Reconstitution of Delipidated Bacteriorhodopsin with Endogenous Polar Lipids*

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Delipidated bacteriorhodopsin has been reconstituted with endogenous polar lipids from Halobacterium halobium. The vesicles (diameter, 250-500 Å) formed are very stable, relatively homogeneous in bacteriorhodopsin and lipid content, and almost optically clear; a minor turbid fraction can be separated by gel filtration. Bacteriorhodopsin in the reconstituted vesicles has an inside out orientation and, on illumination, translocates protons efficiently from the medium to the interior of the vesicles in the presence of the ionophore valinomycin. In the absence of the latter, both the rate and the extent of light-dependent proton uptake by the vesicles are decreased 3-6- and 5-15-fold, respectively, depending on the salt in the assay medium. Both the stimulation by valinomycin and the proton-translocating activity are higher in NaCl than in KCl. Bacteriorhodopsin in these vesicles, as in purple membrane, undergoes light adaptation as indicated by a red shift (7-8 nm) of the absorption maximum. At low pH, the absorption maximum of reconstituted protein shows a 50-nm red shift, possibly due to protonation of an ionizable group which interacts with the chromophore. The latter group appears to be accessible only from the external medium.

Bacteriorhodopsin, the only protein present in the purple membrane of Halobacterium halobium, undergoes a light-initiated cycle of several reactions which results in an electrogenic extrusion of a proton from the interior of the cell to the surrounding medium (1). The protein consists of a single polypeptide of 248 amino acids to which retinal is bound as a Schiff's base through the ε-amino group of a lysine residue (1, 2). From the known amino acid sequence of bacteriorhodopsin (3, 4) and the electron density map of the protein in purple membrane (5), a model for the folding of the seven α-helical fragments of bacteriorhodopsin in the bilayer has been proposed (6). The molecular mechanism by which bacteriorhodopsin translocates protons and the manner by which the electrochemical gradient of protons provides energy to the cell are not well understood.

The membrane of H. halobium, as well as the purple membrane, contains a set of unusual polar lipids (7, 8). They are all negatively charged and are glycerol ether derivatives instead of the usual fatty acyl esters, the hydrocarbon chains consisting of phytanyl residues (9) (Fig. 1). The purple membrane contains, in contrast to other fractions of the cell membrane, substantial amounts of sulfated polar lipids (7), mainly in the form of glycolipid sulfate, which has been reported to be essential for the formation of osmotically active liposomes of Halobacterium lipids (10).

Recently, complete removal of the endogenous lipids from purple membrane was reported (11). Reconstitution of this delipidated bacteriorhodopsin with exogenous lipids gave vesicle preparations which efficiently translocated protons.

Because of the highly unusual nature of H. halobium lipids, it seemed desirable to study reconstitution of the completely delipidated bacteriorhodopsin with them. Previously, the effect of the endogenous lipids on the catalytic and spectroscopic properties of bacteriorhodopsin has mainly been studied with purple membrane sheets (1), and the attempts which have been made to incorporate purple membrane (12-15) or partly lipid-free bacteriorhodopsin (16) into H. halobium lipid vesicles, have, in general, resulted in preparations with poor proton-translocating activity.

In this paper, we report on the reconstitution of delipidated bacteriorhodopsin into endogenous polar lipid vesicles which, upon illumination, translocate protons very efficiently provided that valinomycin is present to dissipate the inhibitory membrane potential. The bacteriorhodopsin vesicles thus prepared are quite impermeable to ions, stable, almost optically clear, and homogeneous in regard to bacteriorhodopsin and lipid content. Some additional properties of bacteriorhodopsin in these vesicles have been investigated.

EXPERIMENTAL PROCEDURES

Materials

Purple membrane was isolated from H. halobium (strain S9) as described by Oesterhelt and Stoeckenius (17). 14C-labeled purple membrane was obtained from cell cultures (200 ml) grown in the presence of [1-14C]acetic acid (200 μCi) obtained from New England Nuclear. Soybean phospholipids (asolecin), from Associated Concentrates, Woodside, NY, were treated according to the method of Kagawa and Racker (18). Pronase and valinomycin were obtained from Calbiochem. Analytical thin layer chromatography plates, Silica Gel 60 F254, were purchased from E. Merck, and Sepharose CL4B from Pharmacia. Triton X-100 was obtained from Rohm and Haas Co. Carbonyl cyanide m-chlorophenylhydrazone, cholic acid (recrystallized from acetone), chymotrypsin (treated with 1-chloro-3-tosyl-amido-7-tamino-2-heptanone), and sodium tetraphenylborate were purchased from Sigma.

