Expression of the bacterio-opsin gene in *Escherichia coli* has been described in the accompanying papers. We now describe rapid and efficient methods for the purification of the *E. coli*-expressed bacterio-opsin. Bacterio-opsin can be extracted from *E. coli* membranes in a denatured form by using an organic solvent containing chloroform, methanol, water, and triethylamine. The bacterio-opsin, enriched to 30–50% in the extract, can be further purified to 90% by ion-exchange chromatography on DEAE-Trisacryl or hydroxylapatite chromatography in organic solvents or by preparative sodium dodecyl sulfate gel electrophoresis. In appropriate aqueous phospholipid/detergent mixtures, up to 80% of purified protein refolds and binds retinal covalently to regenerate the bacteriorhodopsin chromophore. When reconstituted into phospholipid vesicles, bacteriorhodopsin from *E. coli* shows the expected proton pumping activity in response to illumination.

Recombinant DNA methods allow structure-function studies of proteins by specific amino acid substitutions in the primary sequence. In order to apply these techniques to the study of the integral membrane protein bacteriorhodopsin (bR), we have investigated the expression of the bR gene (1) in a number of vectors and have obtained an adequate level (~1%) of expression in *Escherichia coli* (2, 3). Furthermore, to facilitate site-specific mutagenesis of the gene, we have designed and carried out the total synthesis of the bR gene with suitably placed unique restriction sites (4).

The purification of bacterio-opsin (bO) as expressed in *E. coli* at low levels has been an outstanding problem. Previously, a monoclonal anti-bR antibody (5) was used for a one-step immunoaffinity purification of bO from crude *E. coli* membranes (e-bO) (2, 6). Whereas purification of e-bO to 70–80% was thus achieved in a single step, the procedure entailed losses, and there was a severe limit on scaling up. For a variety of studies that we wish to carry out, milligram amounts of the mutant e-bO proteins are required. Therefore, we have devoted a considerable effort to developing alternative methods to purify e-bO from relatively large amounts of *E. coli* membrane. The present study describes the methods that have resulted. The most significant observation made in this work has been that denatured bO is quite soluble in mixtures of chloroform, methanol, and water at low or high pH. Thus, we have developed organic solvent extraction and chromatographic techniques which are similar to those used to purify proteolipids from myelin and other sources (7–10).

**e-bO as extracted and purified from *E. coli* membranes is probably in a denatured state. Earlier work (11–14) has established conditions under which it is possible to denature and denature bR from *Halobacterium halobium* (h-bR) and then to restore the visible absorption band and proton pumping activity characteristic of the native bR by adding back exogenous retinal and phospholipids. We have refined these methods and are now able to reconstitute e-bR with essentially the same activity as h-bR.**

**EXPERIMENTAL PROCEDURES**

**Materials**

All-trans-retinal, RNase A, DNase I, phenylmethylsulfonyl fluoride, Sephadex LH-60, and electrophoresis-grade SDS were obtained from Sigma. Soybean lipids (L-a-phosphatidylcholine, Type II-S) were obtained from Sigma and were purified by twice precipitating them from ether solution with acetone (15) using 50 μM diethylthreitol as an antioxidant. Octyl glucoside, DMPC, and CHAPS were from Behring. Protein A-conjugated Sepharose 4B was obtained from Repligen; DEAE-Trisacryl was from LKB; and hydroxylapatite (BioGel HTP) was from Bio-Rad. Chlorofrom and methanol were Omnisol-G grade (EM Science). Water was purified using a commercial deionizer. Other solvents were reagent-grade.

**Buffers**—Buffer A contained 0.15 M NaCl, 0.010 M NaPi, pH 7.0, 0.02% (w/v) NaN3; Buffer B contained 0.100 M NaPi, pH 6.0, 0.025% NaN3.

