EFFECTS OF PHOSPHOLIPID COMPOSITION ON ACTIVITIES OF BACTERIORHODOPSIN IN RECONSTITUTED PURPLE MEMBRANE

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1. Introduction

Bacteriorhodopsin in the cell membrane of halobacteria functions as a proton pump to convert light energy into a transmembrane electrochemical potential [1,2]. The protein has been incorporated into phospholipid vesicles which exhibit the characteristic proton-pumping activity [3,4]. We have shown [5] that the kinetics of proton movement in the purple membrane reconstituted into egg yolk phosphatidylcholine (PC) conforms to the pattern found in broken chloroplasts [6]. The effects of lysine modification by fluorescamine on the kinetic parameters of the reconstituted system suggest that there is a light-triggered (on–off) proton leak pathway which is related to, but physically different from, the proton pumping mechanism in the protein [5]. Here we are concerned with the role of the phospholipid in the pumping activity of the reconstituted purple membrane.

The lipid portion of native purple membrane is consisted of ~85% of a bis-dihydrophytyl ether analog of phosphatidylglycerophosphate (PGP) [7]. The possible correlation between this high content of a negatively charged lipid and the proton pump of the native purple membrane has not been investigated. We have now incorporated bacteriorhodopsin into PC vesicles containing increasing amounts of phospholipids with different polar head groups. The kinetic parameters of the proton pumping in the reconstituted systems were analyzed as in [5,6]. The data show that the presence of negatively charged polar head groups in the bilayer increases the efficiency of proton pumping by enhancing the pumping rate and decreasing the leak rate constants. These results suggest that the linkage between proton pumping during the primary photochemical cycle and its associated leak pathway may be adjusted by negative charges on the surface of the membrane.

2. Materials and methods

The procedure in [8] was followed to harvest the *Halobacterium halobium* R_1, and to isolate its bacteriorhodopsin. The purity and the concentration of the protein were established by SDS–polyacrylamide gel electrophoresis and absorbance at 560 nm, respectively, as in [5]. Bacteriorhodopsin was incorporated into sonicated phospholipid vesicles by the octylglucoside dilution method [4]. To a 2 ml solution (75 mM KCl, 1 mM Hepes (pH 8.0)) containing 2 mg bacteriorhodopsin and 2.5% octylglucoside, an equal volume of sonicated suspension of phospholipids (120 mg) in the same solution was added slowly with constant stirring. The mixture was incubated in the dark at 0°C overnight, and diluted 20 times in volume with 150 mM KCl. The reconstituted purple membrane was centrifuged at 100 000 × *g* for 45 min. The resulting pellet was resuspended in 150 mM KCl to proper concentration before assaying.

The kinetics of light-induced proton movement was monitored at pH 5 and analyzed as in [5]. Excellent fittings (correlation coefficients >99.9%) were obtained by assuming that the growth phase of proton movement obeys the equation $\ln(1 - \Delta/\Delta_s) = -k_L t$ (A), where Δ and Δ_s are the extent of proton uptake at time t of illumination and at the steady state, respectively, while k_L is the rate constant for proton leak under illumination. The release of protons in the dark stage follows the decay equation $\ln(\Delta/\Delta_s) = -k_D t$ (B), where k_D is a light-independent decay rate constant. The initial proton pumping rate, R_o, is obtained from the steady-state equation

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$R_o = k_L \Delta_s$ (C). For a reconstituted system with definite composition, both R_o and '$k_L - k_D$' increase with increased light intensity. However, the relationship, $k_L = k_D + mR_o$ (D), in which 'm' may be regarded as a regulatory constant indicating the linkage between the proton pumping (R_o) and the light-triggered leak pathway ($k_L - k_D$) remains unchanged.

The proton permeability of the lipid vesicles of various compositions was tested by rapid injection of successive volumes of standard HCl to the vesicle suspension. The decay of the proton concentration in the medium was followed under conditions comparable to those employed with the reconstituted systems. The proton permeability of the pure PC and the modified PC vesicles proved to be virtually identical.

L-α-Phosphatidylcholine (type III), L-α-phosphatidylethanolamine (type III), L-α-phosphatidic acid (Na-salt) from egg yolk, and bovine heart cardiolipin were obtained from Sigma. All other reagents were of highest obtainable purity. The circular dichroism spectra were obtained with a JASCO J-41C Automatic Recording CD Spectrometer equipped with a data processing attachment.

3. Results

3.1. *Effects of phospholipid composition on the kinetic parameters of proton movement*

The light-induced proton movement, with the membrane potential nullified by valinomycin and K^+, obeys the kinetic equations in section 2, in all the reconstituted purple membrane systems included here.

The incorporation of phosphatidylethanolamine (PE) into sonicated vesicles of (PC) does not alter the observed extent of proton uptake at the steady state (Δ_s, —○—), but causes an increase in the initial proton pumping rate (R_o, —□—) and in the values of k_L and k_D (—△— and —■—, respectively) (fig.1A). A different picture is observed when cardiolipin (CL) or phosphatidic acid (PA) are incorporated into the PC vesicles. Now, both Δ_s and R_o increase, while both k_L and k_D decrease (fig.1B,C). The control experiments show that these changes in membrane permeability are not due to defects introduced when PC is mixed with CL or PA.

