Purification of photochemically active halorhodopsin
(membrane protein/octyl β-D-glucopyranoside/retinal pigment/halobacteria)

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ABSTRACT We have developed a procedure for the purification of halorhodopsin in a photochemically active state. Solubilization of membranes from a bacteriorhodopsin-negative Halobacterium strain with octyl glucoside was followed by chromatography on hydroxylapatite and octyl-Sepharose gels. All steps were carried out in high-ionic-strength solutions. The procedure resulted in 270-fold enrichment with a 35% yield. The eluted pigment had an absorption maximum at 575 nm and an A450/A575 ratio of 2. On removal of the detergent by dialysis, the purified halorhodopsin was chemically bleached, regenerated with [3H]-retinal, and reduced with cyanoborohydride. Such samples showed one main and one satellite band after staining or fluorography of NaDdSO4/polycrylamide gels. The apparent molecular weight of the main band was 25,000. Purified halorhodopsin underwent a photocycle after excitation with pulsed laser light and showed a 9-nm blue shift (at neutral pH) on removal of chloride ion. The pigment also underwent a photoreversible shift at alkaline pH to a form absorbing maximally at 410 nm. All three reactions closely resembled those of membrane-bound halorhodopsin.

Three retinal-containing pigments are synthesized by Halobacterium halobium. These membrane-bound pigments are bacteriorhodopsin (bR), halorhodopsin (hR), and the slow rhodopsin-like pigment (s-rhodopsin; sR), they have similar UV and visible absorption spectra, with maxima at 565 nm (bR), 578 nm (hR), and 587 nm (sR), and all three undergo characteristic photochemical cycles after excitation by actinic light (1–4). Whereas bR, the light-driven proton pump, converts light energy into a protomotive force that can be harnessed by bacterial cells for ATP synthesis (for review see ref. 1), hR is probably involved in electronic transport of Cl− into the cells (5). The physiologic significance of this light-driven process is not known with certainty. Some evidence suggests that sR participates in the phototactic responses of halobacterial cells (3).

Many structural and functional studies require purification of the photoactive chromoproteins. This task is relatively simple for bR, because it is the only protein of the purple membrane that can be fractionated on sucrose gradients (6). In our available halobacteria strains, hR is not so organized and has to be purified by detergent solubilization and subsequent fractionation.

Using a strain deficient in bR [determined by flash spectroscopy as described (4, 7)], we solubilized the membranes in octyl glucoside and high-ionic-strength buffers. Although sR is denatured by the nonionic detergent octyl β-D-glucopyranoside (octyl glucoside), hR is stable at high ionic strength in this detergent (3). We then purified the still photocactive hR by chromatographic methods. We further investigated whether purified hR still undergoes the previously measured photocycle on excitation with actinic light and whether its absorbance band reversibly shifts to the blue in the light at alkaline pH (hR578 ≅ hR410; subscripts indicate wavelength (nm) of the maximum absorbance in the visible range) or in the dark on removal of Cl− (hR578 ≅ hR500) (7–10). (The photochemical reactions of membrane-embedded hR will be fully described elsewhere.)

MATERIALS AND METHODS

Membrane Preparation. H. halobium strain JW-12 was isolated from strain S9 (obtained from L. Jan; see ref. 11), by published procedures (12). Cells of JW-12 synthesize hR, sR, and cytochromes but no bacterioruberins or bR. The cells were grown in 12-liter fermentors and harvested by standard techniques (6). After washing with 4 M NaCl, the cells (about 3 g of protein) were suspended in 150 ml of 4 M NaCl and lysed by dialysis in the presence of 10 mg of DNase at 4°C for 18 hr against 6 liters of 50 mM Hepes (pH 7.0). The lysate was cleared of large debris (containing little or no hR) by centrifugation (6,000 × g, 15 min) and the membranes were then sedimented (260,000 × g, 1 hr). The bluish pellet was suspended in 50 mM Hepes buffer (pH 7.0). The sedimentation and resuspension of membranes was repeated twice in Hepes buffer and then once in 4 M NaCl. Finally, the membranes were stored in 4 M NaCl at 4°C at 10–20 mg/ml.

Protein was determined by microanalytical Biuret method (13) using purple membrane as a standard.

Subsequent operations were carried out in dim or no light at 4°C, unless otherwise stated.