Methods

Preparation of Delipidated Bacteriorhodopsin—Delipidation of bacteriorhodopsin was performed essentially as described earlier (11) except that the initial solubilization of purple membrane by Triton X-100 was carried out at pH 7 (0.1 M Tris-HCl) for 12 h, instead of 2 days at pH 5, followed by ultracentrifugation at 100,000 × g for 45 min (19). The delipidation procedure was routinely performed in the presence of 14C-labeled purple membrane (1 × 106 cpm) in order to monitor complete delipidation of the protein. Delipidated bacterio-
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Proteolysis of Bacteriorhodopsin—Digestion of bacteriorhodopsin in purple membrane and in vesicles with pronase was performed as described previously (11).

Bacteriorhodopsin (0.2 mg/ml; 100 μl) in purple membrane or in vesicles was bleached in the presence of hydroxylamine (0.1 M), prior to addition of chymotrypsin (0.5 μg) and CaCl₂ (5 mM). The samples were incubated for 12 h at 30 °C and the digestion terminated by the addition of phenylmethylsulfonyl fluoride (1 mM). Where indicated, Triton X-100 (0.2%) was present during the incubation. Gel electrophoresis of the digested samples was performed in 15% polyacrylamide gels containing 0.1% sodium dodecyl sulfate as described by Laemmli (27). Prior to electrophoresis, samples containing vesicles were extracted to remove lipids (11).

RESULTS

Reconstitution of Delipidated Bacteriorhodopsin with H. halobium Lipids—Reconstitution of delipidated bacteriorhodopsin with endogenous polar lipids was performed by the cholate dialysis method (11). Upon illumination, these vesicles translocated protons from the medium to their interior, as has been found previously with vesicles reconstituted with SBPL or synthetic phospholipids (11, 12, 16). On the average, 4–6 H⁺ were translocated per molecule of bacteriorhodopsin, this value being much smaller than that obtained with vesicles prepared from SBPL (11). However, as shown in Fig. 2, A and B, both the initial rate and the extent of proton translocation in these vesicles was dramatically increased in the presence of valinomycin. Thus, the rate increased 4–6-fold while the extent of proton translocation increased 7–11-fold. On the other hand, valinomycin brought about only a moderate stimulation (1.5–1.8-fold) of the extent of proton translocation in bacteriorhodopsin/SBPL vesicles (Fig. 2, C and D). Although less pronounced, stimulation of proton translocation in bacteriorhodopsin/HhPL vesicles was also observed in the presence of the lipophilic anion tetraphenylboron (data not shown).

The uncoupler CCCP abolished the light-dependent pH response of the vesicles. The concentration of CCCP required for total uncoupling of the vesicles was decreased 100-fold by the presence of valinomycin. A similar observation has been made previously with purple membrane vesicles (16).

Orientation of Bacteriorhodopsin in Endogenous Lipid Vesicles—The proton translocation experiments described above indicated that a majority of bacteriorhodopsin molecules in the endogenous lipid vesicles had an inside out orientation, as has been found for previously reconstituted bacteriorhodopsin vesicles (11). For more accurate determination of the orientation of the protein, proteolysis of bacteriorhodopsin vesicles was performed. As shown in Fig. 3, most of the protein in the vesicles was cleaved by pronase, and the single large proteolytic fragment produced was the same as that obtained previously after similar digestion of purple membrane. The intactness of the vesicles during digestion was tested by measuring proton translocation by the vesicles, both in the absence and presence of valinomycin. The initial rate and the extent, as well as the response to valinomycin, was fully retained after a 5-h incubation in the absence of pronase and subsequent incubation with pronase.

A second test of the orientation of bacteriorhodopsin in the reconstituted vesicles involves cleavage by chymotrypsin. The enzyme cleaves bacteriorhodopsin in the apomenbrane to form two fragments (28), one containing amino acid residues 1–71 (Fragment C-2) and the second, 72–248 (Fragment C-1). The currently postulated transmembranous helical arrangement of bacteriorhodopsin in the bilayer predicts that the chymotryptic cleavage site should be on the side opposite to that of the carboxyl terminus (6). The above vesicles, which...
have the carboxyl terminus exposed to the outside, were treated with chymotrypsin after bleaching of bacteriorhodopsin. As expected for the inside out orientation, most of the protein was unaffected (Fig. 3B, channel 2). However, cleavage occurred when Triton X-100 was present (Fig. 3B, channel 3). The small amount of cleavage observed in the absence of Triton X-100 (Fig. 3B, channel 2) could imply either that a portion of the vesicle preparation is disrupted during the bleaching of bacteriorhodopsin and/or subsequent incubation with chymotrypsin for 12 h, or that the vesicles contain a minor population of the protein with opposite orientation.2

