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Biochemistry

Delipidation of bacteriorhodopsin and reconstitution with exogenous phospholipid

(*Halobacterium halobium*/purple membrane/proton pump/retinal/inside-out orientation)

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ABSTRACT **Solubilization of the purple membrane from *Halobacterium halobium* with the detergent Triton X-100 followed by gel filtration in deoxycholate solution gave bacteriorhodopsin that was more than 99% free from endogenous lipid. The delipidated bacteriorhodopsin was reconstituted with exogenous phospholipids to form vesicles which on illumination efficiently translocated protons. The direction of proton pumping was from the outside to the interior of the vesicles, indicating that the orientation of bacteriorhodopsin in the vesicles was opposite to that in the bacterial membrane. This orientation was confirmed by cleavage of the carboxyl terminus of the protein by proteolysis from the outside of the vesicles.**

The cell membranes of a number of extremely halophilic bacteria, such as *Halobacterium halobium*, contain highly organized patches of a purple membrane which functions as a light-driven proton pump (1, 2). The purple membrane contains a single protein, bacteriorhodopsin (M_r, 26,788), each molecule of which has one molecule of retinal covalently linked as a Schiff base. Bacteriorhodopsin, whose amino acid sequence is now known (3, 4), contains seven α-helical rods about 40 Å in length, which are largely embedded in the membrane (5). The orientation of the protein in the purple membrane is such that the carboxyl terminus is on the cytoplasmic side (6, 7). The lipids of the membrane all have dihydrophytanol side chains that are ether linked to a glycerol backbone that is attached to a polar headgroup.

Reconstitution of the proton pumping activity of delipidated bacteriorhodopsin with exogenous phospholipids is necessary for precise chemical and biochemical studies of this system. The activity was first reconstituted by Racker and Stoeckenius (8, 9) without removal of the endogenous phospholipids of the purple membrane. Subsequently, a number of studies on solubilization of the purple membrane and exchange or removal of the endogenous phospholipids have been reported (10–15). However, complete delipidation and satisfactory reconstitution of the proton pumping activity have so far not been achieved. In this paper we report on the preparation of bacteriorhodopsin that is more than 99% free from endogenous lipid and its reconstitution with added phospholipids. The reconstituted vesicles show light-dependent proton translocation to the extent of 50–70 protons per molecule of bacteriorhodopsin, which is several times higher than that of vesicles prepared directly from purple membrane sheets. The direction of proton translocation in these vesicles is from outside to inside as previously found for vesicles prepared from the purple membrane (8, 9) and is the opposite of that in intact bacterial cell envelopes. The "inside-out" orientation of bacteriorhodopsin in the reconstituted vesicles has been confirmed by proteolysis of the carboxyl terminus which is exposed on the outside of the vesicles.

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MATERIALS AND METHODS

Purple membrane was isolated from *H. halobium* (strain S9, originally obtained from W. Stoeckenius) as described (16). Soybean phospholipids (asolectin) were obtained from Associated Concentrates and were treated as described by Kagawa and Racker (17). Cholic acid and deoxycholic acid (from Sigma) were recrystallized from acetone. *n*-Octyl β-D-glucoside was the gift of Bruce Burnett or was synthesized (18). Triton X-100 was from Rohm and Haas Chemical Co. [^{3}H]Cholic acid, [^{3}H]deoxycholic acid, [^{3}H]Triton X-100, [^{3}H]inulin, and [^{32}P]phosphate all were from New England Nuclear; [2-^{14}C]-mevalonic acid lactone was from Amersham. Other materials were obtained as follows: soybean phosphatidylcholine, L-α-dioleoylphosphatidylcholine, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and sodium tetraphenylborate (Φ_4B^-), Sigma; L-α-dimyristoylphosphatidylcholine, valinomycin, and Pronase, Calbiochem; mercuripapain, Worthington; and *o*-iodosobenzoic acid, Pierce.

Protein and organic phosphate were assayed by published procedures (refs. 19 and 20, respectively). The protein content of the vesicles was taken to be the same as the amount of protein used for reconstitution. The bacteriorhodopsin concentration in suspensions of purple membrane was calculated by using a molar extinction coefficient of 6.3×10^4 (6).