Hydroxylapatite Chromatography. Prepared membranes (250 mg of protein) were solubilized in 115 ml of 3 M NaCl/15 mM octyl glucoside (Calbiochem–Behring)/50 mM Tris(hydroxymethyl)aminomethane sulfonic acid (Taps) buffer, pH 8.6, at a protein/detergent ratio of 1:2 (wt/wt). The suspension was incubated at 4°C overnight and then centrifuged (200,000 × g, 1 hr) to sediment un solubilized material. The supernatant was applied to a column (7.7 × 2.5 cm) of hydroxylapatite (Bio-Rad, DNA grade), previously equilibrated with 3 M NaCl/15 mM octyl glucoside/50 mM Taps buffer, pH 8.6. The column was washed at a flow rate of 11 cm/hr with 75 ml of the same buffer and developed with 20 ml of 3 M NaCl/100 mM octyl glucoside/50 mM Taps buffer, pH 8.6, to elute hR. In order to use less detergent, the elution was completed by reusing the original buffer. Fractions with an A450/A575 ratio of <1 were pooled and concentrated 3-fold in an Amicon CF50A Centriflo cone. The proteins that remained bound to the column could be removed by treating the column with 5 mM octyl glucoside/0.25 M phosphate buffer, pH 7.0.

Hydrophobic Interaction Chromatography. To the pooled and concentrated hR fractions from the hydroxylapatite column was added an equal volume of saturated (NH4)2SO4, and the

Abbreviations: bR, bacteriorhodopsin; hR, halorhodopsin; sR, slow rhodopsin-like pigment.
mixture was stored overnight at 4°C and then cleared by centrifugation \((200,000 \times g, 1\) hr). The supernatant \((12\) ml) was applied to a column of octyl-Sepharose CL-4B (Pharmacia, 25 \(\times 1.5\) cm) that had been equilibrated with 1.5 M NaCl/50% saturated \((\text{NH}_4)_2\text{SO}_4/15\) mM octyl glucoside/25 mM Taps buffer, pH 8.6. hR was eluted with the same buffer at a flow rate of approximately 14 cm/hr. Fractions with an \(A_{280}/A_{575}\) ratio of \(\leq 2.2\) were pooled and dialyzed for 48 hr against several changes of 3 M NaCl/50 mM Hepes buffer, pH 7.0. Proteins that remained on the column after the elution could be removed with 5 mM octyl glucoside/50 mM Hepes buffer, pH 7.0.

**Bleaching and Regeneration of hR.** To 2.6 ml of a purified hR solution \((A_{680}, 0.5)\) in 3 M NaCl/50 mM Hepes buffer, pH 7.0, was added 650 \(\mu\)l of 2 M hydroxylamine in 3 M NaCl (pH 7.0). hR was bleached at 40°C in white light \(\left(2 \times 10^6\right. \text{ erg cm}^{-2}\text{sec}^{-1}, 1\) erg = 0.1 \(\mu\)J) for 4 hr with a second addition of hydroxylamine after 2 hr of illumination. The bleached halopin was then dialyzed against two 2-liter portions of 3 M NaCl/50 mM Hepes buffer, pH 7.0, for 24 hr and sedimented \((300,000 \times g, 14\) hr). The pellet was then suspended in 1 ml of 3 M NaCl/50 mM Hepes buffer, pH 7.0. To the bleached sample, sufficient amounts of all-trans-[\(^{1}H\)]retinal \(\left(100\text{ mcCi}/\text{mmol} \ (1\text{ Ci} = 37\text{ GBq})\right)\) were added to allow for 40% regeneration, the maximum attainable with this preparation. The sample was kept at 20°C overnight and unreacted retinal was removed by washing once with 0.2% bovine serum albumin (fraction V, Sigma) in 3 M NaCl/50 mM Hepes buffer, pH 7.0. The washing consisted of mixing the hR with the bovine serum albumin solution and then sedimenting the hR \((300,000 \times g, 12\) hr). This process was repeated twice in the same buffer but without bovine serum albumin.

**Reduction of Regenerated Labeled hR.** The regenerated and washed sample was reduced at acid pH according to a previously published method for bovine rhodopsin (14). To 5 parts of hR in 3 M NaCl/50 mM Hepes, pH 7.0, was added 1 part of 10% sodium cyanoborohydride in water (previously centrifuged at 30,000 \(\times g, 20\) min), 2 parts of 1 M HCl, and 2 parts of 1% NaDodSO\(_4\) in water. The mixture was kept at 20°C for 2 hr and then centrifuged \((200,000 \times g, 1\) hr). The supernatant was discarded (together with less than 10% of the titrum label), and the hR-containing pellet was solubilized by addition of the solubilizing buffer used in electrophoresis (15). The described procedure conveniently reduced, concentrated, and desalted the protein sample simultaneously. A sample of hR that had also been regenerated with \([^{3}H]\)retinal was similarly treated. The previously reported reduction method using ether and sodium cyanoborohydride, pH 5.0 (16), was also used.