**Organic Solvent Mixtures**—Solvent A contained chloroform/methanol/water (TEA 100:100:25:1); Solvent B was Solvent A without water (chloroform/methanol/TEA (100:100:1)); Solvent C was chloroform/methanol/acetic acid (100:100:1); Solvent D was chloroform/methanol/water/acetic acid (100:100:25:4); and Solvent E was chloroform/methanol/water (4:4:1). TEA acetate solutions of various concentrations were prepared by adding equimolar amounts of TEA and acetic acid to Solvent E.

The solvent compositions given above are volume ratios. Except where mentioned, all operations using these organic solvents were carried out in clean, dry glassware with Teflon fittings.

**Methods**

**Growth of Cells**—e-bO was produced by *E. coli* (strain C900) which had been doubly transfected with the plasmids pPL-BO6 and pCL857 (9). Cells were grown at 30°C in 10-l fermentor culture using an...
enriched LB medium (16). Expression of bOl was induced in mid-log growth phase (4–10 g of cells, dry weight/liter of culture) by raising the temperature of the culture to 42 °C for 10 min. Cells were harvested by centrifugation (15 min, 5,000 × g, 4 °C) and then frozen. Thawed cells were resuspended in phosphate-buffered saline containing MgCl2 (0.1 M) (CSM), DNase (20 µg/ml), RNase (20 µg/ml), and the protease inhibitor phenylmethylsulfonyl fluoride (200 µg/ml) and were lysed using a French pressure cell (10,000 p.s.i.). Membranes were pelleted by centrifugation (60 min, 40,000 × g, 4 °C). Pellets of whole cells or membranes were stored at −70 °C as lyophilized membranes—Lipids and highly lipophilic proteins, including denatured eOl, were extracted from E. coli membranes or whole cells in Solvent A. Since Solvent A contains a saturating amount of water and therefore separates into two phases with an incremental addition of water, the wet pellet was mixed initially with water-free solvent (Solvent D) until a single liquid phase was obtained. Alternatively, membranes or whole cells were lyophilized and then mixed directly with Solvent A, and the pellet and solvent were homogenized thoroughly (1 min, Tekmar SDT Tissueizer with Teflon bearing). After centrifugation (20 min, 8000 × g, room temperature), the clear solution was decanted and the pellet was re-extracted with the same solvent. Typically, membranes (~5 g, wet, or 1 g, dry weight) were extracted three times with Solvent A, each time with 40 ml. The solven extracts were combined (total volume, 120 ml), and then a phase separation was effected by the addition of 220 ml of Buffer B. After centrifugation (10 min, 8000 × g, 4 °C), the supernatant was washed with water, and the resulting solid interphase, which adhered to the walls, was washed with distilled water (2 × 100 ml). The wet pellet was stored at −20 °C.

Delipidation of eOl Extract—Solvent B was added to the wet pellet until a single liquid phase containing some insoluble material was obtained. The latter was then removed by centrifugation. The total supernatant solution (5 ml) was loaded on a Sephadex LH-20 column (1.5 × 50 cm) and eluted with Solvent A at 20 ml/h. The 280 nm absorbing peak at the void volume contained lipid-depleted protein. Alternatively, the phospholipids were removed from the wet eOl pellet by extraction with chloroform/methanol (72:28). A sufficient volume of solvent was added to dissolve all of the water in the wet pellet (~40 ml of solvent/ml of water). The lipid-depleted protein was collected by filtration (Whatman 1) and redissolved in Solvent A.

Hydroxylapatite Adsorption Chromatography in Organic Solvent—Dry hydroxylapatite (Bio-Gel HTP, 0.5 g) was washed with Solvent D (2 × 5 ml) and then with Solvent C (2 × 5 ml). Lipid-depleted extract of E. coli membranes in Solvent A (5 ml, 2–5 mg of protein/ml) was mixed with 2 volumes of the acidic solvent (Solvent C). A flocculent precipitate which formed was removed by centrifugation. The supernatant solution was mixed with half of the washed hydroxylapatite (1 g, wet weight). The hydroxylapatite suspension was poured into a column stopped with glass wool, on top of a previously poured layer of the other half of the washed hydroxylapatite. After the loading solution had flowed through the column, the column was washed with 5 ml of Solvent C and then with 5 ml of Solvent D. Finally, bOl was eluted with 10 ml of Solvent D to which water had been added to a concentration (15% volume/volume) of 0.5 ml were collected; those containing protein (as monitored by 280 nm absorbance) were pooled. Addition of 3 volumes of 0.2 M NaHPO4 resulted in a phase separation, and bOl was recovered as a waxy solid at the interface. The bOl was washed twice with water and then redissolved by adding 1 ml of Solvent B.