Reconstitution of Bacteriorhodopsin into Vesicles at Various Lipid to Protein Ratios—Reconstitutions were performed by using different ratios of endogenous polar lipids and delipidated bacteriorhodopsin, and the resulting preparations were tested for the initial rate and extent of proton translocation. The results (Fig. 4) showed that while a minimum lipid to protein ratio of 20:1 (w/w) is required to obtain vesicles capable of proton translocation, maximal proton translocation, both extent (Fig. 4B) and initial rate (Fig. 4B), is obtained when the lipid to protein ratios are in the range 40:1 to 80:1 (w/w).

All bacteriorhodopsin preparations reconstituted using the above-mentioned different ratios of lipid to protein showed an absorption maximum at 555 nm except for the sample in which delipidated bacteriorhodopsin was dialyzed without the addition of endogenous lipids. In the latter case, $\lambda_{\text{max}}$ was 538 nm, as for the delipidated bacteriorhodopsin in deoxycholate. Therefore, association of the endogenous lipid with bacteriorhodopsin brings about the red shift (from 538–555 nm) in the bacteriorhodopsin spectrum and also stabilizes bacteriorhodopsin. (Following dialysis, bacteriorhodopsin is completely recovered in the presence of lipids, while about 50% of the chromophore is lost in their absence.)

Fractionation of Reconstituted Bacteriorhodopsin Vesicles on Sucrose Density Gradients—The reconstituted vesicles (lipid:protein, 40:1) were subjected to sucrose density gradient centrifugation, and the fractions were analyzed for bacteriorhodopsin and lipid phosphate. The results are shown in Fig. 5A. The majority of the vesicles appeared as a distinct purple band at a density of about 1.07 g/ml (Fig. 5A). A minor population of the vesicles appeared as a faint turbid purple

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2 Another possibility is that the small fraction of vesicles undergoing cleavage are those that appear in peak 1 of Fig. 6 (see below). This turbid fraction may contain denatured protein.
Gradient fractionation of vesicle preparations reconstituted with lipid to protein ratios of 80:1 or 40:1 in sucrose density gradient fractions of the 80:1 preparation than in those from the 40:1 preparation. These results indicate that a more homogeneous vesicle preparation, with respect to stimulation by valinomycin, is obtained by using a higher lipid to protein ratio.

Fractionation of Bacteriorhodopsin-containing Vesicles by Gel Permeation Chromatography—As described above, sucrose density gradient fractionation of reconstituted bacteriorhodopsin vesicles showed that the main vesicle population was contaminated by a minor, more dense, and turbid fraction. The latter showed low activity in proton translocation and gave a small response to valinomycin. This fraction was readily separated from the major vesicle population by gel filtration on a Sepharose CL4B column. The elution profile of bacteriorhodopsin-containing vesicles from such a column is shown in Fig. 6. Two peaks with proton-translocating activity were found. The first (Peak I), eluting with the void volume, contained vesicles with high turbidity and a much lower proton uptake than those found in Peak II, where more than 70% of the chromophore was recovered. Furthermore, the stimulation of proton translocation by valinomycin was very low in Peak I relative to Peak II.

When the material in Peak I and Peak II, together with nonchromatographed vesicles, were subjected to sucrose density centrifugation, Peak I from the Sepharose column appeared on the gradient as a single band at the same position as the vesicles with high density of the nonchromatographed sample (inset, Fig. 6). The vesicles in Peak II co-migrated with the major band of the nonchromatographed vesicle preparation. The amount of the turbid material (Peak I) varied from preparation to preparation, although it never exceeded 20% of the total vesicle preparation. Its amount was strikingly reduced if freshly prepared bacteriorhodopsin was used for reconstitution, rather than a preparation of delipidated protein which had been stored frozen. It would thus appear that the material in Peak I contains denatured bacteriorhodopsin monomers or aggregates in association with endogenous lipids. Fractionation of vesicles on Sepharose CL4B thus gave a

![Diagram](https://example.com/diagram.png)
veicle population which was more homogeneous and had very low turbidity. The latter fact was also shown by the absorption spectra (Fig. 7) of the vesicles as recorded against either hydroxylamine-bleached vesicles (curve a) or against water (curve b).

