minimize scattering). A vesicle suspension containing ~50 μg of bacteriorhodopsin was added to a 2.2-ml aliquot of 150 mM KCl, pH 5.2, for the measurement of proton movement in the presence of 5 μg of valinomycin.

*Dansylation of bacteriorhodopsin.* Dansylation of bacteriorhodopsin was performed according to the procedure of Harris *et al.* (23). Purified purple membrane containing 10 mg of bacteriorhodopsin was suspended in 67 ml of 20 mM phosphate buffer, pH 8.0. To this solution, a volume of 0.8 ml of 20 mM dansyl chloride in ethanol was added. The reaction was allowed to proceed for 18 h at 0°C in the dark. The modified membrane was collected and then washed with 0.1 M NaCl by centrifugation (48,000*g*) and resuspension three times. The washed membrane was finally resuspended in 0.15 M KCl. The extent of incorporation of dansyl group per mole of bacteriorhodopsin was determined by difference spectroscopy, using a molar extinction coefficient of 5000 at 330 nm (24). The reconstitution of dansyl-labeled bacteriorhodopsin was accomplished by the same method used for unmodified protein.

*Proton movement of reconstituted vesicles.* The light-induced proton movement of reconstituted vesicles was measured by the pH changes of the medium as described in a previous report (18). A 750-W slide projector equipped with proper shutter and filter systems was used as the light source. The temperature of the suspension was maintained by circulating constant-temperature water through a Gibson-Medical oxygen chamber with a thermo jacket.

As discussed in our previous communication (19), the kinetics of the proton movement under the conditions of nullified membrane potential (excess valinomycin + K<sup>+</sup>) may be satisfactorily described by a set of simple equations. The growth phase of proton uptake obeys the equation

$$\ln(1 - \Delta/\Delta_s) = -(k_I + k_D)t, \quad [1]$$

<sup>3</sup> Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; CHOL, cholesterol; PA, phosphatidic acid; BR, bacteriorhodopsin.

where  $\Delta$  and  $\Delta_s$  are the extent of the uptake at the time  $t$  after illumination and at the steady state, respectively. Assuming that the nonspecific proton leakage of the membrane ( $k_D$ ) remains the same under illumination, the additional inhibition to proton uptake in the light stage may be represented by the rate constant  $k_I$ . The release of protons in the dark stage follows the decay equation

$$\ln(\Delta/\Delta_s) = -k_D t, \quad [2]$$

where  $\Delta$  represents the remaining uptake at time  $t$  after the termination of illumination. The initial proton-pumping rate,  $R_0$ , may be determined either directly from the limiting slope of the pH vs time curve, or indirectly from the steady-state approximation:

$$R_0 = (k_I + k_D)\Delta_s \quad [3]$$

However, the direct measurements of  $R_0$  may be affected by delay of electrode response (19, 20). The study of the effect of light intensity on the proton movement of a reconstituted system with definite composition shows that  $k_D$  is independent of light intensity and  $k_I$  and  $R_0$  are related by

$$k_I = mR_0, \quad [4]$$

in which  $m$  may be regarded as a regulatory constant expressing the coupling between light-activated proton-pumping inhibition,  $k_I$ , and initial proton-pumping rate,  $R_0$  (16, 17). The direct analysis of the growth curve usually only gives the sum of  $k_I$  and  $k_D$ . This sum, expressed as  $k_L$ , may be regarded as the total retardation to the apparent rate of proton pumping under illumination.

**Fluorescence polarization of labeled bacteriorhodopsin.** Upon excitation at 340 nm, dansylated bacteriorhodopsin emits fluorescence at 510 nm. The steady-state fluorescence polarization was determined from the values of fluorescent intensity polarized parallel ( $I_{||}$ ) and perpendicular ( $I_{\perp}$ ) to a vertically polarized excitation beam. A homemade fluorometer which consists of two Schoeffel GM 250 monochrometers, an Oriel 6137 150-W Xenon light source, a Keithley 610 B electrometer, a temperature-controlled cell holder, and a proper polarization arrangement was used for fluorescence measurements.

**Chemicals.** Dansyl chloride, valinomycin, cholesterol ( $\Delta^5$ -cholesten-3-ol), egg yolk L- $\alpha$ -phosphatidic acid, egg yolk L- $\alpha$ -phosphatidylcholine, and dipalmitoylphosphatidylcholine were obtained from Sigma Company.<sup>4</sup> Octylglucoside was purchased from Calbiochem Company. All other reagents used were of analytical grade.