Iso-exchange Chromatography on DEAE-Trisacryl in Organic Solvent—DEAE-Trisacryl was washed successively with 4 volumes each of water, methanol, and Solvent E. It was then packed into a 0.9 × 10-cm column. The column was equilibrated in Solvent E containing 50 mM Tris-acetate buffer (pH 8.0), 0.5 M NaCl, 2 mM MgCl2, and 0.5 mM DTT; the flow rate was 1 ml/h. The column was loaded with 50 ml of 10-mg/ml solution of Solvent C was loaded on the column. The column was washed with 50 ml of buffer (pH 8.0, 50 mM Tris-acetate, 0.5 M NaCl, 2 mM MgCl2) containing bOl was pooled. The 280 nm absorbance of the effluent reached a flat base line. Elution was started using a linear gradient in Tris-acetate concentration (50–200 mM, using 2 column volumes each of initial and final concentrations in a gradient mixer chamber). Fractions of 2 ml were collected and analyzed by SDS gel electrophoresis and immunoblot analysis using CSM and CSM modified to contain reconstituted bOl in a 1 ml total volume was added to 100–250 µl of aqueous SDS solution (10% (w/v)). The exact amount of SDS was chosen to give a final SDS:protein ratio of 5:1 (w/w). If a phase separation occurred, Solvent B was added until the solution was homogeneous. The solvent was added until the volume of aqueous solution reached 250 µl, and then the mixture was added to a lyophilizer, making sure that the vacuum reached 100 millitors for at least 1 h. To the dry powder was added to give a final SDS concentration of 0.5% (protein concentration, ~1 mg/ml). After vortexing gently to dissolve the SDS and protein, insoluble material was removed by centrifugation.

Preparative SDS Gel Electrophoresis—Preparative electrophoresis in Laemmli slab gels was performed as described (12, 17) except that bOl was recovered from the gels by electroelution (18). The bOl band was excised from the slab gel and minced. The gel pieces were placed in the large well of an electroelution chamber (ISCO) and covered with Laemmli gel running buffer (17). The protein was electrophoresed into the other well of the chamber (100 V, 4 h). The recovered bOl solution (0.2 ml) was dialyzed for 48 h against 0.2% SDS, 1 mM NaP, pH 6.0, 0.02% NaN3.

Preparation of hOl and h–bOl—h–bOl was prepared as purple membrane from H. halobium (strain S9) using an established procedure (19). Delipidated h–bOl was prepared from purple membrane as described (12, 13) or by the following procedure. An aqueous suspension of purple membrane in distilled water (5–10 mg/ml, 0.1 ml) was mixed with 0.9 ml of Solvent B. Then, 10 µl of 10 M aqueous hydroxylamine was added to cleave the retinylidene-protein Schiff's base bond. Next, a phase separation was brought about by adding 1 ml of Buffer B. After vortexing and centrifugation, retinaloxime and halobacterial lipids partitioned predominantly (~90%) into the chloroform phase, whereas bOl was recovered as a solid at the solvent interface. The partially delipidated bOl was washed several times with water, and then the wet pellet was redissolved in 1 ml of Solvent B. Repetition of the phase partitioning and recovery of the protein interface (twice) resulted in the removal of at least 99% of the retinaloxime (assayed by UV-visible spectrophotometry) and 97% of the endogenous phospholipid (assayed by total phosphorous determination (20)).

Immunofinity-purified eOl—This was prepared as described in an accompanying paper (2).