Preparation of Delipidated Bacteriorhodopsin. A suspension of the purple membrane in water (10 mg of bacteriorhodopsin) was centrifuged. The pellet was resuspended, with the aid of a glass rod, in 0.5 ml of 5% (vol/vol) Triton X-100/0.1 M sodium acetate, pH 5.0 (1) and the mixture was allowed to stand, with occasional vigorous agitation (vortex mixer), in the dark at room temperature for 2 days. As found previously (1), most of the purple membrane was solubilized at this stage. The solution then was centrifuged at $8000 \times g$ for 1 min, and the cooled supernatant solution was applied to a column of Bio-Gel A-0.5m (1.5 × 100 cm) that had been preequilibrated with 0.01 M Tris·HCl/0.15 M NaCl/0.25% deoxycholic acid/0.025% NaN_3 titrated to pH 8.0 with NaOH. The column was eluted in the dark at 4°C with the above equilibration buffer. Fractions containing delipidated bacteriorhodopsin were rapidly frozen (−78°C) and stored in the dark at −20°C.

Analysis of Residual Lipids. Analysis of the lipids remaining in bacteriorhodopsin after delipidation was facilitated by using purple membrane labeled with ^{14}C or ^{32}P by described methods (11). Extraction of total lipids was performed according to Bligh and Dyer (21). The lipids were separated by two-dimensional thin-layer chromatography as described by Kushwaha *et al.* (22) with the modifications of Wildenauer and Khorana (11).

Reconstitution with Phospholipids. Phospholipids (7 mg) in $CHCl_3$ were dried under a stream of N_2, dissolved in ether,

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Φ_4B^-, tetraphenylborate.

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and dried once more under the N_2 stream. Residual solvent was removed with a vacuum pump (≈50 μm Hg) for at least 10 hr. To the dried lipid was added 0.8 ml of 2% sodium cholate in 0.15 M KCl at pH 8.0. The mixture was sonicated until it was clear and then was rapidly mixed with the delipidated bacteriorhodopsin (0.8 ml of the gel filtration buffer containing 0.19 mg of protein per ml) at 4°C. The clear solution was immediately dialyzed against 0.01 M Tris·HCl, pH 8.0/0.15 M NaCl/0.025% NaN_3 at room temperature for 2–3 days (two 1-liter changes per day) and then dialyzed for 1 more day against 0.15 M NaCl/0.025% NaN_3 (two 1-liter changes). Aliquots (50 μl) were assayed for proton pumping activity in 0.15 M NaCl or 2 M NaCl (1 ml) at pH 6.2 and 30°C, as described by Racker and Stoeckenius (8). Vesicles prepared with the above phospholipid-to-protein ratio were used in all the subsequent experiments except those reported in Table 2.

Pronase Digestion. Vesicles containing delipidated bacteriorhodopsin were concentrated by ultrafiltration in a CF25 Centriflo cone (Amicon, Lexington, MA) to a protein concentration of 0.75 mg/ml. To this solution (0.2 ml) were added 10 mM $CaCl_2$ (20 μl) and Pronase (3 μg). The mixture was incubated at 37°C for about 6 hr. The same conditions were used for digestion of purple membrane sheets.

Electrophoresis. Gel electrophoresis was performed in 15% polyacrylamide gels containing 0.1% sodium dodecyl sulfate ($NaDodSO_4$) as described by Laemmli (23). An organic solvent extraction effectively removed the large amount of phospholipid which otherwise interfered with the electrophoretic analysis of protein from reconstituted vesicles. Vesicles (20–50 μl) were mixed with methanol (1 ml), vortexed thoroughly, and centrifuged at 8000 × *g* for 2 min. The pellets were vortexed with 1 ml of toluene/ether, 2:1 (vol/vol). After a further centrifugation, the new pellets were dried in a vacuum centrifuge and dissolved in loading buffer containing 2% $NaDodSO_4$ for electrophoresis.

