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PHOSPHOLIPID SUBSTITUTION OF THE PURPLE MEMBRANE

THE STOICHIOMETRY OF LIGHT-INDUCED PROTON RELEASE BY PHOSPHOLIPID-SUBSTITUTED PURPLE MEMBRANES

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Summary

The method of Warren et al. (1974, Proc. Natl. Acad. Sci. U.S. 71, 622—626) was employed to substitute the polar lipids of the purple membrane of *Halobacterium halobium* by different phosphatidylcholine species. Substitution at pH 6.5 yields proteolipid complexes in the form of bent open sheets which have a protein to lipid phosphorus ratio similar to the natural membrane, i.e. about 1 : 10 (mol/mol). The extent of substitution increases with the length of the fatty acid chain of the phosphatidylcholine used.

The spectral properties of bacteriorhodopsin are only slightly affected by substitution of 95% of the lipid, except that the photocycle is slowed down appreciably. Due to this slow rate the M_{412} intermediate of the cycle accumulates in the light. Associated with this accumulation is a net light-induced proton release, which proved insensitive to uncoupler. A comparison between the net proton release and the amount of M_{412} accumulated, studied as a function of pH, shows that no fixed stoichiometry exists between the two processes.

Phospholipid substitution by egg phosphatidylcholine at pH 7.5 or by egg phosphatidylethanolamine leads to preparations of purple membrane with 15 or 25 mol of phospholipid per mol of bacteriorhodopsin, respectively. These preparations seem to consist of closed membrane structures. They take up protons in the light in an uncoupler-sensitive way.

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Abbreviation: SF 6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidene malononitrile.

Introduction

The purple membrane is synthesized by extremely halophilic bacteria like *Halobacterium halobium* under conditions of minimal oxygen tension and strong illumination [1]. It can easily be isolated as membrane sheets by differential centrifugation of the cell lysate and purification on a sucrose gradient [2,3]. Purple membrane contains only one protein, bacteriorhodopsin. This is responsible for the colour, which is attributable to retinal covalently linked to a lysine residue of the protein [3,4]. Bacteriorhodopsin functions in the intact cell as a light-driven proton pump [5,6], enabling the cell to maintain an electrochemical potential gradient of protons ($\Delta\tilde{\mu}_H+$) of considerable magnitude in the light [7]. This gradient can be used for energy-consuming processes like ATP synthesis [8—10] or transport of amino acids [11,12].

Like visual rhodopsin, bacteriorhodopsin undergoes a photochemical reaction cycle, although it is not permanently bleached. With its retinal originally in the all-trans form [13], bacteriorhodopsin successively gives rise to the intermediates K, L, M, and O, absorbing light maximally at 590, 550, 412, and 660 nm, respectively, before it returns to its initial form, bR, absorbing at 570 nm [14—17]. Probably the M_{412} intermediate is the last of the cycle, and the O_{660} intermediate [18] is not located in the main cycle [15], since although O_{660} is formed more slowly than M_{412}, it decays at the same rate as M_{412} to bR_{570} [17], and is not observed under conditions where the rate of the photocycle is slowed down [15,17]. The Schiff base between retinal and lysine is protonated in bR_{570} and the K_{590} and L_{550} intermediates, becomes deprotonated upon formation of the M_{412} intermediate, and is reprotonated upon completion of the cycle, as shown by resonance Raman spectroscopy [19], and as can be inferred both from deuteron-isotope effects on the photocycle [20], and from measurements of pH changes in solution [14,21,22]. Recently Lozier et al. [22] presented experimental evidence that, almost concomitant with the bleaching to M_{412}, about one proton is released by the protein per M_{412} species formed.

Bacteriorhodopsin is an extremely rigid protein with a large α-helix content. It spans the purple membrane [23—25], in which it is embedded in a lipid environment mainly of diphytanyl ether analogues of phospho- and glycolipids [26,27], typical of all extremely halophilic bacteria [28]. To study the influence of this particular lipid environment on the properties of bacteriorhodopsin, we applied to the purple membrane the method of lipid substitution as developed by Warren et al. [29] for the sarcoplasmic reticulum ATPase. Substitution of different phosphatidylcholine species resulted in complexes with the same protein to lipid phosphorus ratio as found in the original purple membrane. In such substituted complexes, however, the photochemical reaction cycle was slower. This enabled us to measure the steady-state concentration of the M_{412} intermediate in the light and to compare it with the net light-induced proton release. The stoichiometry between these two processes turned out not to be constant, but to be a function of pH.

Materials and Methods

Purple membranes. Strain R_1 of *H. halobium* was grown purple as described previously [8,7]. The purple membrane was isolated and purified according to the method of Oesterhelt and Stoeckenius [3]. In some experiments purple membrane was used obtained by extensive differential centrifugation of the cell lysate only. Such preparations turned out to be impure, both with regard to lipid and to protein content, compared with purple membrane purified on a sucrose gradient (see below, Table I).

The concentration of bacteriorhodopsin in suspensions of purple membrane was determined spectroscopically, assuming a molar extinction coefficient of 63 000 $M^{-1} \cdot cm^{-1}$ at 570 nm [30]. The light minus dark differential molar extinction coefficient of M_{412} ($\Delta E = E_{M_{412}} - E_{bR_{570}}$) was assumed to be 27 000 $M^{-1} \cdot cm^{-1}$ at 412 nm [14]. The same value was obtained by a combination of data from refs. 14 and 17, i.e. 0.5 ms after bleaching (before O_{660} formation) the ratio of the value of ΔE at 570 and 412 nm, respectively, equals —2.0 [17], and the values of ΔE equals —54 000 $M^{-1} \cdot cm^{-1}$ at 570 nm [14]. The amount of protein in the purple membrane was determined according to the method of Lowry et al. [31], with bovine albumin as a standard. The phosphorus content of the purple membrane was determined according to the micro method of Chen et al. [32]. For analysis of the polar lipids, the lipids were extracted from known microamounts of purple membrane [33,26] and fixed quantities of the extract were applied to Merck DC-Fertigplatten. Polar lipids were then separated by thin-layer chromatography using a solvent mixture (by volume) of chloroform/methanol/water in the ratio 65 : 35 : 4 [34], and were detected with a cupric phosphoric acid spray [35]. Phospholipids were detected with an acid molybdate spray [36], glycolipids with an orcinol sulfuric acid spray [37].