**Electrophoresis and Fluorography.** NaDodSO\(_4\)/polyacrylamide gels were run according to the method of Laemmli (15), using a 12–20% acrylamide gradient. If necessary, samples were desalted by dialysis before solubilization for 2 hr at room temperature. Gels with reduced radioactively labeled samples \((25,000\text{ cpm per lane})\) were prepared for fluorography with 1 M sodium salicylate (17) and exposed to Kodak X-Omat AR film for 5 days at -70°C.

**Spectroscopy.** Rapid transient absorbance changes were measured with equipment essentially as described (18). We assayed hR by measuring the transient absorbance changes at 600 nm after stimulation with pulsed actinic light (nitrogen-pumped dye laser at 520 nm, Molecron, Sunnyvale, CA). All assay measurements were made after 1:10 dilution of the sample into 3 M NaCl/50 mM octyl glucoside in 50 mM Hepes buffer (pH 7.0) followed by centrifugation \((200,000 \times g, 1\) hr) to remove insoluble material. It was assumed that the photocycle reaction and the quantum efficiency remained the same at each stage of purification.

UV and visible static absorption spectra were measured with an Aminco DW-2a spectrophotometer equipped with a Nicolet 1074 data acquisition computer.

**RESULTS**

**Membrane Preparation and Solubilization.** Our membranes from *H. halobium* strain JW-12 contain hR, sR, and cytochromes as the major pigments. Assay of hR by flash spectroscopy was simplified because octyl glucoside eliminates the photoactivity of sR (3). By relating the transient absorbance spectrum of the final purified sample to its absorption spectrum, we calculated that the prepared membranes contained about 0.1 absorbance unit \((575\) nm) of solubilized hR per mg of membrane protein. Assuming a molar extinction coefficient of 50,000 (similar to that for hR or other rhodopsins), this is equivalent to 2 nmol/mg of protein or 300 nmol of hR from one 12-liter fermentation flask. The concentration of hR in these membranes was approximately 4 times higher than in envelope vesicles that we prepared by the sonication method (19).

**Purification of hR.** The hydroxylapatite chromatography profile (Fig. 1) had several noteworthy features. Although a negligible amount of protein \((\geq 2%)\) eluted with the flow-through volume, this step removed considerable amounts of cytochromes (as judged by the Soret absorption band), resulting in a 6-fold purification of hR, and concentrated it if the sample was applied at comparatively low detergent concentration: the pooled fractions were 3.5-fold more concentrated in hR than the starting material. The pulse of high octyl glucoside buffer greatly accelerated elution of hR. If eluted at a constant octyl glucoside concentration of 15 mM, the protein mobility on the column was severely retarded and the material emerged less concentrated. The reason for the trailing of the hR, which was responsible for most of the loss during purification, is not known. Even if larger volumes of the higher detergent concentration buffer were applied to the column, the hR trailed and the later fractions were much less pure than the initial fractions. Pooling and rechromatography of these later fractions did not result in samples of the purity of the early fractions of the first run.

The second purification step, hydrophobic interaction chromatography on octyl-Sepharose, removed the remaining cytochromes together with other proteins and yielded hR fractions with an \(A_{280}/A_{575}\) value of 2 (Fig. 2). The contaminating

![Figure 1](image1.png)

**FIG. 1.** Elution profile of hR from a hydroxylapatite column. ●, absorbance at 280 nm; ○, absorbance at 575 nm. (Inset) Absorption spectrum of pooled fractions. Elution of proteins in the void volume and of adsorbed proteins is not shown.
proteins had a stronger affinity for octyl-Sepharose than hR although hR mobility was somewhat retarded during the elution process. This binding could not be enhanced by increasing the concentrations of (NH₄)₂SO₄ or NaCl because higher salt concentrations in the buffer led to a phase separation between the pigment–detergent complex and the salt solution. Stronger binding of hR could be achieved with deoxy-agarose (Miles) but elution from such columns did not result in any improvement in the separation.

The final preparation was approximately 270-fold enriched for hR (Table 1).