<sup>4</sup> Reference to brand or firm name does not constitute endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

## RESULTS

Under the conditions of nullified membrane potential (excess of valinomycin + K<sup>+</sup>), light-induced proton movement of reconstituted bacteriorhodopsin (BR) vesicles with different lipid compositions were measured in the temperature range of 5 to 30°C and analyzed by the kinetic scheme mentioned under Materials and Methods. The proton leak rate constant ( $k_D$ ) and the total inhibition to proton uptake ( $k_L$ ) as well as the extent of translocation ( $\Delta_s$ ) were determined directly from the decay and the growth curves, respectively. The light-activated inhibition rate constant ( $k_I$ ) was then calculated from the difference between  $k_L$  and  $k_D$ . This analysis was performed for the protein reconstituted in egg yolk phosphatidylcholine (PC), in PC + dipalmitoylphosphatidylcholine (DPPC), in PC + cholesterol (CHOL), in PC + phosphatidic acid (PA), and in PC + CHOL + PA vesicles. The effects of temperature on the kinetic parameters are shown in Fig. 1.

*Initial proton pumping rate ( $R_0$ ).* The initial proton-pumping rate of reconstituted systems, in general, increases as temper-

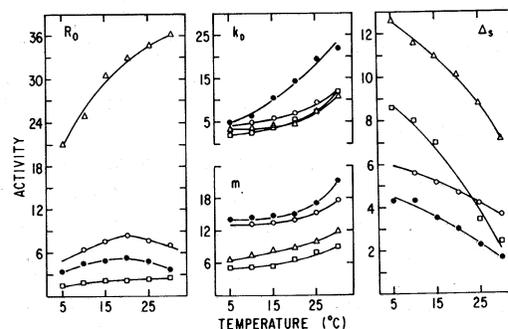


FIG. 1. Temperature dependence of kinetic parameters. Bacteriorhodopsin was reconstituted in pure egg yolk PC vesicles (●), in PC vesicles containing 10% cholesterol by weight (□), in PC vesicles containing 5% cholesterol and 10% PA by weight (△), and in PC vesicles containing 10% PA by weight (○). The proton movement of reconstituted systems at different temperatures were analyzed according to the kinetic model mentioned. The units used to express  $R_0$ ,  $k_D$ ,  $m$ , and  $\Delta_s$  are nmol H<sup>+</sup>/mg protein/min; 10<sup>-3</sup> s<sup>-1</sup>; 10<sup>-3</sup> mg/nmol H<sup>+</sup>; and nmol H<sup>+</sup>/mg protein, respectively.

ature is raised from 5 to 20°C. Above 20°C, the  $R_0$  values of PC and PC + PA systems decrease as temperature increased further. However, this bell-shaped dependence is abolished by the presence of cholesterol in reconstituted vesicles. In agreement with our previous observation (17), the presence of PA does increase  $R_0$  of PC vesicles. However, addition of CHOL in PC vesicles decreases the initial pumping rate. Yet, when both PA and CHOL are present, maximum stimulation of  $R_0$  is obtained.

It has been reported that bacteriorhodopsin exhibits a temperature-dependent aggregation in DPPC and DMPC (dimyristoylphosphatidylcholine) vesicles (25). At temperatures below the lipid-phase transition, bacteriorhodopsin appears to form patches with the same hexagonal lattice observed in native purple membrane (26). Above the phase transition, the lattice disaggregates and the protein molecules are monomeric provided the molar ratio of lipid to protein is greater than 100 (27). The aggregation of BR is mainly determined by a concentration-dependent protein-protein interaction which also affects the extent of light adaptation (28). Thus, it is conceivable that the state of aggregation may influence the  $H^+$ -pumping of BR. However, in the present study, BR was reconstituted in the presence of a large excess of lipid (molar ratio BR:lipid  $\simeq$  2000). Furthermore, the major phospholipids used (PC, PA) have an invariant phase in the temperature range of present study (29). Thus, the observed temperature effects may not be accounted for by the possible monomer-aggregate changes of bacteriorhodopsin. In agreement with our previous observation (17), characteristic circular dichroism of aggregated bacteriorhodopsin was not observed in the present systems (data not shown).

Although the photochemical events of the retinal group in bacteriorhodopsin is undoubtedly providing the primary initiation of proton translocation, the actual pumping rate may also be controlled by the events associated with a transmembranous proton-conduction pathway. Detailed structural models for proton-conduction chains in bacteriorhodopsin have

been proposed (30). The operation mechanism of such arrangements have also been discussed in a general sense (31). It is expected that the activity of a transmembranous structure should be affected by its environment. The effects on  $R_0$  mentioned in Fig. 1 and in previous reports (18, 19) are consistent with the presence of such a structure in functional bacteriorhodopsin.