Phospholipids and other materials. L-Dilauroyl, L-dimyristoyl, and L-dipalmitoyl phosphatidylcholine were obtained from Calbiochem. Egg phosphatidylcholine was either purified on a silicic acid column (Biosil A, 200—325 mesh, Biorad Laboratories) [38] or an aluminium oxide column (Aktiv, neutral, Aktivitätsstufe I, Merck) [39], or was purchased as grade I from Lipid Products, U.K. The three preparations gave identical results, provided that no column material or neutral lipid material was present after the purification on the respective columns. Egg phosphatidylethanolamine was purified on a silicic acid column [38,40]. Sodium cholate was obtained from Sigma and purified by treatment of the salt with active charcoal (B.D.H.) and recrystallization of the acid from 70% (by volume) ethanol [38]. The compound 3,5-di-*tert*-butyl-4-hydroxybenzylidene malononitrile (SF 6847) was a gift from Dr. Y. Nishizawa, Sumimoto Chemical Industry, Osaka (Japan). Gramicidin was obtained from Calbiochem.

Phospholipid substitution procedure. The method of the substitution of the polar lipids of the purple membrane for different phosphatidylcholine species or for egg phosphatidylethanolamine was essentially as described by Warren et al. [29] for the sarcoplasmic reticulum ATPase. 5—10 mg of the purple membrane (containing 200—400 nmol of bacteriorhodopsin plus 1—2 mg of polar lipids) were incubated for 1 h at room temperature with 25 mg

of the particular lipid, 100 mM Tris · HCl buffer, 150 mM KCl, and 30—60 mg sodium cholate in a medium of 3 ml (final pH 6.5). The mixture was layered on a linear 20—60% sucrose gradient and was centrifuged for 16 h at 100 000 × *g*. The purple band was collected, washed with 150 mM KCl, and the substitution procedure repeated for 1 h with only one half the amount of cholate present as compared with the first incubation. After purification on the sucrose gradient, the purple band was collected, washed with 150 mM KCl, and dialyzed against 150 mM KCl for 24 h during which time the medium was replaced twice. In some cases a prolonged incubation (2—3 h) at higher pH (7.5) was employed for the substitution (see below).

Extent of lipid substitution. The extent of lipid substitution in the different preparations of unknown lipid composition was estimated from the intensities and areas of the spots on the thin-layer chromatogram of the diphytanyl ether analogues of phosphatidylglycerol phosphate and the main glycolipid compound present, which are the two major polar lipids of the purple membrane [26—28]. Since it is impossible to obtain absolute data on lipid composition according to this method, we delipidated equal amounts of unknown and native purple membrane, and applied the following relative quantities to the plates: 1.0 parts of the former, and 0.1, 0.3 and 1.0 parts of the latter, respectively. The above-mentioned parameters of the unknown were then compared with those of the native purple membrane series.

Preparation of samples for electron microscopy. The method of Stoeckenius and Rowen [1] for preparation of samples of the purple membrane was modified for conditions of lower salt concentration. The different purple membrane preparations present after the dialysis step in 150 mM KCl (see above) were spun down. The pellets were covered with a 4% formaldehyde solution in 150 mM KCl and incubated overnight at 4°C. They were then washed twice with 150 mM KCl and exposed for 1 h at room temperature to a solution either containing 2% OsO_4, 150 mM KCl and 100 mM Tris · HCl, final pH 7.0, or to a solution containing 2% $KMnO_4$ and 150 mM KCl, pH 6.5. After two washings the cells were post-stained for 30 min with a 2% aqueous solution of uranyl nitrate. The samples were washed, dehydrated with acetone, and embedded in Epon. Sections were cut with glass knives and post-stained on grids with uranyl acetate and lead citrate.

Spectroscopic measurements. A Cary 1605 spectrophotometer equipped with a low light-scattering cuvette house and a device for side illumination of the sample cuvette was used for recording of ultraviolet/visible spectra of the purple membrane preparations. Steady-state concentrations of the M_{412} intermediate of bacteriorhodopsin in the light were measured with side-illumination of the cuvette using light from a 24 V, 150 W slide projector passed through a Corning 3-69 cut-off filter. The photomultiplier was protected from this light by a combination of Corning 7-59 and 4-96 filters. Intensities of the illuminating light were measured with a Y.S.I.-Kettering model 65A radiometer. The maximal light intensity within the cuvette was 600 W/m^2. Light intensity was varied with neutral filters of defined light transmission.

pH measurements. The pH of suspensions of the different purple membrane preparations in a 3 ml thermostat vessel was measured with a Radiometer GK 2312C combined glass electrode connected to a Radiometer PHM 64 pH

meter. The signal was fed to a Varian A 65 recorder. Changes in pH observed in the vessel were transformed to absolute changes by calibrating the scale of the recorder by addition of known microliter amounts of an approx. 1 mM HCl solution. The concentration of the latter solution was determined exactly by measuring its pH value at 20°C, after previously calibrating the pH measuring system at pH 4.0 and pH 7.02 (two-buffer method). The half time of response of the combined pH measuring system was estimated to be 0.8 s. The vessel was illuminated by means of a 24 V, 150 W slide projector. The light was passed through a 3-69 Corning cut-off filter to minimize light effects on the electrode. The maximal light intensity obtainable within the cuvette was 1100 W/m^2. Light intensity was varied by using neutral filters of defined light transmission.

Results

Substitution of the polar lipids of the purple membrane

The analytical data of different purple membrane preparations in which the lipids were substituted twice for different phosphatidylcholine species, or once for egg phosphatidylethanolamine are summarized in Table I. The extent of lipid substitution was estimated from the intensity of spots 1 and 4 on thin-layer plates (Fig. 1). These two spots correspond to the main phospholipid analogue, phosphatidylglycerol phosphate, and the main glycolipid of the purple membrane, respectively. These lipids can be separated satisfactorily from phosphatidylethanolamine, but sometimes a small overlap is observed between spot 1 and phosphatidylcholine (spot 7, Fig. 1). Nevertheless, the extent of substitution can be estimated roughly (Table I, column 1). The data given are those of the purple membrane polar lipids which have been exchanged as a percentage of the total polar lipids originally present in the same amount of native purple membrane. The accuracy of the exchange data in Table I is about 10%. No preferential substitution of either phospho- or glycolipid analogues from the purple membrane could be observed. Incubations of 1 h with different species of phosphatidylcholine at pH 6.5 yield purple membrane preparations with approximately the same quantity of lipid-phosphorus molecules/mol of bacteriorhodopsin as the original purple membrane (Table I). From the known composition of the polar lipids of the purple membrane [26—28] it was calculated that a ratio of about 10 mol of lipid phosphorus/mol of bacteriorhodopsin in the native membrane corresponds to about 7.4 mol of polar lipid/mol of bacteriorhodopsin.

