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BACTERIORHODOPSIN VESICLES

AN OUTLINE OF THE REQUIREMENTS FOR LIGHT-DEPENDENT H^+ PUMPING

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Summary

A systematic study was performed to determine under which conditions bacteriorhodopsin can be applied as an energy generator in reconstituted systems. It is concluded that reconstitution of an active light-driven proton pump is possible over a wide range of conditions.

High extents (per bacteriorhodopsin molecule) of proton uptake by reconstituted vesicles are found at a high lipid to protein ratio, after long sonication and at high pH. No active proton pump is obtained if reconstitution is attempted at high pH with neutral phospholipids or at low ionic strength with negatively charged lipids. Attention was also paid to the requirement of a crystalline array for active pumping; most likely, monomeric bacteriorhodopsin molecules can effectively pump protons.

Introduction

In the plasma-membrane of some halophilic bacteria, like *Halobacterium halobium*, a purple coloured protein called bacteriorhodopsin [1] is present in the form of a two-dimensional crystal [2]. This protein is involved in a photophosphorylation system present in the halobacteria [3] and it was suggested by Stoeckenius [4] that it functions as a light-driven proton pump. The function of bacteriorhodopsin as a generator of an electrochemical potential gradient ($\Delta\tilde{\mu}_{H^+}$) has now become firmly established on the basis of measurements of light-dependent proton movements in reconstituted systems consisting of

Abbreviation: CD, circular dichroism.

bacteriorhodopsin and lipids only [5—7] and electrical measurements on reconstituted bacteriorhodopsin membranes [8—11].

Bacteriorhodopsin as a $\Delta\tilde{\mu}_{H^+}$ generator can be used for the study of bioenergy-converting systems [12—15]; this has already resulted in valuable information on these energy-converting systems. This application of bacteriorhodopsin can be extended in principle to active transport, energy-linked transhydrogenase etc. For this reason it is important to investigate the conditions under which bacteriorhodopsin can generate a $\Delta\tilde{\mu}_{H^+}$ across the membrane of a reconstituted vesicle.

In vivo, bacteriorhodopsin is present in the cell membrane as a two-dimensional crystal embedded in lipids that have ether-linked phytanyl groups [16,17] and exposed to a medium with approximately neutral pH [18—20] and a salt concentration of 4 M. We will demonstrate in this paper that the above mentioned conditions are not essential for the activity of the bacteriorhodopsin proton pump. An outline of the conditions in which bacteriorhodopsin can be used as a $\Delta\tilde{\mu}_{H^+}$ generator is given.

Materials and Methods

Halobacterium halobium (Strain R_1) was kindly donated by Dr. Oesterhelt. It was grown according to the method of Danon and Stoeckenius [3] and purple membranes were isolated according to Oesterhelt and Stoeckenius [21]. The concentration of bacteriorhodopsin, present in the form of purple membranes, was determined spectrophotometrically at 570 nm, using an extinction coefficient of 63 000 $M^{-1} \cdot cm^{-1}$ [22]. Soybean phospholipids were prepared according to ref. 23 by acetone precipitation of asolectin (Associated Concentrates).

Purified soybean phosphatidylcholine and soybean phosphatidylethanolamine were isolated according to ref. 24, using a silicic acid column (Biosil A, 200-325 mesh.). Egg phosphatidylcholine was isolated according to Pangborn [25]. Phospholipids from *H. halobium* were isolated according to ref. 26. Phosphatidylserine (from ox-brain), cardiolipin (from bovine heart) and dicetylphosphate were obtained from Sigma (St. Louis, Mo. U.S.A.) and pure cetyltrimethylammonium-bromide from Koch-Light. All other materials were reagent grade.

Lipids were stored at −80°C in $CHCl_3$ or $CHCl_3/CH_3OH$ (2 : 1, v/v) under argon atmosphere. Absorption spectra were recorded at 20°C in a Cary 17 spectrophotometer. Circular dichroism (CD) spectra were recorded at 20°C in a Dichrographe Roussel-Jouan Modele C.D. 185 in 1-cm quartz cuvettes. The amount of exciton coupling was determined from the difference of the amplitudes of the CD spectra at 535 nm minus 605 nm.