Reconstituted Bacteriorhodopsin Vesicles Containing Internal Radioactive Markers. Vesicles that contained bacteriorhodopsin were loaded with $^{32}P_i$ or [^{3}H]inulin as follows. For $^{32}P_i$-loaded vesicles, a buffer consisting of 9 vol of 0.01 M Tris·HCl, pH 8.0/0.15 M NaCl/0.025% NaN_3 and 1 vol of 50 mM NaP_i at pH 8.0 was used throughout. Delipidated bacteriorhodopsin and soybean lipid in cholate solution were mixed as above with [^{32}P]Na_2HPO_4 (250 μCi; 1 Ci = 3.7×10^{10} becquerels). The mixture (5 ml) was dialyzed against buffer (100 ml) containing $^{32}P_i$ (5 mCi) for 20 hr. Dialysis was then continued without $^{32}P_i$ in the buffer for 2 days. The resultant vesicle solution contained no external $^{32}P_i$. For [^{3}H]inulin-containing vesicles, a similar procedure was followed with buffer containing 0.1 mM inulin. After the final dialysis against unlabeled buffer, the vesicles were passed through a short column of Bio-Gel P-60 in buffer containing no inulin, to remove external [^{3}H]inulin. The release of radiolabeled markers from the vesicles during proteolysis was monitored by chromatography of samples (25 μl) on Bio-Gel P-2 contained in a pasteur pipet; the dialysis buffer was used as the eluant. Control experiments showed that five freeze-thaw cycles or treatment with 1% Triton X-100 released the contents of the vesicles.

Digestion of Pronase-Treated Vesicles with Papain. Vesicles containing bacteriorhodopsin were digested with Pronase under conditions such that removal of the carboxyl-terminal fragment was optimal. After passage through Bio-Gel P-60, the vesicles (0.54 mg of protein per ml) were digested with papain at a substrate-to-enzyme ratio of 10:1 at 37°C for 20 hr.

Chemical Cleavage of Bacteriorhodopsin from Pronase-Treated Vesicles. The bacteriorhodopsin was freed from lipid by methanol precipitation followed by extraction with toluene/ether, 2:1 (vol/vol). Samples (250 μg) were dissolved in 70% HCO_2H (100 μl) and treated with cyanogen bromide (10 μl of 200 mg/ml) or pipetted onto 1 mg of iodosobenzoic acid (24). After 20 hr at 25°C, the solvent was removed and the residue was redissolved in formic acid/ethanol/10% aq. $NaDodSO_4$, 3:7:2 (vol/vol), dried, and redissolved in 8 M urea. Portions were examined by electrophoresis in the system of Swank and Munkres (4, 25) using 12.5% polyacrylamide gels.

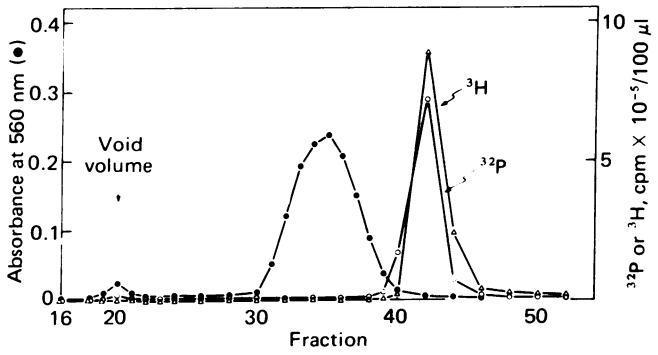

FIG. 1. Removal of endogenous lipid from bacteriorhodopsin. ^{32}P-Labeled purple membrane (2 mg; 3.0×10^7 cpm) was suspended in buffer (0.1 ml) containing ^{3}H-labeled Triton X-100 (3.8×10^7 cpm) and the solution was applied to a column of Bio-Gel A-0.5m (1.0 × 100 cm) in the presence of 0.25% deoxycholate. Fractions (1.2 ml) were collected at a flow rate of 7 ml/hr.