The extent of lipid substitution increases with increasing fatty acid chain length of the phosphatidylcholine species. With egg phosphatidylcholine and dipalmitoyl phosphatidylcholine the extent of lipid substitution was found to be 95 and 90%, respectively. These numbers are still lower than the 99% substitution that one would expect from a statistical lipid distribution during the two substitution procedures. Apparently, the stiff nature of the purple membrane and the strong protein-protein interactions in it [23—25] make lipid substitution more difficult than in the case of the sarcoplasmic reticulum ATPase, where statistical substitution does occur [29]. The poor substitution for shorter chain phosphatidylcholine species might be because such

TABLE I

PROPERTIES OF PHOSPHOLIPID-SUBSTITUTED PURPLE MEMBRANES

For details of formation and for analytical methods see Materials and Methods.

Preparation of purple membrane (native or lipid-substituted as indicated)	Percent exchange of original polar lipids	Lipid phosphorus content (mol/mol of bacteriorhodopsin) ***	Percent sucrose (w/v) at which preparation bands	A_{280nm}/A_{565nm}	Apparent molecular weight (mg protein/mmol bacteriorhodopsin)	Membrane structure (according to electron microscopy, Fig. 2, see text)	Light-induced proton movement (Figs. 4 and 8)
Native membrane	—	10 (2)	48	1.71	25 000	flat sheets	release
Dilauroyl phosphatidylcholine	70	9 (1)	47	1.85	24 000	bent open sheets	release
Dimyristoyl phosphatidylcholine	80	10—11 (3)	47	1.89	24 000	bent open sheets	release
Dipalmitoyl phosphatidylcholine	90	9—11 (3)	47—45	2.00	22 500	bent open sheets	release
Egg phosphatidylcholine	95	11--12 (3)	45	2.20	23 000	tendency to closed membranes	release
Egg phosphatidylcholine *	95	15—17 (2)	37	2.30	26 500	cylindrical vesicles	uptake
Egg phosphatidylethanolamine **	80	25—30 (3)	30—35	2.62	28 000	spherical vesicles	uptake
Native membrane (sucrose-gradient step omitted)	—	12—15 (3)	—	1.95—2.10	38 000—53 000	not tested	release or uptake
Cbolate-treated native membrane	—	6 (2)	52	1.90	25 000	flat sheets	release

* Obtained by 2 h incubation at pH 7.5.

** The native lipids were only exchanged once in this preparation, as compared with twice in the other lipid-substituted preparations.

*** Values between brackets refer to the number of different preparations of which the lipid phosphorus content was determined.

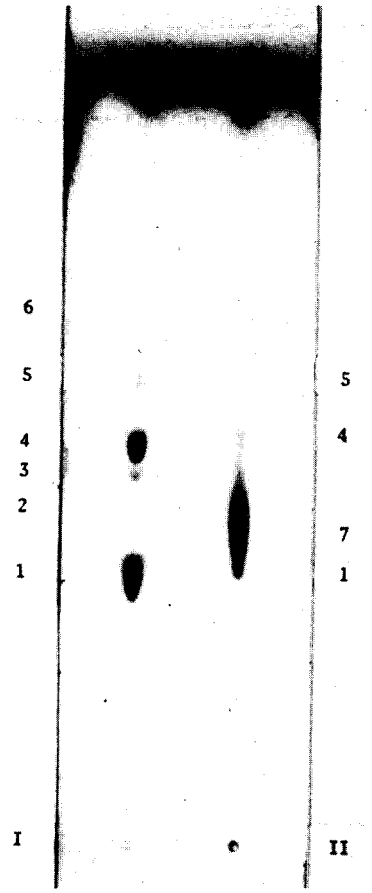

Fig. 1. Thin-layer chromatogram of the polar lipids of: (I) purple membrane and (II) egg phosphatidylcholine-purple membrane. Membranes with equal amounts of bacteriorhodopsin were delipidated (see Materials and Methods). The plate was sprayed with the cupric-phosphoric-acid spray [35]. Spots 1 and 3 could be identified as phospholipids; spots 4 and 5 as glycolipids. Spot 7 is phosphatidylcholine.

species are too short to provide the membrane span of 45 Å needed by bacteriorhodopsin [25].

Incubations with egg phosphatidylcholine at higher pH (7.5) or with egg phosphatidylethanolamine increased the total number of phospholipid molecules/mol of bacteriorhodopsin, without improving the extent of lipid substitution (Table I). The increase in phospholipid content was accompanied by some formation of a compound of bacteriorhodopsin absorbing maximally around 470 nm. This compound might be identical to that observed by Oesterhelt et al. [13] under different conditions. At incubations at higher pH values than 6.5 not all of the 470 compound formation reversed upon removal of excess cholate and phospholipids (see also Lozier et al. [22]). Table I includes data on the lipid-phosphorus content of purple membrane not purified on a sucrose gradient, and on purple membrane treated twice with cholate The former preparation is still impure, but the latter is partially delipidated in comparison to sucrose gradient purified purple membrane.

The ratio of absorbances of the purple membrane at 280 and 570 nm is used as a criterion for the purity of the preparation [3,41]. This parameter is given, for the different preparations, in Table I, column 4. However, one has to assume that the contribution of light scattering to the absorbance is constant for the different preparations in order to make valid use of this parameter. This is clearly not the case, since the size and form of the membranes vary from preparation to preparation (see below). Therefore, we measured an apparent molecular weight for each preparation, defined as the protein present (in mg) [31] per mol of bacteriorhodopsin, determined by using a molar extinction coefficient of 63 000 at 565 nm (Table I, column 5). This parameter has a fairly constant value for the different sucrose gradient-purified preparations of purple membrane. The variation in numbers between different preparations, rather than representing real differences, is caused by the inaccuracy of the method of protein determination (>10%).