Renaturation and Chromophore Regeneration of bOl—bOl was renatured using procedures similar to those described previously (12–14). Typically, 0.2–0.4 ml of an aqueous SDS solution of bOl (0.5% SDS; protein concentration, 1 mg/ml) was added to 0.5 ml of Buffer B containing DMPC and CHAPS, each at a concentration of 2% (w/v); and the volume was brought to 1.0 ml by the addition of water. The actual protein concentration was determined precisely by measuring the UV absorbance at 280 nm (ε280 = 6.6 × 104 cm−1 M−1 (13)). Renaturation (1.5 h) in 10 µl of solvent was added, and the solution was kept overnight at room temperature.

Analytical SDS-PAGE and Scanning Gel Densitometry—Gel electrophoresis was performed in 12% polyacrylamide gels according to Laemmli (17). Gels were stained with Coomassie Blue, and protein bands were scanned using the laser scanning gel densitometer (LKB Model 2202 equipped with peak integrator option).

UV-visible Absorbance Spectra and Light-Dark Adaptation—Spectra were obtained on a Beckman DU-7 spectrophotometer in 1-cm path length quartz cells. For dark adaptation, bOr was kept in the dark at 25 °C for 24 h prior to obtaining the spectrum. Subsequently, light adaptation was accomplished by illumination (10 min, 20°C) with the 300-watt quartz halogen output of a Kodak Type 800 slide projector using an orange Schott filter (GG475). The spectrum was obtained within 2 min after removal of the sample from the illumination.

Proton Pumping Assay—bOl was renatured as described above except that the phosphate buffer concentration in the rehydration/ regeneration mixture was decreased to 1 mm (pH 6.0). Also, for all bOl samples used in proton pumping measurements, the SDS concentra- tion was decreased to 1% (w/v). In the regeneration mixture in Solvent B, the time of incubation of the mixture, the concentration of renatured bOl was determined spectrophotometrically (ε280 = 5.2 × 104 cm−1 M−1).

The Asave of a 1 mg/ml solution of pure bOl in Solvent A is 2.7 (1-cm path length) or much larger than the value of 2.0 for pure bOl in aqueous SDS at pH 8 (13). The value for bOl, which has an unusually high specific absorption, was used even for impure samples, although it would be expected to result in a significant underestimation of total protein concentration.
Purification of Bacterio-opsin Expressed in E. coli

**RESULTS**

Fig. 1 diagrams the procedures we have developed to purify e-bO from membranes prepared by French press treatment of *E. coli* cells. In order to obtain a well-controlled comparison of these purification methods, a single fermentor batch of cells was divided into several portions of known cell mass. These portions were then subjected to different purification procedures.

**Solvent Extraction of e-bO from E. coli Membranes**—Fig. 2 (lane *B*) shows the total proteins in *E. coli* membranes. e-bO is present in too small an amount to be observed at this stage. Extraction of *E. coli* membranes or cells with Solvent A gives the patterns shown in lanes *C* and *D*, respectively. A prominent band in these lanes, corresponding in mobility to h-bO, is mostly e-bO as shown by immunoblot with the monoclonal antibody BR114.

Minor variations in treatment of the starting material affected the amounts of contaminant proteins which were co-extracted, but had little effect on the total amount of e-bO obtained. Thus, for example, the e-bO band on the gel is similar whether the extraction was performed directly on whole cells (lane *C*) or on membranes (lane *D*). Scanning gel densitometry of the "bO" bands in lanes *C* and *D* after staining with Coomassie Blue showed that their intensities were identical to within 10%. This suggests that the extraction procedure recovers nearly all of the e-bO present in the cells. This conclusion is supported by a comparison of immunoblot assays of e-bO in *E. coli* membranes prior to and following extraction (data not shown). The purity of bO in the membrane extract is estimated to be 40% by scanning gel densitometry of lane *D*. The optical density of the e-bO bands on lanes *C* and *D* was matched by that of a sample of 5 µg of pure h-bO loaded on the same gel (lane *A*). From this value, the level of bO expression in this particular fermentor batch was estimated to be 0.15% of dry cell weight. This value is consistent with the expression levels estimated by immunoblot techniques for other cultures (3, 16).