RESULTS

Delipidation of Purple Membrane. When ^{32}P-labeled purple membrane was solubilized in [^{3}H]Triton X-100 and subjected to gel filtration in the buffer containing 0.25% deoxycholate, the elution profile shown in Fig. 1 was obtained. The delipidated bacteriorhodopsin was monitored by its absorbance at 560 nm. The absorption maximum was at 538 nm (ϵ = 58,000; A_{280}/A_{540}, 1.8–2.0). No significant loss of retinal occurred during chromatography as judged by the absence of free retinal (A_{385}) in any of the fractions emerging from the column. As judged from the distribution of ^{32}P (lipid) and ^{3}H (detergent), at least 99% of the polar lipid and all of the Triton X-100 were separated from bacteriorhodopsin.†

The experiment of Fig. 1 was repeated using purple membrane from cells grown in the presence of [^{14}C]mevalonate. As shown previously (11), ^{14}C was not incorporated into the polypeptide chain under these conditions, but retinal and the other membrane lipids were labeled. After solubilization and gel filtration of the ^{14}C-labeled purple membrane, about 19% of the radioactivity remained associated with the protein. Analysis by thin-layer chromatography showed that 98% of this residual lipid was retinal. Therefore, by this analysis, ≈0.4% of the polar lipid remained associated with the protein.

Delipidated bacteriorhodopsin in 0.25% deoxycholate solution slowly lost retinal at 4°C, but it could be stored frozen at −20°C in the dark for at least 3 months without loss of the chromophore. Deoxycholate could be replaced by 1% cholate during the gel filtration step.

Recently, octyl glucoside has been used to solubilize bacteriorhodopsin (15). When purple membrane solubilized in Triton X-100 or in 5% octyl glucoside was chromatographed on Bio-Gel A-0.5m in buffer containing 1% octyl glucoside and 25 mM sodium phosphate at pH 6.9 (15), not more than 90%

† The recovery of bacteriorhodopsin from the column was usually about 75%. However, when a new column was used, the recovery was only 50%; after two or three runs, the recovery improved.

of the polar lipids (^{32}P-labeled) were removed from the bacteriorhodopsin.

Reconstitution of Delipidated Bacteriorhodopsin into Phospholipid Vesicles. Reconstitution was performed by a modification of the cholate dialysis method (17) using soybean phospholipids or other phosphatidylcholines. ^{3}H-Labeled cholate and deoxycholate were used to monitor the removal of these detergents during reconstitution. The results indicated that ≈0.3% of the cholate and ≈3% of the deoxycholate remained after 3 days of dialysis at pH 8.0 and that no further removal was possible. Therefore, the vesicles contained ≈1 deoxycholate or cholate molecule per 60 phospholipid molecules. When vesicles were formed in the absence of bacteriorhodopsin, the same amount of residual detergent was found, and the addition of 10 times the residual amount to vesicles in the assay buffer did not affect the proton pumping activity. Assuming that the extinction coefficient of bacteriorhodopsin was unchanged during reconstitution, at least 70% of the retinal was still covalently attached to bacteriorhodopsin after reconstitution as determined by difference spectroscopy against a sample of vesicles bleached with hydroxylamine.

To estimate the size of the bacteriorhodopsin-containing vesicles, they were chromatographed on a Bio-Gel A-150m column (1.0 × 50 cm) in 0.01 M Tris·HCl/0.15 M NaCl, pH 8.0. Although some phosphate-containing material appeared at the void volume, the bulk (≈90%) of the material was present in the major second peak. The position of elution (26) and electron microscopy with negative stain indicated that the radius of the majority of the vesicles was between 200 and 500 Å.

Proton Translocation by Reconstituted Vesicles. A typical time course for the light-dependent pH change observed with vesicles reconstituted with soybean phospholipids is shown in Fig. 2*A*. The direction of the light-driven translocation of protons was from the outside to the inside of the vesicles, as is the case with vesicles reconstituted from the purple membrane and phospholipids by sonication (9). Vesicles directly prepared from an equivalent amount of the purple membrane gave the pH change shown in Fig. 2*B*. For the vesicles reconstituted from delipidated material, the initial rate of proton pumping was somewhat greater than that observed with directly reconstituted vesicles and the total number of protons pumped per molecule of bacteriorhodopsin was considerably larger. The pH response was sensitive to low concentrations of the uncoupler CCCP (Fig. 2 *C* and *D*), as has been shown (8) for reconstituted purple membrane vesicles. Φ_4B^- (Fig. 2 *E* and *F*) and K^+ valinomycin (data not shown) stimulated the response.