Unless otherwise described, the reconstitution of bacteriorhodopsin vesicles was carried out as follows. The required amount of lipid was dried in a round-bottom flask on a rotatory evaporator at room temperature. Purple membranes were then added in a medium in which the experiments were to be carried out (usually 0.5—2 mg/ml in 150 mM KCl, pH 6.0). The lipids were resuspended by shaking on a Vortex mixer, with the addition of three glass beads, for 1- 2

min, until visual inspection indicated that all the lipid was removed from the glass wall. Sonication was carried out in a M.S.E. ultrasonifier (20 KHz, 4 μm amplitude) in a glass vessel with cooling in ice under argon atmosphere; 15-s periods of sonication were spaced by 45-s periods of rest. The total sonication time is indicated in the legends to the figures. In the following, the protein present in these reconstituted membrane vesicles will be called bacteriorhodopsin. pH measurements were carried out in a 3.7-ml incubation vessel maintained at 25°C, equipped with a magnetic stirrer. The pH of the medium was continuously measured using an Ingold pH-electrode connected to an amplifier (Vibron Electrometer 33B-2), the signal from the amplifier being continuously recorded on a Servogor linear recorder. Usually, the vessel contained 0.3—1.0 mg bacteriorhodopsin. Illumination was carried out with a 500 W Leitz Slide-projector, equipped with two heat filters. The pH-changes upon illumination reached a steady state after approximately 10 min and were calibrated by the addition of small amounts of either 0.01 M oxalic acid or 0.01 M HCl.

Results and Discussion

An investigation of the conditions in which bacteriorhodopsin can be used as a $\Delta\tilde{\mu}_{H^+}$ generator should, ideally, be carried out by investigating the capacity of this chromoprotein to build up an electrochemical potential difference across the reconstituted vesicle membrane. Indeed, such potential differences have been measured, using the flow-dialysis technique [27] but this measurement of the light-induced $\Delta\tilde{\mu}_{H^+}$ is too laborious to be taken as a screening method. Also, assumptions have to be made on the homogeneity of the reconstituted vesicles and it is difficult to determine the percentages of bacteriorhodopsin that are oriented outward or inward in the membrane. Another possible method, the use of fluorescent indicator dyes [28—30] has the disadvantage that no certainty exists about the percentage of liposomes present in the preparation that give rise to the observed effects. Furthermore, this method has been criticized by various authors with respect to its reliability [31—33], especially concerning the quantitative aspects.

The method used in this paper, determination of the extent of the light-driven proton uptake, has the advantage of measuring the net proton uptake of the whole liposome preparation; consequently, the net orientation of bacteriorhodopsin can be determined. Its major disadvantage, the ignorance of the $\Delta\psi$ component of the $\Delta\tilde{\mu}_{H^+}$ has been minimized by carrying out most of the experiments in 150 mM KCl. Since these reconstituted vesicles are relatively permeable to ions [34,35], the membrane potential is low in the steady state of light-driven proton uptake. Thus under these conditions, within one preparation, the extent of light-driven proton uptake is approximately proportional to the ΔpH and therefore to the $\Delta\tilde{\mu}_{H^+}$.

In the steady state, the magnitude of the extent of proton uptake is determined by the difference between the pump activity of bacteriorhodopsin and the passive leak of the vesicles. The activity of the pump may be influenced by changes in: (i) the light intensity [36]; (ii) alteration of the properties of the pump (e.g. by diethyl ether [22] or the salt concentration [37]); (iii) the aggre-

gation state of the bacteriorhodopsin; (iv) the fraction of bacteriorhodopsin present in a reconstituted membrane and the fraction of bacteriorhodopsin present in one of the two possible orientations. The passive leak of the vesicles is determined by: (1) the permeability of the vesicle-membrane to protons; (2) the internal buffering capacity and the internal volume of the vesicles; (3) the permeability of the reconstituted membrane to HCl, K^+ etc.

If in the following the capacity of bacteriorhodopsin as a $\Delta\tilde{\mu}_{H^+}$ generator is to be compared between different experiments, possible changes in one or more of the above mentioned variables should be taken into account.

