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GENETIC AND BIOCHEMICAL RESOLUTION OF THE CHROMOPHORIC POLYPEPTIDE OF HALORHODOPSIN

Elena N. Spudich*, Roberto A. Bogomolni+, and John L. Spudich*

*Department of Anatomy, Albert Einstein College of Medicine, Bronx, NY 10461;
+Cardiovascular Research Institute and Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143

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SUMMARY: Retinal-binding proteins in bacteriorhodopsin-deficient membranes of Halobacterium halobium have been separated on polyacrylamide gels after labeling with (^{3}H)retinal. Four labeled polypeptides are observed: one at an M_r of 19,000, two distinct bands near 25,000, and one at 94,000. Comparing halorhodopsin-containing and halorhodopsin-deficient mutants and using ion translocation measurements and flash spectroscopy to test for the photoactive retinal pigments, we show that the lower of the two radiolabeled bands at 25,000 is the retinal-binding polypeptide of halorhodopsin. The identities of the other three polypeptides are not known, but one of them, probably the upper of the two 25,000 bands, is likely to be s-rhodopsin.

Ion transport and spectroscopic studies of H. halobium membranes deficient in bacteriorhodopsin (bR) have revealed a second light-driven ion pump named halorhodopsin (hR) (cf. review in ref. 1) and, more recently, a third photoactive pigment named s-rhodopsin (sR), which may function as a phototaxis receptor (2). All three pigments have retinal chromophores with broad absorption bands and maxima in the 560nm-590nm region. The absorption maxima of hR and sR differ by only 9nm (3), making it difficult to distinguish them solely on absorption criteria. However, they are readily distinguished by flash spectroscopic assay of their photochemical reaction cycling, which occurs with a ~10 msec half-time for hR and ~800 msec for sR (2).

Recently mutants deficient in hR were isolated (4) and shown to still contain sR (2). These mutants provide an important tool for discriminating hR and sR properties and have enabled us to compare spectroscopic and biochemical properties of hR^+ and hR^- membranes (2,3,4). In this communication we report

Abbreviations: bR, bacteriorhodopsin; hR, halorhodopsin; sR, s-rhodopsin (slow cycling rhodopsin-like pigment); CCCP, carbonyl cyanide-m-chlorophenylhydrazone.

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the incorporation of (^{3}H)retinal into hR^+ and hR^- membranes to identify the hR chromophoric polypeptide.

MATERIALS AND METHODS

Strains and culture conditions. H. halobium strain OD2 is $bR^-hR^+sR^+$ and Flx3 is a derivative strain selected to be $bR^-hR^-sR^+$. Their selection (4) and properties (2,4) have been reported. OD2R and Flx3R in addition are deficient in retinal synthesis (ret^-) and were isolated by selecting white (i.e. carotenoid-deficient) colonies, which are often ret^-. Our screening criterion for retinal-deficiency was that the strains lack phototaxis responses (assayed as in reference 5) and phototaxis is restored by addition of exogenous retinal. Cells were cultured as in reference 6, but grown in the dark to avoid possible differences introduced by growing hR^+ and hR^- strains in the light. Membrane vesicle preparation and hR transport activity were as described (4).

Chemical processing of membranes and polypeptide analysis. (^{3}H)retinal polypeptides were generated as described in the figure legends. To reduce the Schiff base we used a modification of the method of Lanyi and Oesterhelt (7), adding 0.5ml 8% $NaBH_3CN$ in 1M sodium acetate, pH5.0, 3M NaCl, to 0.5 ml of membrane vesicle suspension in 4M NaCl (3 mg protein) in a 10mm rectangular cuvette. Just prior to its use the BH_3CN^- solution was prepared and certrifuged at 58,300g for 1 hr to remove insoluble materials. Addition of 0.1ml diethylether was followed by a brief vigorous agitation and was carried out in dim light. The suspension was maintained in the dark at room temperature for 3 hrs, centrifuged 1 hr at 108,000g, the pellet resuspended in 4M NaCl, and washed by 3 centrifugations with resuspensions in 4M NaCl and finally in 4M NaCl, 0.1M sodium phosphate, pH7.0. The photochemical activity of hR and sR were assayed by flash spectroscopy (2) to monitor the BH_3CN^- reduction step. Addition of diethylether alone did not destroy hR or sR activity and did not result in incorporation of label into membrane polypeptides as assessed by $NaDodSO_4$ (SDS)-polyacrylamide gel electrophoresis.