3.2. *Interdependence of proton pumping and light-triggered leak*

From equation (D), the linkage between the proton pumping that is associated with the primary photochemical cycle, and the light-triggered leak pathway, can be represented by a constant 'm' which is the ratio of '$k_L - k_D$' over R_o, and is independent of light intensity. Fig.2 shows that the independence of 'm' with respect to light intensity is not altered by incorporation of other phospholipids into PC vesicles. However, the value of 'm' decreases significantly upon incorporation of negatively charged phospholipids, but not of PE, into the PC vesicles. CL has 4 acyl chains and 2 phosphodiester functions, $(RO)_2PO_2^-$, per molecule. PA has 2 acyl chains and 1 phosphomonoester function, $ROPO_3H^- \rightleftharpoons ROPO_3^{2-} + H^+$, per molecule. In fig.3, we plotted observed values of 'm' as a function of increased mole fractions of phospholipids in PC vesicles. CL and PA are lipids of quite different structure and would not be expected to result in similar perturbations of the lipid bilayer. The analogous effects of these compounds on 'm' suggest that negative charges on the head groups exert a dominant role in adjusting the linkage between the primary proton pumping and the light-triggered leak pathway.

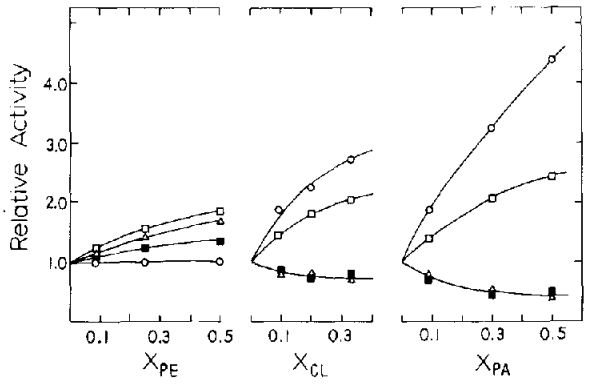

Fig.1. Effects of phospholipid composition on the kinetic parameters of proton pumping in reconstituted purple membrane. Sonicated vesicles containing egg yolk phosphatidylcholine (PC), and PC plus the indicated mole fractions of phosphatidylethanolamine (PE), cardiolipin (CL) or phosphatidic acid (PA) were employed in the reconstitution. The parameters with pure PC under a given constant light intensity had the values: Δ_s = 245 nmol H^+/mg, k_L = 0.0410 s^{-1}, k_D = 0.0124 s^{-1} and R_o = 603 nmol H^+/mg . min. These values were arbitrarily set at 1.0 for comparisons with values obtained with the modified PC vesicles. Effects of added PE, CL and PA on Δ_s (—○—), k_L (—△—), k_D (—■—) and R_o (—□—) are shown.

3.3. *State of aggregation of bacteriorhodopsin under different conditions*

Monomeric bacteriorhodopsin and its aggregates of

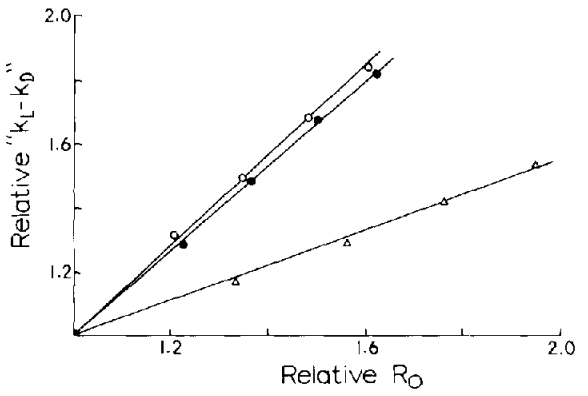

Fig.2. Effects of light intensity of the link between '$k_L - k_D$' and R_O in reconstituted purple membrane. The correlations between '$k_L - k_D$' and R_O under a given light intensity at the sample (~80 J . m^{-2} . s^{-1}) were arbitrarily set as 1.0. Effects of higher light intensities (105, 135, 170 and 195 J . m^{-2} . s^{-1}) are shown for vesicles of PC (–○–), of PC plus PE (– ●–, X_{PE} = 0.25) and of PC plus CL (–△–, X_{CL} = 0.20).