In Fig. 1 the optical absorbance spectra of purple membranes and vesicles reconstituted from purple membranes and either soybean, phospholipids or egg phosphatidylcholine are shown. For comparison, also the absorbance spectrum of pure soybean phospholipid vesicles is shown, which is due to light scattering by the vesicles. It can be seen that upon reconstitution of purple membranes with phospholipids, the absorbance maximum shifts to the blue by about 10—20 nm. During reconstitution some denaturation of the protein occurs, but this usually amounts to less than 10%. Parallel experiments show that upon reconstitution also a change of the tryptophan fluorescence of the bacteriorhodopsin occurs. Both the excitation and the emission maximum shift to a longer wavelength (from 285 to 292 nm and from 320 to 335 nm respectively).

The weight-averaged size of sonicated lipid vesicles is about 300 Å [37,38]. It can be seen in Fig. 1 that the light-scattering properties of protein-containing vesicles differ from those of the lipid vesicles, possibly indicating that in these preparations the protein-containing vesicles are larger than the lipid vesicles.

It has been well established in the literature that the aggregation state of bacteriorhodopsin in the membrane can be inferred from measurement of its CD spectrum [39—41]. Fig. 2 shows the CD spectra of bacteriorhodopsin vesi-

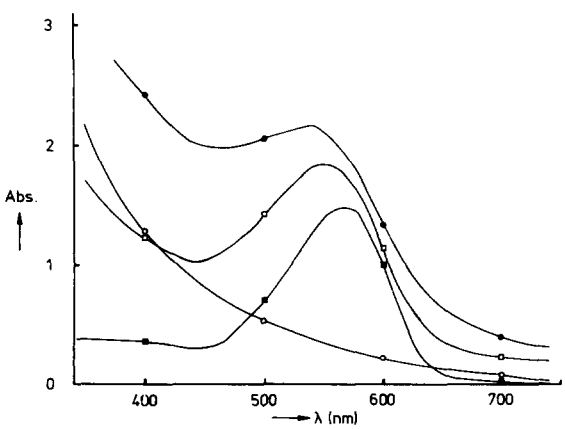

Fig. 1. Redrawing of the optical absorbance spectra of bacteriorhodopsin vesicles. ○———○, 10 mg/ml soybean phospholipids sonicated for 900 s; □———□, 10 mg/ml soybean phospholipids, 1 mg/ml bacteriorhodopsin, sonicated for 900 s; ●———●, 9.5 mg/ml egg phosphatidylcholine, 0.5 mg/ml dicetyl phosphate, 1 mg/ml bacteriorhodopsin, sonicated for 900 s; ■———■, 1 mg/ml bacteriorhodopsin. Medium, 150 mM KCl, pH 6.0.

cles, reconstituted from purple membranes and soybean phospholipids, before and after sonication. From this figure it is clear that the exciton coupling, resulting from interactions between neighbouring bacteriorhodopsin molecules, present in the unsonicated liposomes, disappears upon sonication. The spectra of the two preparations agree quite well with the CD spectra published previously for bacteriorhodopsin existing in a hexagonal array or in the dissolved state, respectively. Table I shows that the disappearance of the exciton coupling in bacteriorhodopsin vesicles depends on the lipid to protein ratio and the sonication time during the reconstitution procedure. Long sonication times or a low protein to lipid ratio favour the disappearance of the exciton coupling. The last column of the table shows the proton uptake capacity of these vesicles. The decrease in exciton coupling is paralleled by an increase of the proton uptake capacity. These results strongly suggest that monomeric bacteriorhodopsin molecules are active proton pumps. Until now, in all reports on conditions in which bacteriorhodopsin was present in a monomeric form no significant light-driven proton pump could be measured [39,40].

Stimulation of the disaggregation of the bacteriorhodopsin by sonication can be understood by realizing that during sonication, continuously small air bubbles are formed which cause high shearing forces in the suspension [42] and therefore a breakage of the protein-protein interactions.

The effect of sonication on the extent of proton uptake, agrees with the results of Racker [5]. We have found that with increasing sonication times, the initial rate of proton uptake increases while the kinetics of the off-reaction remain essentially unchanged (Hellingwerf, K.J., unpublished data). This means that with increasing sonication times the apparent capacity of the active H^+ pump increases. This could be caused by: (i) an increase in the net orientation of bacteriorhodopsin in the direction opposite to the in vivo orientation, (ii) a more homogeneous distribution of the protein over the vesicle population, (iii) an increase in the activity of the pump due to the conformational change which occurs upon reconstitution (see also ref. 43). Indications for a change

TABLE I

DEPENDENCE OF EXCITON COUPLING IN SPECTRA OF BACTERIORHODOPSIN VESICLES ON LIPID TO PROTEIN RATIO AND SONICAYION TIME

Bacteriorhodopsin, 1 mg/ml; soybean-phospholipids, 1—25 mg/ml; medium, 150 mM KCl, pH 6. Pre-illumination before taking the CD spectra was carried out as described in the legend to Fig. 2.