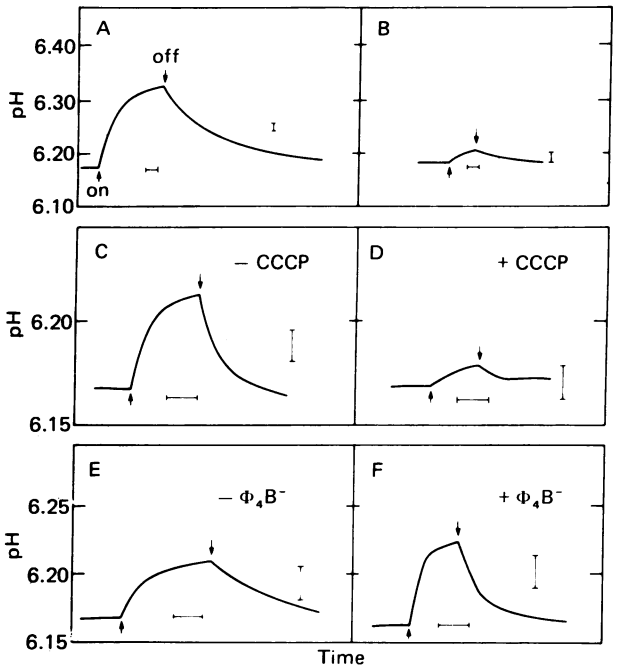

FIG. 2. Light-dependent proton translocation by reconstituted bacteriorhodopsin vesicles. Vesicles in *A* and *C–F* were reconstituted from delipidated bacteriorhodopsin and soybean phospholipids; the vesicles in *B* were made from purple membrane sheets and soybean lipids (9). In all assays, the total volume was 1 ml. The NaCl concentration was 2 M in *A–D* and 0.15 M in *E* and *F*. The protein-to-lipid ratio was 1:50 in *A* and *B* and 1:80 in *C–F*. The protein concentration was 3 μg/ml in *A* and *B*, 1.5 μg/ml in *C* and *D*, and 4 μg/ml in *E* and *F*. CCCP was present in *D* at 10 nM; Φ_4B^- was present in *F* at 10 μM. Proton translocation was measured by the method of Racker and Stoeckenius (8) in which the pH of the external medium is monitored. Horizontal scale = 30 sec; vertical scale = 1 neq of H^+.

Table 1. Proton pumping activities of bacteriorhodopsin reconstituted with different phospholipids

Phospholipid	Initial rate, no. H^+/bacteriorhodopsin molecule/sec	Total H^+ pumped, no./bacteriorhodopsin molecule
Soybean phospholipids	1.50	74.0
Soybean phosphatidylcholine	0.90	24.0
Dioleoylphosphatidylcholine	0.36	5.4
Dimyristoylphosphatidylcholine	0.21	1.9

The proton pumping activity was assayed in 2 M NaCl in 30°C (8).

In Table 1 are compared the proton pumping activities of reconstituted vesicles prepared with different phospholipids. Under the conditions tested, the highest response was observed when soybean lipids were used, and the weakest response was obtained with the saturated phospholipid dimyristoylphosphatidylcholine.

It is highly significant that in the reconstituted vesicles all the bacteriorhodopsin that we could account for had an inside-out orientation. Further confirmation was obtained by a number of proteolysis experimnts.

Table 2 shows the influence of the ratio of soybean phospholipids to bacteriorhodopsin on proton translocating activity. The activity increased with decreasing amounts of protein and reached a plateau when the ratio (wt/wt) of lipid to protein was ≈50. This is most likely due to the lower internal volume/

Table 2. Influence of phospholipid/bacteriorhodopsin ratio on extent of proton translocation in reconstituted vesicles

Ratio (wt/wt)	Protons pumped, no./bacteriorhodopsin molecule
2.5	0.6
5.0	2.0
10.0	12.0
20.0	30.0
40.0	49.0
80.0	60.5
120.0	52.5

The vesicles were reconstituted from delipidated bacteriorhodopsin and soybean phospholipids. Samples were assayed for proton pumping activity in 2 M NaCl at 30°C.