**Properties of Vesicles Reconstituted with Endogenous Lipids**—The bacteriorhodopsin vesicles reconstituted with endogenous lipids proved to be very stable. Thus, proton translocation activity and the stimulation of proton uptake by valinomycin were unaffected after storage at 4 °C in the dark for 4 months. In contrast, bacteriorhodopin in vesicles reconstituted using soybean phospholipids was totally bleached after approximately 1 month under comparable conditions. The two types of vesicles also behaved differently in regard to bleaching of bacteriorhodopsin on illumination in the presence of hydroxylamine (Fig. 8). The time required for bleaching of the chromophore in soybean phospholipid vesicles was much shorter than that required for vesicles reconstituted with endogenous lipids (Fig. 8). Furthermore, in the presence of high concentrations of salt, a significantly longer illumination time was required for bleaching of the chromophore in the endogenous lipid vesicles, whereas bleaching of the reconstituted vesicles prepared from soybean phospholipids was unaffected by salt. It may be added that the time required for bleaching of the chromophore in purple membrane on illumination in the presence of hydroxylamine is approximately 100-fold greater than that of bacteriorhodopsin in endogenous lipid vesicles (data not shown).

Electron microscopy of the vesicles after negative staining indicated that the diameter of the vesicles ranged between 250 and 500 Å, a result which is consistent with the chromatographic behavior of the vesicles on Sepharose CL4B.

**Light Adaptation of Bacteriorhodopsin in Reconstituted Vesicles**—Bacteriorhodopsin in purple membrane undergoes light adaptation on illumination as evidenced by a 10-nm red shift in its absorption maximum (1). Bacteriorhodopsin as reconstituted into vesicles has now been found to display a red shift as well on illumination. Thus, the absorption spectrum of bacteriorhodopsin reconstituted with endogenous lipids is shifted by 7–8 nm while that of bacteriorhodopsin reconstituted soybean phospholipids is shifted by 5–6 nm on illumination (Table I). In contrast to bacteriorhodopsin in purple membrane, the absorption maximum of both dark- and light-adapted bacteriorhodopsin in vesicles was influenced by high salt concentration as well as by an increase in pH, the effects depending on the lipids present (Table I).

In the case of the coupled vesicles obtained from reconstitution of bacteriorhodopsin with the endogenous lipids, the possibility was considered that the formation of a membrane potential and/or a pH gradient could influence the light adaptation of the chromophore. The presence of either valinomycin or CCCP did not affect the shift in the absorption maximum of the protein after illumination.

**The Effect of Salt on Proton Translocation by Bacteriorhodopsin Reconstituted with Endogenous Polar Lipids**—Proton translocation activity of bacteriorhodopsin reconstituted with endogenous polar lipids was measured in the presence of a wide concentration range of NaCl and KCl (Fig. 9).

Both the extent (Fig. 9A) and the rate (Fig. 9B) of proton translocation by the vesicles were at a maximum at 0.5 M NaCl (59 H+/bacteriorhodopsin and 6.5 H+/bacteriorhodop-

![Fig. 7. Visible spectrum of bacteriorhodopsin in endogenous polar lipid vesicles. The spectrum of vesicles recovered in Peak II from Sepharose CL4B (Fig. 6) was recorded against (a) vesicles bleached in the presence of hydroxylamine (0.1 M) and (b) water.](image)

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Purple Membrane</th>
<th>BR-HhLV</th>
<th>BR-SBLV</th>
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<td>0.15 M KCl</td>
<td>560</td>
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<td>+9</td>
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<tr>
<td>10 mM Tris-HCl, pH 8</td>
<td>560</td>
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<td>+9</td>
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<tr>
<td>1.1 M NaCl</td>
<td>561</td>
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The Effect of pH on the Absorption Spectrum of Bacteriorhodopsin in Endogenous Lipid Vesicles—The pH-dependent changes in the visible spectrum of bacteriorhodopsin in endogenous lipid vesicles are shown in Fig. 10. Increasing the pH from 7.3 to 11 caused only a minor blue shift of the absorption maximum, with no loss of the chromophore. At pH 11.6, a partial loss of the chromophore was detected; and at pH above 12, a complete loss of the chromophore was found. This alkali-dependent bleaching of bacteriorhodopsin was only partially reversed after neutralization of the sample.