Bacteriorhodopsin/phospholipid (w/w)	Sonication time (s)	Exciton coupling (%)	Proton uptake (nequiv./mg bacteriorhodopsin)
1 : 1	0	100	n.d.
1 : 1	90	63	n.d.
1 : 1	450	25	8
1 : 5	0	100	n.d.
1 : 5	90	37	72
1 : 5	450	15	112
1 : 25	0	100	n.d.
1 : 25	90	28	98
1 : 25	450	0	170

n.d., not determined.

in the conformation of bacteriorhodopsin upon reconstitution were obtained from the change in absorbance maximum and the change in fluorescence of the tryptophan residues of bacteriorhodopsin. As shown above, no evidence was obtained for a less effective operation of the bacteriorhodopsin proton pump if the protein molecules are present in the form of monomers. This is in contrast with the results of Hartmann et al. [44], who did report a less efficient operation of the bacteriorhodopsin proton pump in a condition where the bacteriorhodopsin was prevented to form the hexagonal array.

The increase in light-dependent proton-uptake capacity of bacteriorhodopsin with decreasing protein to lipid ratio, is shown more clearly in Fig. 3. The increase levels off when the protein to lipid ratio falls below 0.01 (w/w), where it can be calculated, assuming a homogeneous bacteriorhodopsin distribution, that about one molecule of bacteriorhodopsin is present per vesicle. In a separate experiment it was shown that the percentage quenching of 9-amino-6-chloro-2-methoxyacridine fluorescence upon illumination of bacteriorhodopsin vesicles, was independent of the lipid to protein ratio (Arents, J.C. and Hellingwerf, K.J., unpublished data). This indicates that the $\Delta\tilde{\mu}_{H^+}$, generated by bacteriorhodopsin may be, in contrast to the extent of proton uptake, independent of the protein to lipid ratio. Probably, the increase in extent with increasing lipid to protein ratios can be accounted for by the increase in the internal buffering capacity per bacteriorhodopsin molecule without a change in the intrinsic properties of the proton pump.

Since it is known that the functioning of many halophilic proteins is restricted to media with a very high salt concentration [45], it is interesting to study the effect of changes in the salt concentration on the reconstitution of the bacteriorhodopsin proton pump. Variation of the salt concentration during

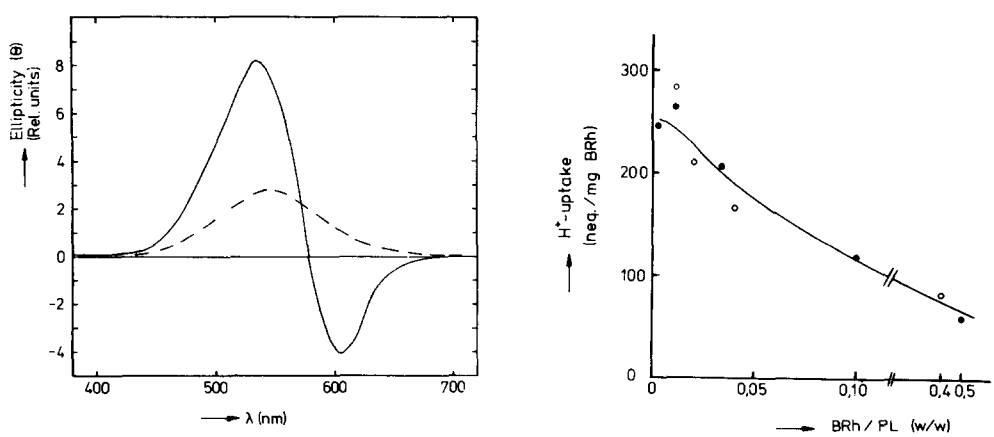

Fig. 2. CD spectra of bacteriorhodopsin vesicles, soybean phospholipids, 20 mg/ml, bacteriorhodopsin 1 mg/ml, 150 mM KCl, pH 6.0. ———, sonication time 0 s; - - - - -, sonciation time 450 s. The bacteriorhodopsin vesicles were preilluminated by illumination with a 500 W Leitz slide projector, equipped with one heat filter, for 2 min.