Except for the above-mentioned permanent formation of the 470 compound of bacteriorhodopsin in egg phosphatidylcholine-purple membrane where substitution was carried out at high pH values, the absorption spectrum of bacteriorhodopsin in the different complexes is hardly influenced by the exchange procedure. Only a slight shift of the absorption maximum of the light-adapted form towards lower wavelengths (565 nm) is observed. It is assumed that the molar extinction coefficient of bacteriorhodopsin at the absorption maximum is in all cases 63 000 $M^{-1} \cdot cm^{-1}$. This assumption seems to be justified by the fact that for most complexes formed a constant ratio of protein to bacteriorhodopsin was found (Table I, column 5). All the different purple membrane preparations still have a light-adapted form (due to all-*trans*-retinal [13]) and a dark-adapted form (partially due to 13-*cis*-retinal [13]). At room temperature the half time of decay from the light-adapted to the dark-adapted form was about 1 h for all preparations except the dipalmitoyl phosphatidylcholine-purple membrane, where it was almost twice as long. The rate of the photochemical reaction cycle of bacteriorhodopsin in the different phospholipid-substituted preparations was slowed down. Values at room temperature for the half time of decay of the M_{412} intermediate were found to lie between 30 and 80 ms, compared to 4 ms for that of the normal membrane [17]. No O_{660} intermediate of the photocycle could be detected in phospholipid-substituted purple membranes (Sherman, W.V. and Bakker, E.P., unpublished observations). This confirms that this intermediate is not observed under conditions where the photocycle is slowed down artificially [15,17].

Electron microscopy of the phospholipid-substituted purple membrane

Originally we followed the procedure of Stoeckenius and Rowen [1] and used 2% $KMnO_4$ to stain the different preparations of the purple membrane. However, with this reagent satisfactory staining was obtained with the native membrane and cholate-treated membranes only. Better staining was obtained with 2% OsO_4. Even with this reagent, however, it was hard to stain membranes with a high content of saturated lipids and a low content of glycolipids.

The flat sheet-like structure of the native purple membrane [1,41] is not lost in the cholate-treated preparation (Fig. 2A). But the size of the sheets may be smaller in the latter, and they may be somewhat more bent. In the

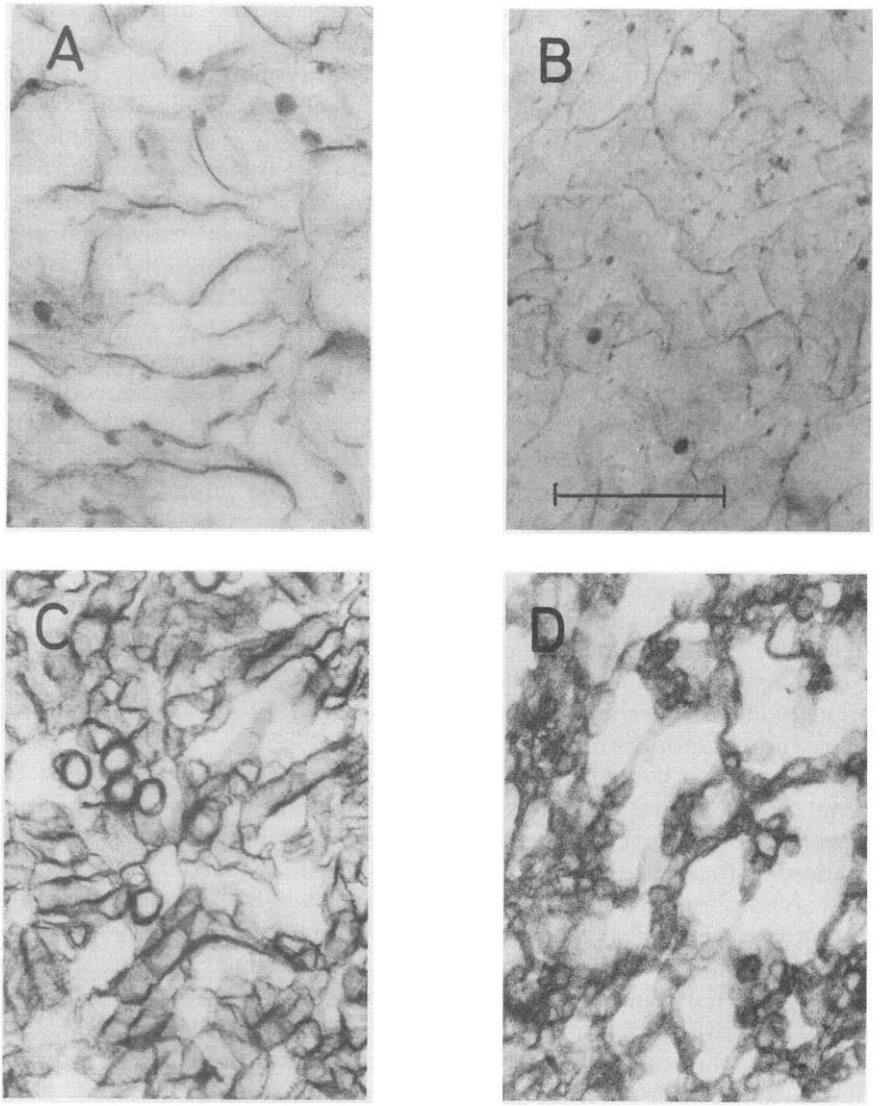

Fig. 2. Electron micrographs of thin sections of different phospholipid-substituted purple membranes. For preparation of the samples, see Materials and Methods. (A) Cholate-treated purple membrane, (B) Dilauroyl phosphatidylcholine-purple membrane, (C) Egg phosphatidylcholine-purple membrane, formed at pH 7.5, (D) Egg phosphatidylethanolamine-purple membrane. Magnification 44 000; length of bar 1000 Å. These preparations have a molar ratio of phospholipid to bacteriorhodopsin of 6, 9, 15, and 25, respectively.

dilauroyl phosphatidylcholine-purple membrane a tendency to form bent sheets is more obvious (Fig. 2B). Similar structures were observed in preparations of the purple membrane which contain dimyristoyl phosphatidylcholine or dipalmitoyl phosphatidylcholine. These three preparations all contain about 10 mol of polar lipids/mol of bacteriorhodopsin (Table I). The egg phosphatidylcholine-purple membrane with a value for this ratio of about 12 (Table I) consists of structures resembling closed membranes (not shown). The surfaces

TABLE II

THE STOICHIOMETRY BETWEEN LIGHT-INDUCED PROTON RELEASE AND M_{412} FORMATION BY DIFFERENT PHOSPHOLIPID-SUBSTITUTED PURPLE MEMBRANES UNDER CONDITIONS OF CONTINUOUS ILLUMINATION

20—50 nmol of bacteriorhodopsin were present in a medium of 3 ml containing 150 mM KCl., final pH 6.5—6.7. Temperature 23—25°C. Light intensity 500 W/m^2. For further details, see legend to Fig. 6.