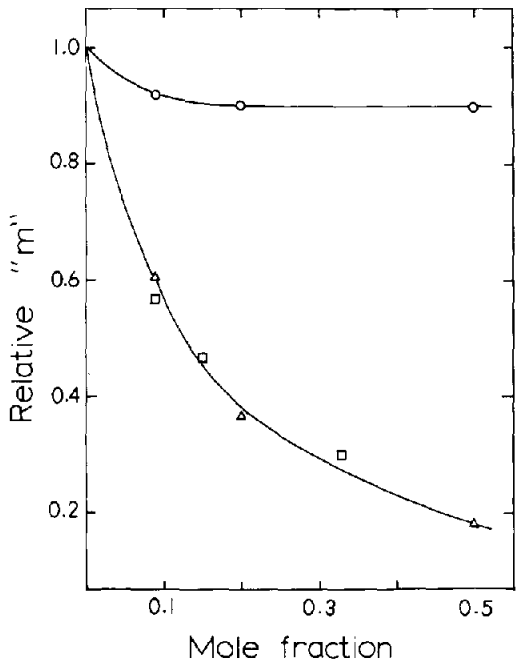

Fig.3. Effects of phospholipid composition on the link between '$k_L - k_D$' and R_O. At a fixed light intensity the values of the correlation 'm' were determined for vesicles of PC containing increasing mole fractions of PE (–○–), CL (–□–) or PA (–△–), and these values of 'm' were compared to that for vesicles of pure PC set as 1.0.

higher order (hexagonal lattice) can be distinguished by circular dichroism measurements [9,10]. The hexagonal lattice structure of the protein in isolated purple membrane exhibits exciton bands with a crossover occurring at 574 nm, while the monomeric form in detergent dispersion shows only a positive CD around its absorption maximum at 567 nm. Bacteriorhodopsin changes from lattice to monomeric state in vesicles prepared from synthetic lipids using Triton X-100 dialysis technique [11], and it has been suggested that the monomers can pump protons [12].

To explore possible effects of lipid composition on bacteriorhodopsin conformation in the reconstituted purple membrane, we have studied the CD spectra of the various systems mentioned above. We utilize a high lipid/protein ratio (~2000:1), and native lipids of low transition temperatures, which should favor the monomeric state of bacteriorhodopsin in the reconstituted purple membrane. Fig.4 discloses that the system involving CL incorporated into PC vesicles, which exhibits pronounced proton pumping activity, shows no detectable circular dichroism spectrum in the 450–650 nm region. The same observation has been made for the other systems that show pumping activity, with pure or mixed PC vesicles.

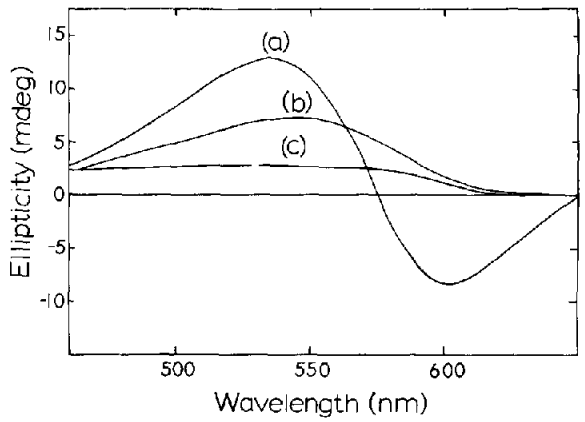

Fig.4. Circular dichroism spectra of bacteriorhodopsin under different conditions. Effects are shown for the isolated purple membrane in: (a) water (hexagonal lattice); (b) 2.5% octyl glucoside solution (monomer); and (c) vesicle of PC + CL in a 3:1 molar ratio. The CD spectra of bacteriorhodopsin in other functionally active reconstituted vesicles are similar to (C). Bacteriorhodopsin concentration, 0.8 mg/ml; light pathlength, 0.1 dm; spectra were obtained after repetitive scanning by microprocessor to eliminate random noise. All spectra were obtained at ~24°C.

Significantly, the circular dichroism properties of bacteriorhodopsin in the amide absorption region remain unchanged in these systems (not shown).

4. Discussion

Evidence for the interaction between PGP head group and bacteriorhodopsin in native purple membrane has been obtained from ^{31}P NMR studies [13]. Conformational changes in bacteriorhodopsin induced by electric-fields have just been reported [14]. These interesting findings do not provide information on the consequences for proton pumping of charge-induced protein–lipid interactions.

These results, as well as [5], are consistent with the following hypothesis.

We assume that bacteriorhodopsin consists of two segments:

I, where conformational changes in protein driven by light absorption result in the primary transmembrane proton movement (pumping);

II, where conformational changes in the protein result in proton movement in the opposite transmembrane direction (inherent leak).

The primary changes in conformation in segment I are transmitted to segment II by changes in conformation of a link segment. Hence, pumping rate (R_o) controls inherent leak ($k_L - k_D$), and 'm' is the measure of this control. It is possible that electrostatic interactions between negatively charged head groups in the bilayer and positively changed groups in the protein partly affect the ability of the link segment and of segments I and II to undergo the conformational changes associated with pumping activities. Minor effects on the non-specific leak (k_D) are also expected in this picture.

It may be that a more efficient light energy converter is attained in the presence of negatively charged head groups in the bilayer, which would account in physiological terms of the relatively high content of the PGP analog found in native purple membrane.

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