Fig. 3. Effect of variation in the lipid to protein ratio during reconstitution on the extent of light-driven proton uptake of bacteriorhodopsin vesicles. Soybean phospholipids, 2—60 mg/ml; bacteriorhodopsin, 0.2—2 mg/ml; 150 mM KCl, pH 6.0. Sonication time, 900 s, (○———○), 450 s, (●———●).

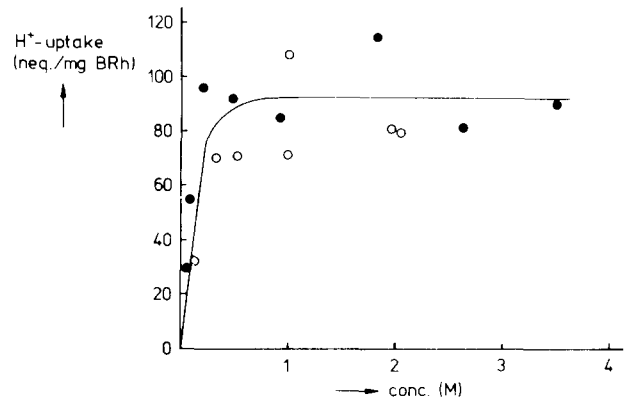

Fig. 4. Salt-dependence of the reconstitution of the light-driven proton pump. Reconstitution of the bacteriorhodopsin vesicles was carried out in media with the indicated salt concentrations. Soybean-phospholipids, 20 mg/ml; bacteriorhodopsin, 1 mg/ml. Sonication time, 90 s. Light-dependent proton uptake was measured in the same medium. pH = 6. ●———●, KCl; ○———○, NaCl.

reconstitution of bacteriorhodopsin vesicles from soybean phospholipids and purple membranes has relatively little influence on the extent of the light-dependent proton uptake capacity of the reconstituted vesicles (Fig. 4). A large decrease occurs only at salt concentrations below 150 mM. In parallel experiments it was shown by sucrose-density gradient centrifugation, that the incorporation of the protein into the vesicles deteriorates at salt concentrations below 100 mM, decreasing to zero in distilled water. Vesicles prepared from purple membranes and soybean phosphatidylcholine show the same dependence on salt concentration. With egg phosphatidylcholine, however, the decrease in extent of proton uptake only occurs below about 10 mM. When reconstitution is carried out in a medium with a low ionic strength (e.g. 250

TABLE II

SALT DEPENDENCE OF THE RECONSTITUTED LIGHT-DRIVEN PROTON PUMP

Reconstitution of the bacteriorhodopsin vesicles was carried out in 250 mM sucrose, pH 6.0. Soybean phospholipids, 20 mg/ml; bacteriorhodopsin, 1 mg/ml; sonication time, 900 s. Light-dependent proton uptake was measured in media with the indicated composition, plus 10 μM valinomycin if potassium was present. Preincubation was 4 h at 25°C.

Medium composition	Proton uptake (nequiv./mg bacteriorhodopsin)
250 mM sucrose	24
248 mM sucrose + 1 mM KCl	39
232 mM sucrose + 10 mM KCl	46
83 mM sucrose + 100 mM KCl	61
0 mM sucrose + 1000 mM KCl	96
248 mM sucrose + 1 mM NaBr	36
232 mM sucrose + 10 mM NaBr	48
83 mM sucrose + 100 mM NaBr	56
0 mM sucrose + 1000 mM NaBr	92

mM sucrose), closed bacteriorhodopsin vesicles are formed, but under these circumstances the hexagonal array of bacteriorhodopsin molecules remains largely intact, as indicated by the CD spectra. This indicates involvement of electrostatic repulsion between the soybean phospholipids and the purple membranes during reconstitution, since the critical salt concentration depends on the lipids used for the reconstitution.