**Delipidation of e-bO Extract**—Removal of lipids from the crude e-bO extract was found to be important for obtaining reproducible results in the ion-exchange and hydroxylapatite purification procedures discussed below. Delipidation of the e-bO pellet was performed by two alternative methods. In one, the lipids were removed by extraction of the pellet with chloroform/methanol (7:28) at neutral pH. This solvent

1. The choice of the specified phospholipid mixture and of the three detergents comes from our earlier studies (12-14) and those of Racker et al. (21). SDS is required for solubilization of bO and in moderate amounts for regeneration of the chromophore (12). In addition, CHAPS or cholate is required as a second detergent for phospholipid solubilization (12-14). DMPC is the most effective phospholipid for regeneration of the chromophore (14). Soybean phospholipids are used in the preparation of vesicles because it was previously found that vesicles prepared from this phospholipid mixture gave much higher proton pumping activity than did vesicles prepared from other phospholipids (11). The use of octyl glucoside comes from the dilution procedure of Racker et al. (21).

2. A 40-µl aliquot of this was injected into 1 ml of an argon-purged salt solution (2 M NaCl). This vesicle suspension was incubated on ice under argon for 1 h. The resulting vesicles were shown to be 20-50 nm in diameter as determined by negative stain electron microscopy. Light-induced pH changes were measured as described previously (22).

3. Extraction of E. coli membranes or cells with Solvent A gives the patterns shown in lanes *C* and *D*, respectively. A prominent band in these lanes, corresponding in mobility to h-bO, is mostly e-bO as shown by immunoblot with the monoclonal antibody BR114.

4. The optical density of the e-bO bands in lanes *C* and *D* after staining with Coomassie Blue showed that their intensities were identical to within 10%. This suggests that the extraction procedure recovers nearly all of the e-bO present in the cells. This conclusion is supported by a comparison of immunoblot assays of e-bO in E. coli membranes prior to and following extraction (data not shown). The purity of bO in the membrane extract is estimated to be 40% by scanning gel densitometry of lane *D*. The optical density of the e-bO bands on lanes *C* and *D* was matched by that of a sample of 5 µg of pure h-bO loaded on the same gel (lane *A*). From this value, the level of bO expression in this particular fermentor batch was estimated to be 0.15% of dry cell weight. This value is consistent with the expression levels estimated by immunoblot techniques for other cultures (3, 16).

5. Delipidation of the e-bO extract was performed by two alternative methods. In one, the lipids were removed by extraction of the pellet with chloroform/methanol (7:28) at neutral pH. This solvent extracted the lipids, but did not remove any bO. Alternatively, gel filtration on Sephadex LH-60 of the pellet redisolved in Solvent A removed lipids efficiently while leaving the protein composition essentially unchanged (Fig. 2, lane *E*).

**Hydroxylapatite Chromatography in Organic Solvent**—The delipidated e-bO extract of membranes from 1.3 g of *E. coli* cells, dry weight, was applied to the column described under "Methods." Lowering the pH of the extract prior to loading
on the column resulted in the precipitation of some proteinaceous material, mostly non-bO contaminants, with only a minor (10–20%) loss of e-bO (SDS-PAGE, data not shown). The 280 nm absorbance of eluted fractions is shown in Fig. 3. No protein was eluted from the column during extensive washing with the loading solvent (Solvent C) or with solvent containing 11% water (Solvent D). When the water content was increased to 15% by volume, e-bO eluted in a peak which exhibited a great deal of tailing. Well out into the tail, e-bO was the predominant protein component present (SDS-PAGE, data not shown); whereas nearly all the contaminant proteins remained on the column. After phase separation, washing, and transfer to SDS as described under "Methods," a total of 1.6 mg of protein was obtained. This represents a recovery of 80% of the e-bO in the starting material (Table I). The purity of this e-bO was estimated to be 90% from scanning gel densitometry of lane $F$ of Fig. 2. The hydroxylapatite column has been scaled up successfully, permitting the purification of up to 50 mg of e-bO at a time. The yield and activity of the recovered e-bO decreased when the sample remained adsorbed to the column for more than 1 h. To increase flow rates and thus improve yields with the larger scale columns, it has been useful to mix a nonadsorbing filler (cellulose) with the hydroxylapatite adsorbent.