Acidiﬁcation of the vesicle preparation with HCl resulted in an approximately 50-nm red shift of the absorption maximum of the chromophore (Fig. 10). The apparent pH value for the transition of the spectrum from an absorption maximum of 554 nm (at pH 7.3) to 601 nm (at pH 3.7) was estimated to be 4.5 ± 0.2 (inset, Fig. 10). At pH below 3.5, loss of the chromophore was observed. However, complete restoration of the retinyl-protein complex occurred when the pH of the sample was adjusted to 7.5. At pH 3.9, the vesicles showed a significant proton-translocating activity (0.4 H+/bacteriorhodopsin/s and 18 H+/bacteriorhodopsin), indicating that they were intact. Furthermore, at this pH the K+-loaded vesicles were found to behave as a coupled membrane system; i.e. with no illumination but in the presence of the protonophore, CCCP, an uptake of protons by the vesicles was observed upon the addition of valinomycin.

DISCUSSION

Reconstitution of delipidated bacteriorhodopsin with the negatively charged polar lipids isolated from H. halobium has been found to yield vesicle preparations which, upon illumination, translocate protons from the medium to their interior and, therefore, have an inside out orientation of bacteriorhodopsin. The same orientation has previously been shown for vesicles prepared either by cholate dialysis method from delipidated bacteriorhodopsin or by sonication of purple membrane using synthetic lecithins or soybean phospholipids (11, 12, 26). However, the endogenous lipid vesicles now prepared have a number of characteristics which are advantageous for bacteriorhodopsin studies. One important property of these vesicles is that they translocate very few protons unless the ionophore valinomycin is present. Under optimal conditions, the proton uptake by these vesicles was stimulated more than 15-fold by valinomycin. In contrast, valinomycin stimulation of proton translocation by bacteriorhodopsin in SBPL vesicles, which normally translocate protons efﬁciently, was only 1.5-1.8-fold (Fig. 2). This difference in response to valinomycin is probably due to a much lower ion permeability of the endogenous lipid vesicles relative to SBPL vesicles. It has been estimated that translocation of only a few protons, if not compensated by the ﬂow of other ions, is sufﬁcient to create a substantial membrane potential (29). Therefore, it is very likely that the very low proton uptake by bacteriorhodopsin-HhPL vesicles, in the absence of valinomycin, is the result of a rapid formation of an inhibitory membrane potential. That a preformed membrane potential also could inhibit proton translocation by bacteriorhodopsin-HhPL vesicles was indicated by the very low proton uptake of the vesicles in the presence of valinomycin and high external K+ concentration (Fig. 9). It should be added that inhibitory effects on enzyme activities by a membrane potential rapidly formed as a result of proton translocation has been reported for other energy-transducing proteins; for example, mitochondrial nicotinamide nucleotide transhydrogenase and cytochrome oxidase, when incorporated into well-deﬁned lipid vesicles (30). The substantial proton translocation observed in H. halobium cells and cell envelope vesicles (31) in the absence of ionophores might be due to, as in the case of mitochondria (32), the presence of

Fig. 9. Effect of salt on proton translocation by bacteriorhodopsin-containing vesicles. Proton-translocating activity of bacteriorhodopsin vesicles reconstituted with endogenous polar lipids was determined as described under “Experimental Procedures” at the salt concentrations indicated.

Fig. 10. pH-dependent spectral shifts of bacteriorhodopsin in endogenous lipid vesicles. Spectrum of dark-adapted bacteriorhodopsin in vesicles was recorded at room temperature at various pH values indicated on the figure. Inset, titration of vesicles with microliter additions of HCl (0.1 M) or KOH (0.1 M).
other active transmembrane processes coupled to the translocation of protons (31).

Additional features of the vesicles reconstituted with endogenous H. halobium polar lipids were as follows. Analysis of maximally active vesicle preparations, reconstituted with lipid to protein ratios of 40:1 to 80:1, by sucrose density centrifugation shows that all lipids co-migrate with bacteriorhodopsin (Fig. 5). In contrast, vesicles made from SBPL comprise a large fraction of free lipids and several separate fractions of protein-containing vesicles.4 Although the majority of the endogenous lipid vesicles migrate as a rather distinct band in a sucrose gradient, a minor population of the vesicles are found at a higher density. The latter turbid fraction can be efficiently removed from the bulk of vesicles by gel filtration, and an almost clear preparation suitable for spectroscopic investigation is thus obtained. These vesicles are very stable, no loss in proton-translocating activity being observed after 4 months of storage; whereas bacteriorhodopsin in SBPL vesicles is totally bleached after 1 month under comparable conditions. The diameter of the bacteriorhodopsin-containing H. halobium lipid vesicles ranged between 250 and 500 Å as judged by electron microscopy. This size of the vesicles was considerably smaller than that reported previously for SBPL vesicles (11). Finally, spectral properties of bacteriorhodopsin in HnPL vesicles more closely resemble those of bacteriorhodopsin in purple membrane than those of bacteriorhodopsin in SBPL vesicles.