The purity of hR at the various stages was also judged by NaDodSO₄/polyacrylamide gel electrophoresis. Purified samples with an A₃₂₅0/A₅₇₅ ratio of 2 showed one major protein band with a faint satellite band right above it; both bands migrated slightly below the hR standard (Fig. 3). Because hR runs anomalously on electrophoresis gels and we assume that hR is similar to bR in this respect, we find an apparent molecular weight of 25,000 for the retinal-binding polypeptide of hR, in agreement with other work (20). Another faint band with an apparent molecular weight of 40,000 was visible in such preparations. It was possible to remove this protein (and improve the A₃₂₅0/A₅₇₅ ratio to 1.8:1) (Fig. 3, lane 5) by rerunning the material on a small column of hydroxylapatite under the same conditions as for the first column but without increasing the detergent concentration.

After removal of octyl glucoside by dialysis, purified hR is quite stable in the absence of NaCl; it can be stored in 50 mM Heps (pH 7) at 4°C in the dark for at least 3 wk without loss of absorbance. Addition of octyl glucoside considerably decreases the stability and at room temperature hR lost its absorbance in less than 1 day, even in the dark. In 3 M NaCl/50 mM Heps, pH 7, purified hR is stable for months at 4°C; with octyl glucoside, the half-life is several weeks. Removal of detergent by dialysis against 3 M NaCl and buffer caused an approximately 4-nm red shift of the absorption maximum and was apparently also accompanied by changes in the aggregation of the protein because a large proportion of the hR could then be sedimented by prolonged centrifugation (300,000 × g, 12 hr). Preliminary observations indicate that some octyl glucoside remained bound to the protein.

It was possible to remove the carotenoids, when these were present in solubilized membranes, by using octyl glucoside and octyl-Sepharose. We solubilized membranes of a bright red strain, S9M001 (isolated as a spontaneous mutant from S9), containing hR and sR, but no hR, and applied this material to a column of octyl-Sepharose under the conditions described here for the purification of hR. The carotenoids bound tightly to the gel while hR eluted without a spectroscopically visible contribution by the carotenoids. They could be removed from the column with 2% Triton X-100 in 25 mM Taps, pH 8.6.

Reconstitution of Bleached Halo-Opsin with Retinal. The purified pigment was bleached with hydroxylamine and white light. The absorbance of hR at 575 nm decreased by 82% in 4 hr. When orange light was used instead of white light, considerable amounts of hR were converted to hR₄₁₀ (see below), which did not form retinaloxime and halo-opsin. After termination of the bleaching process and removal of unreacted hydroxylamine, addition of all-trans retinal to the sample resulted in regeneration of a pigment with an absorbance maximum at 579 nm (Fig. 4), which is close to that of membrane-embedded hR at 575 nm (2). Under our conditions, 42% of the bleached color was regained and the regenerated pigment was stable on addition of octyl glucoside. With the regeneration of the absorbance band, the photocycling activity of hR also developed.

Purified hR could be reduced by sodium cyanoborohydride in the dark at pH 5.0 in the presence of ether (16). It was important to mix the ether cautiously with the hR-containing solution, which was best done by slowly inverting the container.

**Table 1. Summary of purification scheme for hR**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein, mg</th>
<th>ΔA₆₀₀</th>
<th>ΔA₆₀₀ ÷ 10²</th>
<th>Purification factor</th>
<th>Yield, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cells</td>
<td>5,200</td>
<td>0.88</td>
<td>0.16</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Membranes</td>
<td>280</td>
<td>0.70</td>
<td>2.7</td>
<td>17</td>
<td>80</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>23</td>
<td>0.41</td>
<td>17.8</td>
<td>110</td>
<td>47</td>
</tr>
<tr>
<td>Octyl-Sepharose</td>
<td>7</td>
<td>0.32</td>
<td>42.7</td>
<td>270</td>
<td>36</td>
</tr>
</tbody>
</table>

*ΔA₆₀₀ cm⁻¹ × total volume of material, calculated from transient absorbance changes at 600 nm.*
FIG. 4. Regeneration of purified hR with retinal. Both reference and sample cuvettes contained 0.5 ml of bleached washed halo-opsin. To the sample cuvette only was added a substoichiometric amount of all-trans-retinal (2 nmol). Traces: a, 1 min; b, 30 min; c, 240 min. (Inset) Absorbance increase at 579 nm (relative units).

Simply overlaying the sample with ether was not sufficient, whereas vigorous mixing of the two phases led to chromophore extraction. The reduction of hR in the presence of ether could be monitored spectrosopically by following the disappearance of the hR absorbance band and the appearance of a band at 330 nm, characteristic of retinylidene pigments (14). The reduction at low pH could not be followed spectrophotometrically because of the precipitation of the reactants but, for both methods, the radioactive protein bands coincide with the stained protein bands from nonreduced samples (Fig. 3). As was the case with the nonlabeled dye-stained protein band, the radioactive band was also accompanied by a radioactive sat-

elitte band. Both bands were again positioned slightly below a bR standard that had been treated in the same way.