Ion-exchange Chromatography on DEAE-Trisacryl—Delipidated extract from 0.72 g of E. coli cells, dry weight, was applied to the column (see "Methods"). As with the hydroxylapatite procedure, some contaminants had been removed by lowering the pH of the e-bO extract prior to loading it on the column. A profile of the eluted fractions is shown in Fig. 4. The e-bO was the last major peak to elute from the column at a TEA acetate concentration of 150 mM. Other peaks corresponded to major contaminants in the loaded sample, as can be seen from the SDS-PAGE analysis in Fig. 5 (upper). This gel also shows that two protein contaminants with electrophoretic mobilities close to that of bO eluted from the ion-exchange column prior to bO (in fractions 15–24 of the column run in Fig. 4). These protein contaminants do not react with BR114 antibody in an immunoblot analysis (Fig. 5, lower); they are thus clearly identified as non-bO contaminants in the organic solvent extract. Following phase separation, washing, and transfer to SDS, 0.78 mg of e-bO was recovered, representing an overall yield of 70% (Table I). The purity of the recovered e-bO was estimated to be 95% based on scanning gel densitometry of the band in the SDS-PAGE analysis (Fig. 2, lane $G$).

The DEAE-Trisacryl column has been successfully scaled up to prepare as much as 50 mg of e-bO at a time, with no decrease in purity or yield.

Preparative SDS-PAGE—This procedure was employed as an alternative to, or in combination with, column chromatographic methods. To test its usefulness as a single step, the solvent extract of membranes from 0.22 g of cells, dry weight, was transferred to SDS solution as described under "Methods" and electrophoresed on a 0.3 × 16 × 18 cm vertical slab gel. Coomassie staining of the outermost lanes of the gel gave a pattern similar to lane $D$ of Fig. 2. Following excision of the e-bO band from the unstained portion of the gel, electrodialysis, and dialysis, a total of 0.23 mg of protein was recovered. This represents a yield of 70% of the e-bO estimated to be present in the starting material. The recovered protein appeared as a single band upon subsequent SDS-PAGE analysis.

**TABLE I**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Method of preparation</th>
<th>Yield*</th>
<th>Purity*</th>
<th>Regeneration efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>e-bO</td>
<td>1. Solvent extraction/PAGE</td>
<td>70</td>
<td>99%</td>
<td>78%</td>
</tr>
<tr>
<td></td>
<td>2. Solvent extraction/hydroxylapatite column</td>
<td>80</td>
<td>90%</td>
<td>57%</td>
</tr>
<tr>
<td></td>
<td>3. Solvent extraction/DEAE-Trisacryl column</td>
<td>70</td>
<td>95%</td>
<td>72%</td>
</tr>
<tr>
<td></td>
<td>4. Detergent extraction/imunoaffinity column/PAGE</td>
<td>10</td>
<td>99%</td>
<td>70%</td>
</tr>
<tr>
<td>h-bO</td>
<td>1. Solvent extraction/PAGE</td>
<td>60</td>
<td>99%</td>
<td>96%</td>
</tr>
<tr>
<td></td>
<td>2. Solvent extraction/hydroxylapatite column</td>
<td>70</td>
<td>99%</td>
<td>85%</td>
</tr>
<tr>
<td></td>
<td>3. Solvent extraction/DEAE-Trisacryl column</td>
<td>90</td>
<td>99%</td>
<td>85%</td>
</tr>
</tbody>
</table>

*Yields are expressed as total protein in the purified material (based on A$_{280}$) divided by the estimated amount of bO in the starting material (whole E. coli cells for e-bO; purple membrane for h-bO). All e-bO yields refer to material purified from the same batch of E. coli. Yield calculations were based on a value of 1.5 mg of e-bO estimated to be present in each gram of these cells, dry weight (see "Results").