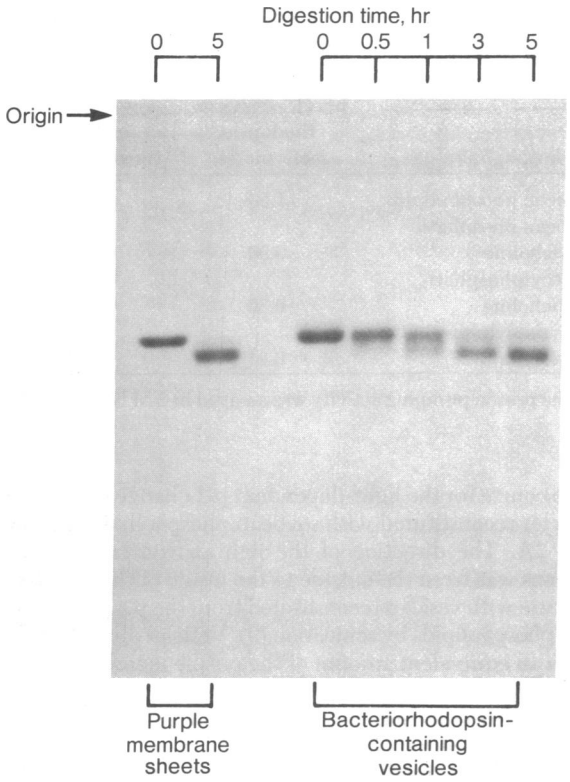

FIG. 3. Proteolysis of purple membrane sheets and bacteriorhodopsin-containing vesicles with Pronase. At the indicated intervals, samples containing 7.5 μg of protein were removed, extracted to remove lipid, and prepared for electrophoresis on a 15% polyacrylamide slab gel (23).

protein ratio in those vesicles containing large amounts of protein.

Orientation of Bacteriorhodopsin in Reconstituted Vesicles. Gerber *et al.* (6) have shown that bacteriorhodopsin in purple membrane sheets and in vesicles reconstituted from the sheets is cleaved by Pronase at the carboxyl terminus. A number of experiments were carried out to determine the orientation of bacteriorhodopsin in the vesicles prepared in the present work. The action of Pronase on vesicles reconstituted from delipidated bacteriorhodopsin and on purple membrane sheets was compared. In both cases, most of the bacteriorhodopsin was cleaved to a slightly shorter product: ≈10 amino acids were removed (Fig. 3). That the vesicles were not permeable to the protease was shown by using vesicles containing internal markers ([^{3}H]inulin and $^{32}P_i$). No release of radioactivity into the medium was observed after prolonged digestion of the vesicles with Pronase.

In a subsequent experiment, the reconstituted vesicles were digested with papain after cleavage with Pronase. The sequential digestions removed a total of about 20 amino acids to yield a product that comigrated on $NaDodSO_4$ electrophoresis with the product derived from purple membrane cleaved with papain alone. Because the latter cleavage is known to be at the carboxyl terminus (3), it was likely that the Pronase cleavage of the bacteriorhodopsin in vesicles was also at this end of the molecule. Furthermore, chemical fragmentation of bacteriorhodopsin from Pronase-treated vesicles followed by separation of the fragments by electrophoresis showed that the carboxyl-terminal fragment [CNBr-6 (4)] and the analogous *o*-iodosobenzoic acid fragment were absent. Finally, digestion of the bacteriorhodopsin-containing vesicles with carboxypeptidase A sequentially released only the expected COOH-terminal amino acids Ser (1.0), Thr (1.0), and Ala (1.5–2.0), confirming that the COOH terminus is exposed on the outside of the vesicles.

From these data, we conclude that bacteriorhodopsin in vesicles uniformly has an inside-out orientation.