Photochemistry of Purified hR. The photocycle of purified hR (in 3 M NaCl, neutral pH) closely resembled that of membrane-bound hR (1, 4), although the kinetics were altered (Fig. 5). In the intact membrane, relaxation of the 600-nm decrease has a half-time of 10 msec; the purified hR had a relaxation half-time of 27 msec, decreasing to 4 msec on addition of octyl glu-

FIG. 5. Flash-induced absorbance changes of purified hR ($A_{575} = 0.85$) 1.5 msec after actinic flash without (A) and with (B) octyl glucoside. (Inset) Kinetic trace at 600 nm, average of 16 flashes, 20°C, without (A) and with (B) octyl glucoside.

FIG. 6. Chloride-sensitive red shift of hR at 20°C. Traces: a, hR in 3 M NaCl/50 mM Hepes, pH 7 (absorbance maximum, 573 nm); b, hR in 50 mM Hepes, pH 7 (absorbance maximum, 564 nm); c, difference spectrum (4-fold amplification).
coside. The red-shifted intermediate (around 680 nm), which is a characteristic of membrane-bound hR (data not shown), is in particular enhanced in the presence of the detergent, presumably because of the changed kinetic constants of the transient intermediates.

Removal of chloride ion caused a 9 nm blue shift accompanied by a 10% decrease in the extinction coefficient (Fig. 6). The difference spectrum of the two forms, \( \Delta R(573 - R(664) \), had a significant negative peak at about 655 nm resulting from an unexpected increase in the red absorption that accompanied the spectral transition of the main band (Fig. 6). This absorbance increase could have originated from a second (smaller) absorbance band at 610 nm–630 nm that was obscured by the main band at 564 nm. The observed blue shift of 9 nm for purified hR was less than the 15-nm shift that we observed with membrane-embedded hR, where the peak shifted from 578 nm to 563 nm on removal of chloride ion.

On illumination with orange light at alkaline pH, the visible absorbance peak of hR (in octyl glucoside) shifted to 410 nm, reversing on illumination with blue light (Fig. 7). We have observed this phenomenon in intact membranes (8), and it has also been reported by Ogurusu et al. (21). The changes at 275 nm may be caused by perturbation in the charge densities around aromatic residues or retinal (or both) or by a change in the interaction of these chromophores.

**DISCUSSION**

Our experiments show that a photochemically active hR can be isolated in good yield from halobacterial membranes. Because the photochemical properties of the purified hR closely resemble those of membrane-bound hR with respect to absorption spectrum, phototcycle, light-induced blue shift, and Cl\(^{-}\)-dependent red shift, we conclude that hR is largely unaffected by the purification process. Because the photochemical characteristics of sR disappear on solubilization and do not reappear, hR is the only spectroscopically detectable pigment after purification. However, we cannot exclude the possibility that a nonactive contaminant is present. The satellite band could be denatured sR but we believe that it is more likely an artefact generated during the purification of hR. Similar double electrophoresis bands are occasionally seen in hR samples. Recently another report about the purification of irreversibly denatured hR in low yields was published (22). In our opinion, the results are not unambiguous, because of the simultaneous presence of sR in halobacterial membranes (3, 8). Halorhodopsin and sR have similar absorbance bands and possibly similar molecular weights (20). Therefore, neither of these two criteria is sufficient to identify hR and functional criteria should be included.

Although the changes in the absorbance band of hR are relatively small when the amphiphilic environment is changed, we have noticed that the position of the hR absorbance band is somewhat variable even when the solvent composition is constant. It may well be that the position of the hR absorbance band reflects the degree of cis-trans isomerization of its retinal chromophore. For instance, it is known that the detergent-induced blue shift of hR is due to the increased proportion of 13-cis-retinal (23).

The photochemical activity of purified hR does not change significantly on removal of octyl glucoside, and this result suggests that hR can be reconstituted into liposomes. If this becomes possible, the significance of our purification procedure lies in the prospect of studying hR spectroscopically, structurally, and functionally.

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**Fig. 7.** Reversible photobleaching of hR in 50% (NH\(_4\))\(_2\)SO\(_4/1.5\) M NaCl/15 mM octyl glucoside/50 mM Tris, pH 8.6. Trace a, formation of blue-shifted species with orange light (9 min, Corning 3-69 filter and projector with 250-W lamp); b, reversal to original spectrum with blue light (1 min, 5-60 filter). Difference spectra were generated by electronic subtraction of static absolute absorption spectra recorded at 20°C.