When reconstituted bacteriorhodopsin vesicles are tested for light-driven proton uptake at different salt concentrations the extent of proton uptake increases up to salt concentrations of 1—4 M (Table II; see also ref. 5). Since at salt concentrations above 100 mM the electrical potential across the vesicle membrane must be very small, this cannot be explained by an increase in concentration of counterions. A possible explanation is a decrease in the passive permeability of the vesicle membrane for protons with increasing salt concentrations [46]. If this is true, the net orientation of reconstituted bacteriorhodopsin decreases going from 0.2 to 4 M NaCl or KCl (Fig. 4).

These data all agree with a mechanism of reconstitution in which the cytoplasmic side of the purple membrane carries an excess negative charge at neutral pH (see also ref. 47). This cytoplasmic side is predominantly oriented to the outside of the reconstituted vesicle, due to mutual repulsion of negative charges, present on either the added lipids or the purple membranes. This would also explain the results of Huang and Stoeckenius [48] who observed a randomization of the orientation of bacteriorhodopsin if a high concentration of Mg^{2+} was included in the reconstitution mixture, since it is known that Mg^{2+} is very effective in shielding negative charges.

Bacteriorhodopsin vesicles, which actively take up protons under the influence of light, can be prepared in various salt solutions other than NaCl, KCl or NaBr. Among those tested were: Na_2SO_4, K_2SO_4, $MgSO_4$, KNO_3, LiCl and CsCl; no significant differences in the extent of proton uptake between these vesicles were detected. However, when salts like NH_4Cl, which are highly "uncoupling" [49], are used no light-dependent proton uptake is observed. Accurate extent measurements are limited to media of salts from strong bases and acids, because of the technical problem of pH shift in solutions of salts of weak bases and acids as a result of the heating due to illumination. ΔpH measurements, on the other hand, can be carried out in such media using flow dialysis (Westerhoff, H.V., unpublished data) and show a normal bacteriorhodopsin function.

Fig. 5 shows the pH-dependence of the bacteriorhodopsin-proton pump. Depending on the reconstitution conditions, different pH profiles are observed. With soybean phospholipids and a short sonication time, the activity of the proton pump is independent of pH in the range tested (Fig. 5A). With a longer sonication time, besides a general increase, an increase of the proton uptake capacity is observed with increasing pH up to pH 8 (Fig. 5B). Bacteriorhodopsin vesicles reconstituted with egg phosphatidylcholine show an optimum in the extent of light-driven proton uptake at about pH 5. Here the decrease of the extent with increasing pH is caused by inactivation of the bacteriorhodopsin at the higher pH values, as evidenced by the spectra. No difference is observed when the vesicles are reconstituted at one and tested at different pH values (Fig. 5C) or when they are reconstituted at the pH values at which the light-dependent proton-uptake capacity is to be measured (Fig. 5D). In agree-

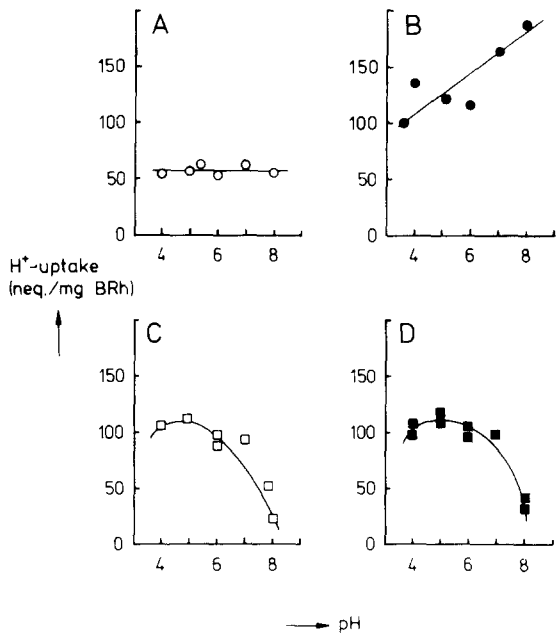

Fig. 5. pH dependence of the bacteriorhodopsin-proton pump. A. 20 mg/ml soybean phospholipids; 1 mg/ml bacteriorhodopsin; 150 mM KCl; sonication time, 90 s. B. 20 mg/ml soybean phospholipids; 1 mg/ml bacteriorhodopsin; 150 mM KCl; sonication time, 375 s. C. 20 mg/ml egg phosphatidylcholine; 1 mg/ml bacteriorhodopsin; 150 mM KCl; sonication time, 225 s. In A, B and C reconstitution and proton uptake measurement were carried out at the indicated pH values. D. 20 mg/ml egg phosphatidylcholine; 1 mg/ml bacteriorhodopsin; 150 mM KCl; sonication time, 225 s. The bacteriorhodopsin vesicles were made at pH 6.0 and tested at the indicated pH values after 1 h preincubation at 25°C.