Preparation of purple membrane	Addition	Proton release (H^+/bacteriorhodopsin in mol/mol)	M_{412} (as a mol % of total bacteriorhodopsin)	Stoichiometry (H^+ released/M_{412} formed in mol/mol)
Dipalmitoylphosphatidylcholine	None	0.40	60	0.66
	SF 6847 (10^{-5} M)	0.38	60	0.63
	gramicidin (10 μg/ml)	0.37	60	0.61
Dimyristoyl phosphatidylcholine	None	0.16	28	0.57
Dimyristoyl phosphatidylcholine	None	0.20	35	0.57
Egg phosphatidylcholine	None	0.14	30	0.47
	SF 6847 (10^{-5} M)	0.13	30	0.43
Cholate-treated native membrane	None	0.11	13	0.84

of these structures are not smooth, however, and many angles can be observed. Since such preparations release protons in an uncoupler-insensitive way (see below, Table II), it was concluded that their membranes are not really closed. The egg phosphotidylcholine-purple membrane preparation containing 15 mol of phospholipid/mol of bacteriorhodopsin (Table I) shows an ultrastructure of closed cylindrical vesicles (Fig. 2C). Egg phosphatidylethanolamine-purple membrane with a corresponding ratio of 25 (Table I) has a closed spherical structure, typical of small liposomes. The vesicle diameter of this preparation seems to be about several hundred Å.

In order to rule out the possibility that the differences in appearance of the different preparations were caused by inhomogeneities within the preparation, several parts of the different pellets were cut and examined. No major differences could be observed within one preparation. Moreover, it should be kept in mind that the different preparations, except for the egg phosphatidylethanolamine-purple membrane, are homogeneous to begin with, since they form a narrow band in a sucrose gradient (Table I). Therefore, it seems reasonable to conclude that the differences observed in the appearance of the different preparations (Fig. 2) are real.

Light-induced proton release by membranes with a low lipid content

The different purple membrane preparations with a molar ratio of phospholipid to bacteriorhodopsin of about 10 or less release protons upon illumination. This is shown for the dipalmitoyl phosphatidylcholine-purple membrane in Fig. 3. At pH 6.7 in the absence of buffer and with the suspension in open contact with air, overshoots are observed both in light-induced proton release and in dark-induced proton uptake (cf. ref. 30). We conclude that such overshoots are due to the relarively slow approach to equilibrium of the reac-

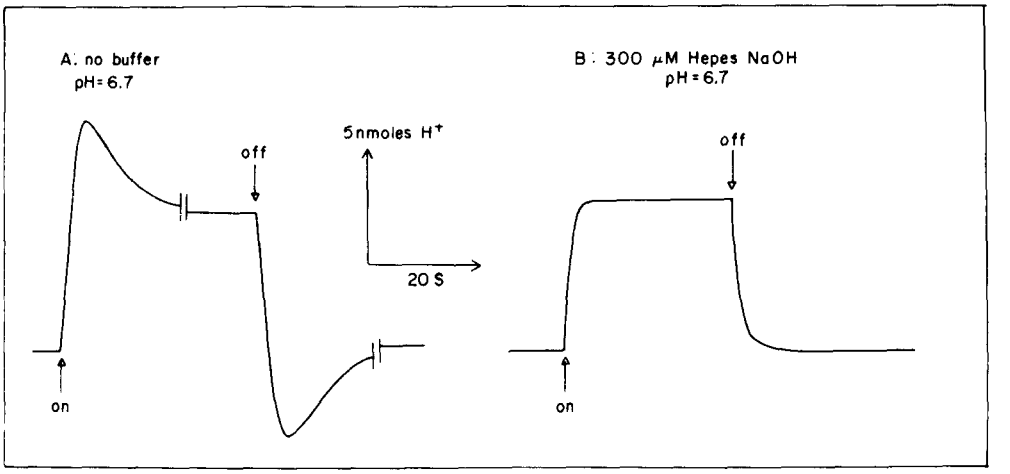

Fig. 3. Light-induced proton release by dipalmitoyl phosphatidylcholine-purple membrane. 18 nmol of bacteriorhodopsin were present in a medium of 3 ml containg 150 mM KCl (A), or 150 mM KCl plus 300 mM HEPES/NaOH buffer, final pH 6.7 (B). The suspension was illuminated with light of intensity 1000 W/m^2.

tion $H_2CO_3 \rightleftharpoons HCO_3^- + H^+$ ($pK_a = 6.37$ at 25°C), since (a) overshoots are also observed upon addition of acid or base to the suspension, but are absent in weakly buffered suspensions (Fig. 3B) or when CO_2 was removed from the suspension (not shown); (b) overshoots are absent above pH 7.5 and below pH 5.5; and (c) the steady-state values are independent of the amount of buffer present (Fig. 3) *. The rate constants of the light-induced proton release and the dark-induced proton uptake are too fast to measure with our present system ($t_{1/2}$ = 0.8 s in buffered suspensions, Fig. 3B).

In Fig. 4, the net light-induced proton release of the different preparations is given as a function of temperature. In all cases a negative temperature coefficient is observed, i.e. at low temperatures more protons are released than at higher ones. At the light intensity applied (approx. 1000 W/m^2, see Materials and Methods) and at pH 6.6, the dipalmitoyl phosphatidylcholine-purple membrane releases the largest quantity of protons (0.5 proton/mol of bacteriorhodopsin at low temperature). The other phospholipid-substituted preparations and the cholate-treated purple membrane release less (all maximally 0.3 proton/mol of bacteriorhodopsin). Untreated purple membrane only releases maximally 0.07 proton/mol of bacteriorhodopsin.

The M_{412} intermediate and light-induced proton release

The negative temperature dependence of net light-induced proton release by different preparations (Fig. 4) suggests a relationship between this process and the deprotonation step of the Schiff base of bacteriorhodopsin upon formation of the M_{412} intermediate [19], since the steady-state concentration

* After completion of this work we became aware of the fact that Scholes and Mitchell [42] described a similar CO_2 artifact with a half-time of about 20 s. These authors state that addition of carbonic anhydrase at 40 μg/ml avoids such artifacts.