DISCUSSION

In this paper we have described an efficient method for the delipidation of bacteriorhodopsin and its subsequent reconstitution with exogenous lipid. After solubilization of the purple membrane in Triton X-100, both the natural lipids and the Triton X-100 were removed by gel filtration in the presence of deoxycholate solution. The removal of endogenous lipids is complete to the extent of at least 99%, and any residual lipid must be at the level of <0.1 mol/mol of bacteriorhodopsin monomer.

A number of studies have been reported previously on lipid exchange, solubilization, and delipidation of the purple membrane. Experiments on delipidation using dodecyldimethylamine-*N*-oxide (11) or the cationic detergent dodecyltrimethylammonium bromide (10) gave bacteriorhodopsin preparations that were not completely free of endogenous lipid. After reconstitution, these preparations had only low proton translocating activity. Attempts to exchange purple membrane lipids with exogenous lipid (27) also led to the incomplete removal of the natural lipid (13). Hwang and Stoeckenius (12) were able to remove ≈80% of the natural lipid with deoxycholate, and after partial removal of the detergent they could reconstitute the delipidated protein with phospholipids to give vesicles with high proton translocating activity. These authors concluded that to obtain this high activity it was necessary to maintain the integrity of the two-dimensional lattice during delipidation. However, the present work shows that dispersed bacteriorhodopsin molecules can be efficiently reconstituted.

It is highly significant that in the reconstituted vesicles all the bacteriorhodopsin that we could account for had an inside-out orientation. This conclusion was first indicated by the highly efficient uptake of protons *into* the vesicles on illumination: the extent of proton uptake was severalfold greater than that found when purple membrane is directly reconstituted (Fig. 2). Further confirmation was obtained by a number of proteolysis experiments. The factors controlling the "sidedness" of the protein after reconstitution are obscure. Happe *et al.* (28) reported that, under certain conditions, vesicles that pump protons into the medium can be obtained. However, the efficiency of proton translocation in those preparations was very low. In the present case, an intact carboxyl terminus was not required for "inside-out" orientation because purple membrane sheets that had been treated with papain could be taken through the entire delipidation and reconstitution procedure to yield vesicles with proton pumping activity similar to those prepared from native bacteriorhodopsin.

The state of the delipidated bacteriorhodopsin (crystalline, trimeric, or monomeric) in deoxycholate or in the bilayer of the reconstituted vesicles is not yet known. The delipidated protein runs in the included volume of Bio-Gel A-0.5m, which rules out a structure consisting of large aggregates. Further work is needed to distinguish between a monomeric and a trimeric structure in deoxycholate. The average number of bacteriorhodopsin molecules in the most active vesicles (Table 2) was ≤25, which makes an extented lattice improbable. Because there is current concern over the possible activity of the protein monomer (14, 29), this point is worthy of further study.

A number of observations indicate that the tertiary structure of bacteriorhodopsin is essentially preserved in the detergent. Thus, the visible absorption spectrum of the deoxycholate so-

lution clearly shows interaction of the retinylidene chromophore with the protein. This preservation of the tertiary structure is likely a consequence of strong interactions between the seven α-helical segments present in bacteriorhodopsin (5). Our preliminary results suggest that bacteriorhodopsin that has been cleaved by chymotrypsin between amino acids 71 and 72 can also be reconstituted into functional vesicles. In this case, the two fragments are tightly bound to one another in detergent. Further work is required to clarify both the tertiary and quaternary structures of the protein in solution, but it should be added that, if the above conclusion is correct, it may bear on the assembly into the membrane of this protein and others that traverse the membrane multiple times. Proteins such as bacteriorhodopsin may insert into the membrane only after the synthesis of the polypeptide chain is complete.

The solutions of delipidated bacteriorhodopsin that we have obtained contain simply the protein, buffer components, and deoxycholate, a monodisperse detergent of known molecular structure. This chemically well-defined preparation as well as the vesicles prepared from it with synthetic lipids should be useful for various chemical and biophysical studies.

Note Added in Proof. Recently, Dencher and Heyn (30) have incorporated detergent-solubilized bacteriorhodopsin into phospholipid vesicles without prior removal of the purple membrane lipids. These vesicles pumped protons efficiently from outside to inside, and the pumping activity was abolished by CCCP. Furthermore, these authors concluded that bacteriorhodopsin monomers pump protons.

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