Several laboratories have reported on direct incorporation of purple membrane into endogenous lipid vesicles by sonication (12–15). In general, these attempts yielded preparations with low proton-translocating activity which may be the result of a random orientation of bacteriorhodopsin and/or ineffective vesicle formation. Reconstitution of partially lipid-depleted bacteriorhodopsin with polar lipids from H. halobium as reported by Hwang and Stoeckenius (16) resulted in a preparation with negligible proton-translocating activity. The authors concluded from freeze-fracture electron micrograph of the preparation that the vesicles were not spherical and that the vesicle walls consisted of crystalline domains with an equal inside out and right-side out orientation of bacteriorhodopsin particles. Thus, the discrepancy between the present investigation and that by Hwang and Stoeckenius (16) might be that a fully dispersed bacteriorhodopsin preparation is necessary for reconstitution with H. halobium polar lipids to give functional vesicles with a uniform orientation of the protein.

It is well established that in purple membrane sheets, bacteriorhodopsin is present as trimers which, in turn, form a hexagonal lattice (1). Upon illumination, the visible absorbance of the chromophore displays a red shift of 10 nm (1). Recently it has been proposed that the extent of the red shift of the chromophore after illumination may serve as a convenient indicator to distinguish between aggregated (trimeric) and monomeric states of the protein (33). In the present work, a 7-nm shift of the absorption maximum of bacteriorhodopsin in the endogenous lipid vesicles after illumination was observed. This shift is larger than that observed for monomeric bacteriorhodopsin in other systems. Further, pH and salt (Table 1) seemed to influence the light-induced spectral shift of the chromophore; and in the presence of both valinomycin and CCCP, the Absmax shift was increased in 10 nm (data not shown). Whether bacteriorhodopsin in H. halobium lipid vesicles is indeed present partly or largely in an aggregated state requires further study.

Upon acidification, the absorption maximum of bacteriorhodopsin in H. halobium lipid vesicles was red shifted approximately 50 nm, similar to the spectral shift reported for bacteriorhodopsin in purple membrane at low pH (34, 35). It has been suggested that red shift of the absorption maximum of the protein is caused by protonation of an ionizable group in the vicinity of the chromophore rather than titration of the retinyl Schiff’s base (35). The apparent pK, (~4.5) for the spectral shift now reported for the absorption maximum of bacteriorhodopsin in endogenous lipid vesicles was higher than that (3.1) reported for purple membrane sheets (34, 35). This difference in pK, could be due to higher amounts of acidic polar lipids present in these vesicles than in purple membrane. At low pH the intactness of the endogenous lipid vesicles appeared to be retained since significant proton translocation was observed upon illumination. Furthermore, with no illumination but in the presence of the protonophore, CCCP, these K+-loaded vesicles could take up protons after addition of valinomycin, a result which would be expected of an intact coupled membrane system. The instantaneous shift of the absorption of bacteriorhodopsin upon acidification thus indicated that the ionizable group was accessible only from the exterior of the vesicles. That this ionizable group is not on the inside of the lipid bilayer was further supported by the observation that protonation of bacteriorhodopsin containing endogenous lipid vesicles, in the presence of valinomycin, did not result in a substantial red shift of the spectrum, beyond the expected 7–9-nm shift, although the interior of the vesicles should have been acidified under these conditions.

The photocycle of bacteriorhodopsin in vesicles has been reported to show different kinetics from that of the chromophore in purple membrane sheets, and a possible regulation of the photocycle by an electrochemical gradient of protons was suggested (36, 37). Recently, a direct relationship between the steady state concentration of the M+ intermediate of the photocycle and the membrane potential has been demonstrated (38). Whether the inhibition of proton translocation by the coupled bacteriorhodopsin-containing H. halobium lipid vesicles observed in this investigation might be the result of an altered photocycle of the protein remains to be determined.

The underlying mechanism of proton translocation as a result of photochemical events of bacteriorhodopsin is at present far from understood. The delipidation and reconstitution of bacteriorhodopsin with H. halobium polar lipids into functional and homogeneous vesicles may prove to be a suitable system for elucidating the molecular mechanism by which this retinal-protein complex translocates protons.

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