*Pump inhibition and proton leakage.* The relationship between the inhibition process expressed by  $k_I$  and  $R_0$  may be represented by Eq. [4]. According to this equation, the response of the inhibition to proton pumping is measured by the ratio  $m$ . As shown in Fig. 1, the presence of PA and/or CHOL in PC vesicles decreases the value of  $m$  at all the temperatures tested. Furthermore, the decrease caused by CHOL in the membrane is much more pronounced than that induced by the presence of PA.

We have reported that for a given system,  $m$  is independent of light intensity (16, 17). On the other hand, under constant illumination,  $m$  is affected by changes in environmental factors, e.g., pH, monovalent cations, lipid composition (18, 19). From Fig. 1, it is also clear that  $m$  increases with increased temperature. These observations are consistent with the hypothesis that the inhibition process is only indirectly linked to the mechanism of proton pumping ( $R_0$ ) (16–19).

It is thought that the proton leakage of reconstituted vesicles measured by  $k_D$  is independent of light, but dependent on lipid composition (17, 22). The data shown in Fig. 1 demonstrate that  $k_D$  is indeed dependent on the lipid composition of the membrane. Like other rate constants ( $k_I$  and  $k_L$ ),  $k_D$  also increases as temperature increased. The temperature effect on rate constant for a given reconstituted system was found to obey a simple Arrhenius equation. A typical example of this relationship is shown in Fig. 2. From Arrhenius plots, the activation energies of the inhibition process and proton leakage through the membrane in reconstituted systems were determined. The results are summarized in Table I. It is interesting to note that the activation energy ( $E_a$ ) of the inhibition process is always considerably

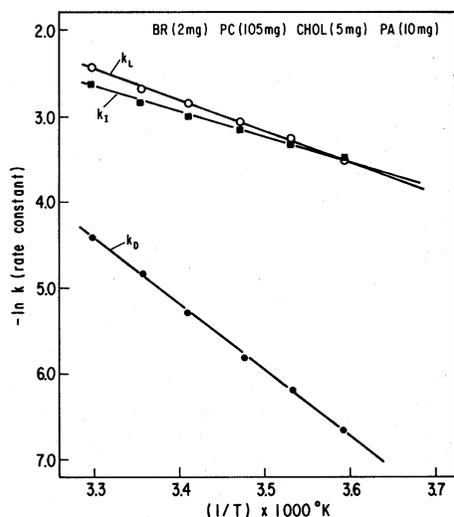


FIG. 2. Arrhenius plot of rate constants. The rate constants  $k_L$  and  $k_D$  of reconstituted bacteriorhodopsin in PC vesicles containing 5% cholesterol and 10% PA by weight were determined directly from recorded pH changes of the medium at different temperatures. The inhibition rate constant  $k_I$  was calculated from the difference between  $k_L$  and  $k_D$ . Similar plots were obtained with other reconstituted systems mentioned in Table I.

lower than that of the proton leakage. The presence of cholesterol tends to increase the activation energy of both processes. The incorporation of PA in PC vesicles decreases the activation energy of the inhibition process without any significant effect on that of leak process. However, the addition of PA to CHOL-containing PC vesicles enhances the effect of CHOL on the  $E_a$  of the leak process, but suppresses its effect on the  $E_a$  of the inhibition process. Clearly, the molecular origins of these two processes are quite different. We have proposed that the inhibition is associated with the protein part in reconstituted systems (16–19). Since bacteriorhodopsin is known to span through the whole membrane, it is expected that the protein-associated inhibition process will be affected by the lipid environment.

**Effects on extent of proton translocation.** According to the kinetic scheme used in the present study, the creation of a sizable  $\Delta_s$  is favored by a faster initial pumping rate ( $R_0$ ), a smaller proton leakage of the

membrane ( $k_D$ ), and a lesser response of the inhibition mechanism to the pumping event ( $m$ ). Based on these criteria, the temperature dependence of factors contributing to the establishment of  $\Delta_s$  in reconstituted systems were analyzed. The observed  $\Delta_s$  of reconstituted systems are shown in Fig. 1. In general,  $\Delta_s$  decreased as temperature increased. For bacteriorhodopsin reconstituted in PC vesicles, the decrease of  $\Delta_s$  is mainly due to the increase of  $k_D$  at higher temperatures. Above 20°C, the decrease of  $R_0$  and the increase in  $m$  also contribute to the observed changes of  $\Delta_s$ . In this system, a change of temperature from 5 to 30°C caused a 63% reduction in observed  $\Delta_s$  (from 430 to 160 nmol H<sup>+</sup>/mg). With PA also present in PC vesicles, a reduction of about 40% in  $\Delta_s$  was observed in the same temperature range. Presumably, the higher  $R_0$ , the lower  $k_D$ , and the smaller response of the inhibition are responsible for the observed changes. In the case of CHOL + PC system, the substantially higher  $\Delta_s$  observed at low temperature must be primarily due to the lower values of  $m$  and  $k_D$ . The relatively rapid increase of  $k_D$  compared to the other kinetic parameters in this system caused a more pronounced reduction (~73%) in  $\Delta_s$  by a change from 5 to 30°C. With both PA and CHOL present in reconstituted PC system, the enhancement of  $R_0$  causes  $\Delta_s$  to decrease moderately in the temperature range (~44%).