*Purity was determined by scanning densitometry of Coomassie-stained bands of SDS-PAGE analysis.

*This value does not reflect the presence of a known impurity with the same electrophoretic mobility as bO.

**Fig. 3.** Hydroxylapatite adsorption chromatography in organic solvent. See text for details. Profile of 280 nm absorbance of fractions eluted is shown. The solvent composition was changed at the indicated points.

**Fig. 4.** Ion-exchange chromatography of e-bO on DEAE-Trisacryl. See text for details. Profile of 280 nm absorbance of fractions eluted (solid line) is shown. The gradient of TEA acetate concentration from 50 to 200 mM is indicated by the rise in the conductivity of eluted fractions (dotted line). Fractions 27–36 (hatched) were pooled.
Purification of Bacterio-opsin Expressed in E. coli

FIG. 5. SDS-PAGE analysis of proteins in DEAE-Trisacryl column fractions. The label at the top of each lane corresponds to the fraction number from the profile in Fig. 4. Upper, Coomassie blue staining; lower, immunoblot using BR114 monoclonal antibody and 125I-labeled protein A from Staphylococcus aureus.

...purified on a single gel.

Measurements of Activity of e-bO—We have evaluated the different purification methods for their effect on protein activity. The most easily measured criterion of activity is chromophore regeneration. With delipidated h-bO, regeneration efficiencies of 90-95% are routinely attained (13). As demonstrated in Fig. 6, e-bO is also capable of binding exogenously added retinal to form a protonated Schiff's base chromophore ($\lambda_{\text{max}} = 560$ nm). The extent of chromophore regeneration is in the range of 55-80% for e-bO samples prepared by four different purification methods (Table I).

Furthermore, the four e-bR samples all exhibit identical absorption maxima, both in the dark ($550 \pm 2$ nm) and subsequent to light adaptation ($552$ nm). Variations in $\lambda_{\text{max}}$ resulted from peak shifts due to excess free retinal present in the regenerated samples. In every case, the shift on light adaptation was $9 \pm 0.5$ nm, indicating that the overlapping retinal absorption band ($\lambda_{\text{max}} = 380$ nm) had shifted the light- and dark-adapted $\lambda_{\text{max}}$ values by equal amounts. We observed an identical 9-nm light adaptation shift for h-bR samples in DMPC/CHAPS mixtures, pH 6; this shift was slightly smaller than the 11-nm value previously observed for h-bR in DMPC/cholate, pH 7 (13).

A further criterion of biological activity of bR is the ability to pump protons across a lipid bilayer in response to illumination. After incorporation into lipid vesicles, e-bR exhibits inward light-induced proton translocation which causes an increase in the pH of the external medium (Fig. 7). The proton pumping activities of e-bR samples produced by the four different purification methods listed in Table I were in the range of 1.1-2.5 H+/bR/s for initial rate and 24-39 H+/bR for total protons pumped (saturating light conditions). For an h-bR sample, the corresponding values were 2.4 H+/bR/s and 30 H+/bR, respectively (Fig. 7). As additional evidence that e-bR is fully functional, we have shown that it undergoes the expected photoreactions to the K, L, M, and O intermediates of the proton pumping cycle, based on a variety of low temperature and time-resolved UV, visible, and Fourier transform IR spectroscopic studies performed in collaboration with Drs. K. J. Rothschild and P. L. Ahl (Boston University Physics Department). In previous in vitro reconstitution studies of purple membrane (23) or delipidated bacteriorhodopsin (11), the protein invariably incorporated into vesicles with a uniformly inside-out orientation.

In previous in vitro reconstitution studies of purple membrane (23) or delipidated bacteriorhodopsin (11), the protein invariably incorporated into vesicles with a uniformly inside-out orientation.