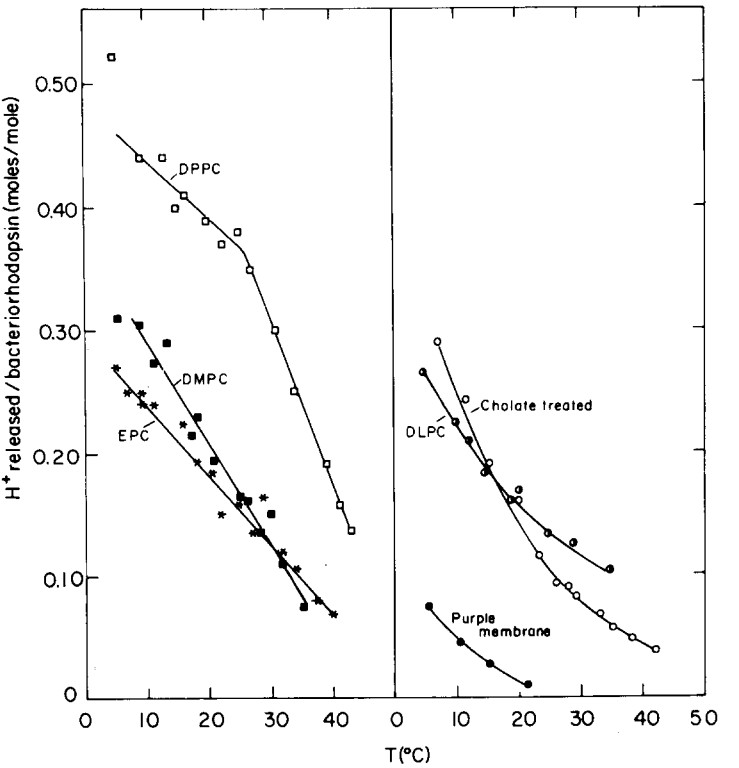

Fig. 4. Light-induced proton release by phospholipid-substituted purple membrane as a function of temperature. 20—50 nmol of bacteriorhodopsin were present in 3 ml medium containing 150 mM KCl, final pH 6.4—6.6. Light intensity 1100 W/m^2. □———□, dipalmitoyl phosphatidylcholine-purple membrane; ■———■, dimyristoyl phosphatidylcholine-purple membrane; ◑———◑, dilauroyl phosphatidylcholine-purple membrane; *———*, egg phosphatidylcholine-purple membrane; ○———○, cholate-treated purple membrane; ●———●, native purple membrane.

of the intermediate in the light also has a negative temperature coefficient (being a function of its rate of formation and decay). It is shown in Fig. 5 that in the light at pH 6.5, in a preparation of dipalmitoyl phosphatidylcholine-purple membrane, measurable concentrations of the M_{412} intermediate indeed exist, as characterized by an absorption maximum at about 410 nm and an isosbestic point at about 455 nm in the difference spectrum of illuminated versus non-illuminated phosphatidylcholine-purple membrane [14,15,17]. In Fig. 6 it can be seen that the net light-induced proton release by dipalmitoyl phosphatidylcholine-purple membrane and the steady-state concentration of the M_{412} intermediate in the light have a very similar dependence on the light intensity. However, the stoichiometry between the two processes is only 0.59 mol H^+ released/mol M_{412} formed. For all preparations tested, this stoichiometry seems to have a value of less than one at pH 6.6 and is hardly affected by uncoupler or gramicidin (Table II). The latter observation rules out the unlikely possiblity (according to the electron microscopy data, see Table I and Fig. 2) that part of the protons released in the light are retained inside closed vesicles. The pH dependence of the stoichiometry between proton release in the light and M_{412} intermediate formation is given in Fig. 7

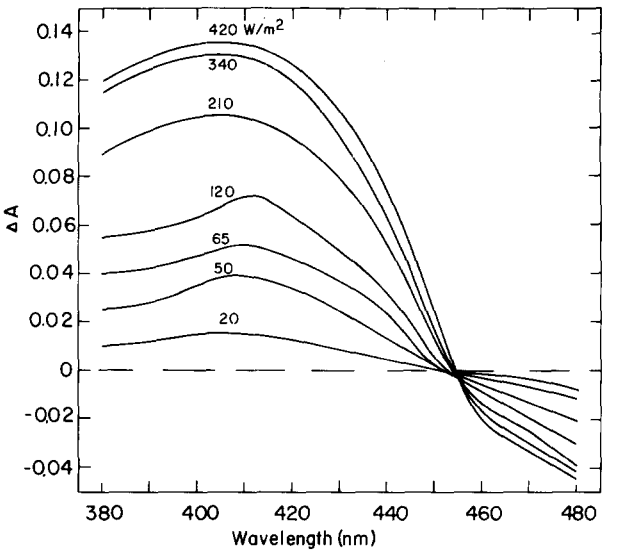

Fig. 5. Spectrum of the M_{412} intermediate under steady-state illumination conditions at different light intensities. A cuvette containing dipalmitoyl phosphatidylcholine-purple membrane (31 nmol bacteriorhodopsin) and 450 μmol KCl in a volume of 3 ml, final pH 6.5, was illuminated from the side with light of the intensity indicated. The spectra given are difference spectra between illuminated and non-illuminated samples. For further details, see spectroscopic measurements (Materials and Methods). Temperature 23°C.

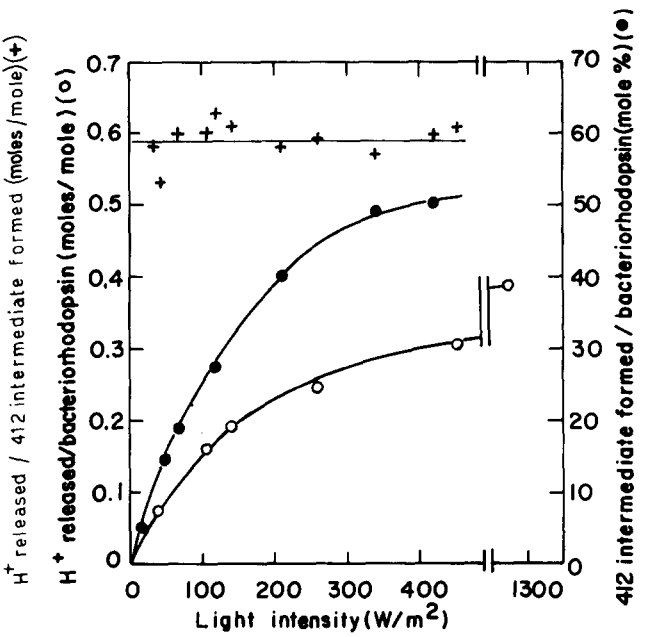

Fig. 6. Relationship between net light-induced proton release and the amount of M_{412} intermediate formed by dipalmitoyl phosphatidylcholine-purple membrane as a function of light intensity. Net proton release was measured at pH 6.5 and at $T = 23°C$, as described in the legend to Fig. 3A), except that 31 nmol of bacteriorhodopsin were present and the light intensity was varied as indicated (o———o). In a parallel experiment with the same suspension the net light-induced formation of the M_{412} intermediate was measured as desribed in the legend to Fig. 5 (●———●). Differential molar extinction coefficient of M_{412} at 412 nm; 27 000 $M^{-1} \cdot cm^{-1}$ [14,17]. Stoichiometries were calculated by relating the experimental points of the net proton release to the curve of the amount of M_{412} intermediate formed at the same light intensity, and the experimental points of M_{412} intermediate formed to the curve of proton release (+———+).