ment with Bakker et al. [43], we also find an irreversible change in absorbance maximum of the bacteriorhodopsin vesicles from 570 nm to 470 nm after prolonged storage at high pH (see also ref. 48). With soybean phospholipids, inactivation of reconstituted bacteriorhodopsin is observed at pH 10 [50]. Bacteriorhodopsin reconstituted with egg phosphatidylcholine can only be used in a pH range smaller than the in vivo range [18—20]. Between pH 4 and 8, the reconstitution process of bacteriorhodopsin is independent of pH; with cardiolipin, at pH 2.5, however, liposomes can be prepared which show a net acidification of the medium upon illumination [51]. Under the conditions described here, always a net proton uptake was observed.

For two reasons it seemed interesting to study the effect of variation of the lipid composition of reconstituted bacteriorhodopsin vesicles on the proton-uptake capacity of these vesicles. Firstly, the lipids surrounding bacteriorhodopsin in vivo are very different from the lipids used for reconstitution of many other energy conversion systems [12—17] and bacteriorhodopsin could be inactivated by the exogenous lipids when it is dissolved from its hexagonal array. Secondly, other energy converting proteins, in reconstitutions with two energy converting proteins, may impose limitations on the lipid composition. The lipids we used all have a relatively high degree of unsaturation, so that they are in the fluid state during sonication at 0°C. This is advantageous in reconstitutions of bacteriorhodopsin with heat-labile proteins.

TABLE III

DEPENDENCE OF THE LIGHT-INDUCED PROTON-UPTAKE CAPACITY OF BACTERIORHODOPSIN VESICLES ON PHOSPHOLIPID COMPOSITION

Vesicles were prepared under the following conditions: bacteriorhodopsin, 0.5 mg/ml; phospholipids, 20 mg/ml; medium, 150 mM KCl, pH 6.0; sonication time, 90 s. Measurements of proton uptake were carried out in 150 mM KCl, pH 6.0. Sonicated purple membranes showed no light-induced pH change.

Lipid composition	Weight ratio	Proton uptake (nequiv./mg bacteriorhodopsin)
H. halobium lipids		60
Cardiolipin		85
Soybean phospholipids		107
Soybean phosphatidylcholine		150
Egg phosphatidylcholine		93
Egg phosphatidylcholine/phosphatidylethanolamine	3 : 1	113
Egg phosphatidylcholine/phosphatidylethanolamine	1 : 1	99
Egg phosphatidylcholine/phosphatidylethanolamine	1 : 5	104
Soybean phosphatidylcholine/phosphatidylserine	1 : 5	75
Soybean phosphatidylcholine/phosphatidylserine	1 : 1	120
Soybean phosphatidylcholine/phosphatidylserine	2 : 1	84
Soybean phosphatidylcholine/phosphatidylserine	5 : 1	92
Soybean phosphatidylcholine/cetyltrimethylammoniumbromide	20 : 1	88
Soybean phosphatidylcholine/cetyltrimethylammoniumbromide	10 : 1	72
Soybean phosphatidylcholine/dicetylphosphate	20 : 1	88
Soybean phosphatidylcholine/dicetylphosphate	10 : 1	80

Table III shows that many different lipid preparations can be used for the reconstitution of the bacteriorhodopsin-proton pump; all lipid preparations used show an extent of light-driven proton uptake, comparable to, or higher than that of bacteriorhodopsin reconstituted with its endogenous lipids. From these data it is clear that the net charge of the lipids or the specific head group are not factors of major importance, determining the reconstitution of the proton pump.

The results of Table III cannot be unambiguously interpreted in terms of changes in the activity of the proton pump, because of the many other factors involved. It is known from the literature that such changes do occur [43, 52,53].

In conclusion, it can be said that bacteriorhodopsin can be used as a $\Delta\tilde{\mu}_{H^+}$ generator in a range of conditions far greater than the in vivo conditions. Care should be taken, however, with the reconstitution below 100 mM salt and/or above pH 7.5.

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