DISCUSSION

The finding that e-bO can be extracted from E. coli membranes into chloroform/methanol-based solvent systems has provided a simple and convenient first step for purification of the E. coli-expressed bO. The procedure is similar to those that have previously been used to isolate proteolipids, for example, from brain (7, 8), from proton-translocating ATPases (9, 10), and from spinach chloroplast reaction centers (24). It has also been demonstrated recently that the mammalian visual pigment rhodopsin can be solubilized in a chloroform/methanol solvent containing trichloroacetic acid (25). Although bO also is not very soluble in mixtures of chloroform and methanol alone, modification of the solvent system resulted in a large increase in solubility. Thus, at least 20 mg/ml pure delipidated bO could be dissolved in Solvent A.

The solvent extraction procedure results in a considerable enrichment of e-bO, from less than 1% of membrane protein to nearly 40% purity. Furthermore, the extraction can be scaled up easily. We can routinely extract 30-50 mg of e-bO from 30 g of fermentor-grown cells, dry weight, using several liters of solvents. For further purification, three distinct procedures have been developed. The first, preparative SDS gel electrophoresis, gives protein of high apparent purity and activity (Table I), but is limited in scale. The second method for purification involves ion-exchange chromatography on DEAE-Trisacryl. Ion-exchange chromatography in chloroform/methanol solvent has been used previously for purification of proteolipids (8, 9). This method also gives protein of high purity and activity and has proven to be particularly amenable to scaling up. A third purification procedure that has been developed uses hydroxylapatite adsorption chromatography in organic solvent. Whereas hydroxylapatite is a well-established adsorbent for purification of proteins in aqueous solution (26), it has not been used for adsorption chromatography in chloroform/methanol solvent mixtures. In our separation, the critical variable appeared to be water content of the solvent, with ionic strength playing a secondary role. The hydroxylapatite procedure is very fast, and its low cost makes it possible to eliminate the possibility of cross-contamination of samples by using each column only once. However, it produces e-bO which is somewhat less pure than that obtained from the ion-exchange column.

Each of the three procedures described yields bO which is substantially free of contaminants, as assayed by analytical SDS gel electrophoresis followed by scanning gel densitometry (see Fig. 2 and Table I). A further demonstration of purity is provided by comparing the 560 nm absorbance of the reconstituted e-bR with the theoretical maximum based on the known extinction coefficient of fully renatured h-bR. This regeneration assay indicates that all three purification procedures produce e-bO with a retinal binding capacity which is in the range of 50-80% of that expected for pure bO (Table I).

The question remains, whether the fraction of the "purified" protein that is incapable of forming a chromophore (~25% based on A280) is a copurified E. coli protein with the same electrophoretic mobility as bO or is actually bO which has somehow been damaged by the purification procedures. In fact, both of these factors appear to be contributing. SDS-PAGE analysis of fractions eluted from the DEAE-Trisacryl ion-exchange column shows that there are indeed proteins in the crude extract with similar electrophoretic mobility as bO, which, however, elute at a lower ionic strength and which fail to react with bO-specific antibody. However, combining ion-exchange chromatography with subsequent SDS-PAGE purification did not increase the chromophore regeneration yield over that obtained with either the ion-exchange step or the SDS-PAGE step alone (Fig. 2, Table I). These results are consistent with the idea that incomplete regeneration is partially due to damage to the bO during purification procedures. The latter explanation is plausible since pure h-bO which is subjected to identical procedures also undergoes some loss of its ability to bind retinal (Table I).

The purification and renaturation procedures which we have developed produce e-bO which undergoes light and dark adaptation and which is capable of pumping protons across a lipid bilayer upon illumination (Figs. 6 and 7). To within experimental error, the λmax and extinction coefficient changes upon light and dark adaptation, as well as the values of initial rate and total number of protons pumped per chromophore, are identical for h-bR and for e-bR purified by any of our procedures.

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REFERENCES