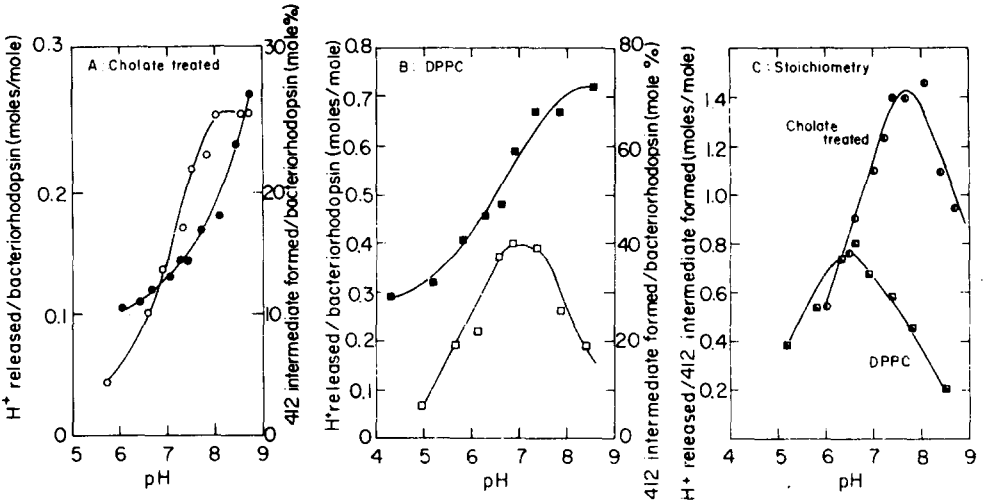

Fig. 7. The pH dependence of the stoichiometry between H^+ release and M_{412} formation. Details as described in the legens to Figs. 3, 5, and 6. Light intensity 500 W/m^2. (A) Cholate-treated purple membrane. (B) Dipalmitoyl phosphatidylcholine-purple membrane. (C) Stoichiometries. Open symbols, proton release; closed symbols, M_{412} formed as a percentage of total bacteriorhodopsin; half-closed symbols, stoichiometry between proton release and M_{412} formation. Circles, cholate-treated purple membrane; squares, dipalmitoyl phosphatidylcholine-purple membrane.

for dipalmitoyl phosphatidylcholine-purple membrane and for cholate-treated purple membrane. The behaviour observed is highly dependent on pH and on the kind of purple membrane examined and suggests that such stoichiometries only have limited meaning. Apparently, part of the protons released upon formation of the M_{412} intermediate are retained by the protein and are not further released into the suspension medium of the purple membrane (see also Discussion).

Light-induced proton uptake by membranes with a high lipid content

Purple membrane with a relatively high lipid content (i.e. the egg phosphatidylcholine-purple membrane formed at pH 7.5, and the egg phosphatidylethanolamine-purple membrane, see Table I), takes up protons from the medium upon illumination (Fig. 8A). Uncoupler reverses this process of proton uptake and gives rise to proton release (Fig. 8B). Apparently, a molar ratio of phospholipid to bacteriorhodopsin of 15 or more is high enough to enable closed vesicles to be formed (Figs. 2C and 2D), and these are able to take up protons upon illumination like liposomes [6]. It is interesting to note that the purple membrane isolated from lysed cells and purified by differential centrifugation only, having a phospholipid to bacteriorhodopsin molar ratio as high as 15 (Table I), also takes up protons upon illumination. Both types of preparation characterised by a ratio of about 15 show fast proton uptake and release only (together with the CO_2 artifacts described earlier) (Fig. 8). However, the egg phosphatidylethanolamine-purple membranes with a ratio of 25 show a biphasic behaviour both in the process of proton uptake and in that of proton release (Fig. 8). Such biphasic behaviour is typical for phospholipid-bacteriorhodopsin liposomes [43]. In a following paper we will show that we can attri-

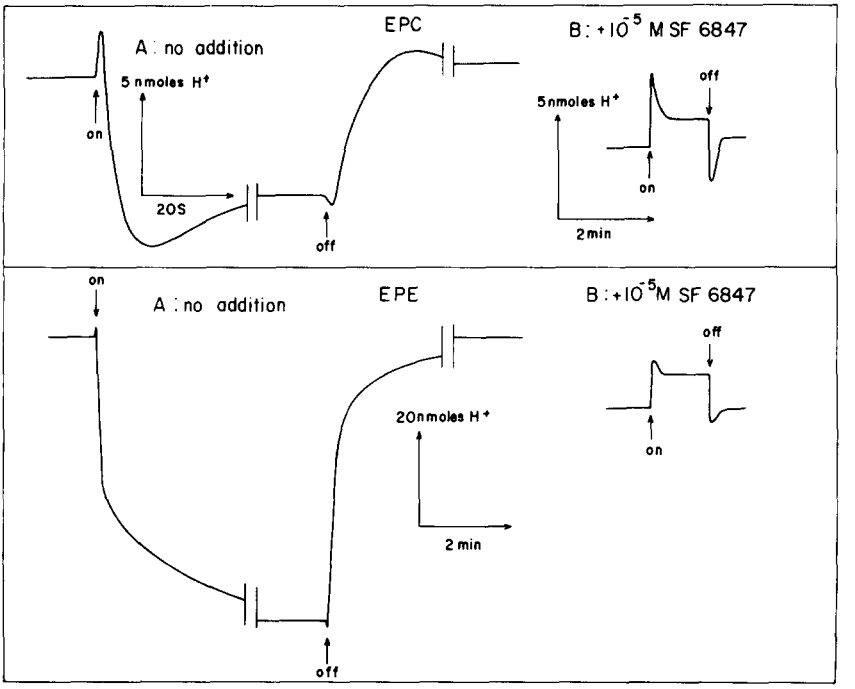

Fig. 8. Light-induced proton uptake by membranes with a high phospholipid content. Upper traces: egg phosphatidylcholine-purple membrane (EPC) formed at pH 7.5. (A) 20 nmol of bacteriorhodopsin in 3.0 ml containing 150 mM KCl, final pH 6.6 (temperature 25°C, light intensity 500 W/m^2). (B) The same in the presence of 10^{-5} M SF 6847. Lower traces: egg phosphatidylethanolamine-purple membrane (EPE). (A) 27 nmol of bacteriorhodopsin in 3.0 ml, final pH 7.0. (B) The same in the presence of 10^{-5} M SF 6847.

bute the fast process to proton binding by the bacteriorhodopsin, and the slow process to proton pumping by this protein across the membrane [44].