TABLE I  
EFFECTS OF COMPOSITION ON  $E_a$

| Composition <sup>a</sup> | kJ mol <sup>-1</sup>  |                      |
|--------------------------|-----------------------|----------------------|
|                          | Proton leak ( $k_D$ ) | Inhibition ( $k_I$ ) |
| PC                       | 42.2                  | 17.0                 |
| PC + 10% PA              | 42.0                  | 12.0                 |
| PC + 5% CHOL             | 48.7                  | 21.2                 |
| PC + 10% CHOL            | 55.1                  | 32.4                 |
| PC + 5% CHOL<br>+ 10% PA | 68.7                  | 22.9                 |

<sup>a</sup> Weight ratio of lipids to protein = 60 during reconstitution.

Although the addition of cholesterol to PC vesicles generates a sizable increase of  $\Delta_s$ , the time needed to reach a steady state is considerably longer than in other reconstituted systems under similar experimental conditions. For example, it took about 30 and 40 s for PC vesicles containing 5% cholesterol to reach 50% of  $\Delta_s$  at 25 and 10°C, respectively. These times were about 22 and 30 s for other systems mentioned in Fig. 1. Since the intensity of the actinic light was kept constant, the observed effects on  $\Delta_s$  indicate the efficiency of light-induced proton pumping is significantly enhanced by the presence of both PA and CHOL in the reconstituted PC systems.

*Fluorescence polarization of dansylated bacteriorhodopsin.* It has been shown that cholesterol has a dual effect on fluidity of phospholipid bilayers. The ordered array of acyl chains in the gel phase is fluidized by the addition of cholesterol, whereas in the liquid-crystalline phase of membranes cholesterol reduces the fluidity (32). Biological membranes, as well as liposomes made up of unsaturated native phospholipids, exhibit increased microviscosity at lower temperature without a distinctive phase transition of the bilayers (33). The incorporation of cholesterol in these systems increases the viscosity and decreases the flow activation energy of the lipid layer. However, it does not have any effect on the phase-transition property of these systems. It is expected that similar effects of cholesterol should be operative in reconstituted bacteriorhodopsin systems.

In Fig. 3, we summarized the effects of temperature on fluorescence polarization ( $P$ ) which is defined as  $(I_{\parallel} - I_{\perp}) / (I_{\parallel} + I_{\perp})$  of dansylated bacteriorhodopsin in reconstituted systems. The polarization of modified protein in PC vesicles exhibits a bell-shaped temperature dependence. The addition of PA decreases the sensitivity of the polarization to temperature changes, but the bell-shaped dependence is maintained. The replacement of part of the PC in vesicles with cholesterol abolishes the bell-shaped dependence and the further addition of PA decreases the rate of change in polarization with respect to variation in temperature. A change in polarization re-

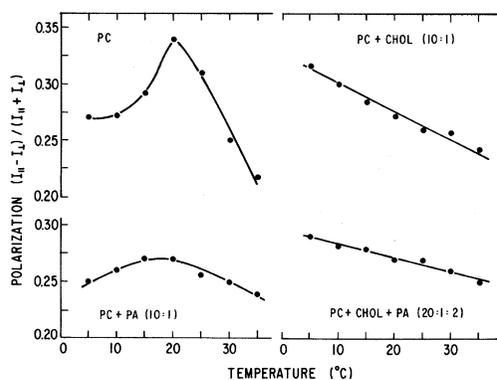


FIG. 3. Temperature dependence of fluorescence polarization of dansylated bacteriorhodopsin in reconstituted systems. Bacteriorhodopsin containing 1.7 dansyl groups per protein molecule was reconstituted into vesicles with lipid composition as mentioned in Fig. 1. The concentration of vesicles used for fluorescence measurements was identical to that used for proton movement experiments.

flects a modification of the molecular motion in the vicinity of the dansyl labels (34). Such a change may also be induced by the association of labeled proteins (35, 36). Since the experimental conditions employed in the present study (high lipid-to-protein ratio) are favorable to the existence of monomeric bacteriorhodopsin in the membrane, the possible complication caused by the aggregation of protein may be disregarded. It should be mentioned that labeled bacteriorhodopsin, before reconstitution, exhibits a rather low fluorescence polarization ( $\sim 0.02$ ) which remains constant over the temperature range of 5 to 35°C.