Discussion

Our results on phospholipid substitution of the polar lipids of the purple membrane by different lecithin molecules clearly show that the diphytanyl ether analogues of phospho- and glycolipids found in the purple membrane [26—28] are not essential for its function. Membranes with only one half mol of the original lipid present per mol of bacteriorhodopsin (e.g. the egg phosphatidylcholine-purple membrane, Table I) have an absorption band centered around 565 nm, exhibit a light/dark adaptation phenomenon similar to the native purple membrane, and undergo a light-induced photochemical reaction cycle with M_{412} as the accumulating intermediate. However, such lipid substitutions do influence the sheet-like structure of the purple membrane (Fig. 2). Upon increasing the phospholipid to protein ratio of the membrane (Table I), closed liposomes are gradually formed (Fig. 2) that are able to take up protons in the light (Fig. 8). In contrast, membranes with a lower phospholipid to protein ratio release protons, due to M_{412} formation (Tables I and II). A question to be answered in the future with the freeze-etching technique [22] is whether

a preferential orientation of bacteriorhodopsin is already present in bent open membrane structures of low lipid content (Fig. 2B and Table I), and if so whether it is the same as that of membranes with higher lipid content able to form closed vesicles (Fig. 2C, D).

The photochemical reaction cycle of bacteriorhodopsin is slowed down by treatment of the purple membrane with cholate, or with cholate plus substituting phospholipids (Table II). It is not known whether this slowing down is caused by residual cholate still present in the preparations, or by the new lipid environment of bacteriorhodopsin. Dipalmitoyl phosphatidylcholine, however, causes a further slowing down of the cycle (Fig. 4 and Table II) at room temperature and also retards dark adaptation. These effects might be due to the solid state of the fatty acids of dipalmitoyl phosphatidylcholine at room temperature. In pure phospholipid vesicles the fatty acid chains melt at about 38—42°C [45]. The break observed at about 28°C in the temperature dependence of the light-induced proton release by the dipalmitoyl phosphatidylcholine-purple membrane (Fig. 4) might indicate a phase transition of the lipid, lowered by the presence of the protein by as much as 10°C. Similar extensive lowerings of the phase transition in dipalmitoyl phosphatidylcholine-substituted membranes have been reported for sarcoplasmic reticulum ATPase [46] and for β-hydroxybutyrate dehydrogenase [47]. Possibly as a results of its incomplete substitution, we could not observe any phase transition for the dimyristoyl phosphatidylcholine-purple membrane (Table I and Fig. 4).

The rate of the photochemical reaction cycle in the substituted complexes is about the same as that of bacteriorhodopsin in intact cells [48,49]. As in intact cells [49,50], but in contrast with isolated purple membrane (ref. 51, and Sherman, W.V., personal communication), the rate is very pH dependent (Fig. 7). This suggests that studies with lipid-substituted purple membrane, as described in this paper, might be more relevant to studies of bacteriorhodopsin in the intact cell than those with isolated purple membrane.

Recently Lozier et al. [22] showed that almost immediately after formation of the M_{412} intermediate in purple membrane one proton is released to the suspension medium. Their technique of measuring pH changes in solution with an indicator dye has the advantage of being fast, but is limited to pH values close to the pK_a of the indicator. Our technique of measuring net light-induced proton release by the purple membrane is relatively slow, but has no limitation as to pH; CO_2 equilibrium artifacts may be avoided (Fig. 3). We found that the stoichiometry between net proton release and M_{412} formation in the steady state in phospholipid-substituted or partially delipidated purple membrane is not at all constant with pH (Fig. 7). If this observation can be assumed to have a bearing on native purple membrane, it would seem misleading to quote a single value for the stoichiometry, viz. 1.0 [14,22].

Our finding that the stoichiometry might be as high as 1.5 (Fig. 7) is extremely thought-provoking. However, whether this observation is real depends very much on the question of whether the value chosen (27 000 $M^{-1} \cdot cm^{-1}$) was correct for the differential molar extinction coefficient of M_{412} at 412 nm (see Materials and Methods). Other references give data from which values can be calculated of 28 000 [15], 33 000 [30] and even 45 000 [16]. A higher values than 27 000 for this extinction coefficient will lead to a lowering of the

values of the stoichiometry of proton release as given in Figs. 6 and 7. If, on the other hand, it is true that this stoichiometry reaches values higher than 1.0 (Fig. 7), light-induced proton release can no longer be explained as a single deprotonation reaction of the linkage between retinylidene and lysine [19]. One is compelled to assume that protons are released by other groups in the membrane. An explanation for this "extra" proton release might be that in the light the protein undergoes a light-induced conformational change, which leads to downward pK_a shifts involving unidentified groups in the apo-part of the protein. Similarly, one might explain the low values of the stoichiometry of proton release observed at high and low pH values (Fig. 7) by assuming that in these cases, in the light, upward pK_a shifts of unidentified groups occur. It is tempting to suppose that the light-induced pK_a shifts towards higher and lower values which lead to association and dissociation of protons, respectively, occur simultaneously in different parts of the protein located on opposite sides of the membrane. Their functions may be binding of protons on one side of the membrane and release of protons on the other [43]. The photocycling moiety of the bacteriorhodopsin would then be the proton carrier between the two parts of the protein.

After the completion of this work several reports relevant to it were published. Hwang and Stoeckenius [52] described results similar to the ones presented here (Table I) on the treatment of the purple membrane with deoxycholate. Happe and Overath [53] demonstrated that bacteriorhodopsin is completely delipidated when the purple membrane is treated with the cationic detergent dodecyl trimethylammonium bromide; the delipidated enzyme is not irreversibly denatured, since it can be recostituted without loss of function. Garty et al. [54] described the pH dependence of the light-induced net proton release by the unmodified purple membrane. Their results are similar to the ones shown in Fig. 7A, except that these authors lowered the pH further, under which conditions they observed a net light-induced proton uptake by the purple membrane. The explanation of their results is similar to the one given above. Happe et al. [55] found that the direction of proton translocation by bacteriorhodopsin in reconstituted systems is dependent upon the pH during the reconstitution procedure. Their results indicate that the orientation of bacteriorhodopsin obtained in the preparation of reconstituted membranes is pH dependent.

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