The bell-shaped temperature dependence of fluorescence polarization suggests a change in the interaction between the protein and its lipid environment at about 20°C. A decreasing of fluorescence polarization below this temperature suggests that the protein gains motional freedom at lower temperatures. The origin of this rather unusual phenomenon is unknown at the present time. The presence of negatively charged surfaces decreases, but does not abolish this tendency. The linear relationship observed in the presence of cholesterol suggests that bacteriorhodop-

sin interacts rather strongly with an ordered lipid environment.

Dansylation of bacteriorhodopsin in purple membrane under the experimental conditions yields a labeled product with  $\sim 2$  dansyl groups located in a region confined between amino acid residues 33 and 56, and has no effect on its proton-pumping activity (23). From the known amino acid sequence of bacteriorhodopsin (37) and the location of retinal binding site (Lys<sub>216</sub>) in the protein (38), it is concluded that both Lys<sub>40</sub> and Lys<sub>41</sub> are probably labeled with dansyl groups. The arrangement of the protein peptide chain in the membrane indicates that the labeled region is positioned at the interface between membrane and its aqueous surroundings (39).

#### DISCUSSION

The kinetic model employed here to describe the proton movement of reconstituted bacteriorhodopsin is consistent with the assumption that there is a light-induced inhibition of proton pumping and that the inhibition must represent a molecular process of bacteriorhodopsin as it responds to the illumination and to the presence of a transmembranous nonequilibrium distribution of protons. Furthermore, this inhibition process is apparently linked to the pumping process, and the quantity  $m$  may be taken as a measure of this link. The observed photoinduced changes in the quantum yield of BR due to transmembrane electrochemical potential and internal and external pH effects in *H. halobium* cells are also consistent with this assumption (40).

In an attempt to provide a structural basis for the kinetic model, we have presented a hypothesis that bacteriorhodopsin consists of two functional domains (17, 19). Light absorption by retinal causes domain I to undergo a series of conformational changes which result in the transmembranous proton pumping characterized by  $R_0$ . The changes in domain I may also propagate, through a link segment, to domain II to activate a series of rearrangements which lead to the inhibition process by modifying the environment of the pump

domain. This modification leads to a decrease in the rate of active proton pumping by either pump "slipping" (14, 15) or a "back pressure" effect (12, 13). It should be mentioned that the possibility of rearranging domain II to provide a mechanism for conducting proton flow in the opposite direction of pumping is also consistent with the experimental results. In any event, the proposed link between domains I and II is indirect, and each domain has sufficient conformational flexibility to accommodate changes in the environment. Thus,  $R_0$  and  $k_I$  can be altered in a different manner (16-19).

In the present study, we have shown that  $R_0$  and  $k_I$  respond differently to the changes in temperature in reconstituted systems. This is evident from the observed temperature dependence of the link between pumping and inhibition as measured by  $m$ . The results reported in this study also support our claim that both domain I and II have sufficient flexibility to accommodate the changes in environment. An increase in the rigidity of lipid phase by the presence of cholesterol causes a substantial decrease in the proton leakage ( $k_D$ ) and a decrease in the response of inhibition ( $k_I$ ) to the proton pumping ( $R_0$ ). These two changes may, in principle, enhance the efficiency of light-induced proton translocation in the reconstituted systems, if  $R_0$  is not inhibited. Unfortunately, the presence of cholesterol also causes a severe reduction in  $R_0$ . The further addition of negatively charged surfaces provided by PA to cholesterol-containing vesicles not only maintains the advantage generated by a rather rigid lipid phase, but also creates a proper environment to substantially enhance the pumping rate. Thus, a large and sustained transmembranous proton gradient is generated in a relatively short period of time. According to our functional domain hypothesis of bacteriorhodopsin, the present data suggest that domain I must span the whole membrane and that domain II as well as the link mechanism are likely to reside mainly in the middle section of the protein.

Bacterial purple membrane is known to contain a high percentage of bis-dihydro-

phytyl ether analogs of phosphatidylglycerol phosphate which are all negatively charged (41). Thus, our present study suggests that an ordered lipid interior and the negatively charged surfaces found in native membrane may be essential for bacteriorhodopsin to function as an efficient proton pump to convert light energy into a transmembranous electrochemical potential for the metabolic needs of *H. halobium*.

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