Samples were dialyzed against H_2O for 2 hr prior to solubilization and then heated at 70°C for 5 min after sample buffer addition before gel electrophoresis (6).

RESULTS

Biosynthesis of the apoprotein moiety of hR but not of sR requires retinal. Both OD2R and Flx3R, because they are ret^-, lack the hR photocycle and ion transport activity, and the sR photocycle and phototaxis. When retinal is added to stationary phase OD2R or Flx3R cultures, phototaxis and the sR photocycle appear within 15 minutes. However, neither the hR photocycle nor protonophore-enhanced, light-driven proton influx (an assay for hR function), can be detected in Flx3R or OD2R cells or vesicles even after 2 hours of incubation with retinal (shown for OD2R in Fig. 1). This result is expected for Flx3R since its parent Flx3 is hR-deficient. The failure to generate hR with retinal in OD2R vesicles as measured by both flash and ion

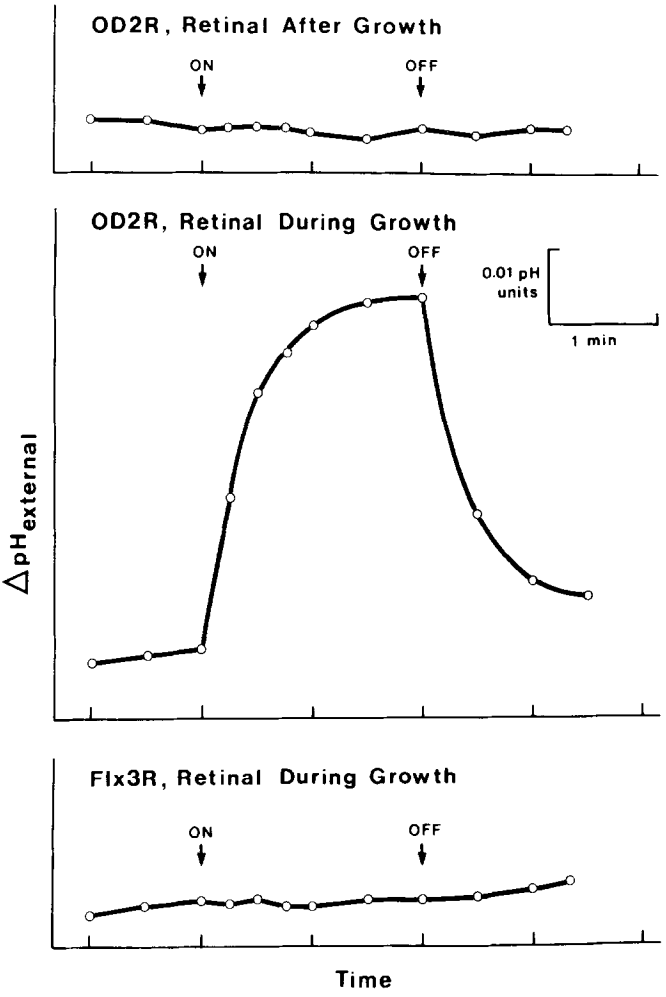

Figure 1. Halorhodopsin-mediated pH changes. Light-driven proton influx in the presence of CCCP measured as in reference 4 using vesicles at 0.3 mg/ml. Vesicles were prepared from: (top) OD2R cells incubated 2 hrs at 37° with 1.4 μM all-trans retinal added from an ethanolic solution (<1% ethanol in the cell culture); OD2R cells (middle) and Flx3R cells (bottom) grown to ~4 X 10^7 cells/ml, 0.05 ml (^{3}H)retinal added to 100ml culture from a 2 mM stock (~400 $\mu C_i/\mu mole$), followed by growth to 1.5 X 10^9 cells/ml. "ON" and "OFF" refers to 2 X 10^6 ergs·cm^{-1}·sec^{-1} orange light from a GE 120V300W ENG lamp filtered through 3-69 Corning and heat filters.

transport assays indicates that the apoprotein is lacking, because we have shown that hR can be regenerated with retinal from the apoprotein in hydroxylamine-bleached membranes (3). Apparently, similar to bR (8), hR requires retinal to synthesize the retinal-binding protein in significant amounts. Adding retinal during growth of the culture, indeed, induces hR activity in OD2R, but not in Flx3R (Fig. 1). The kinetics of hR development

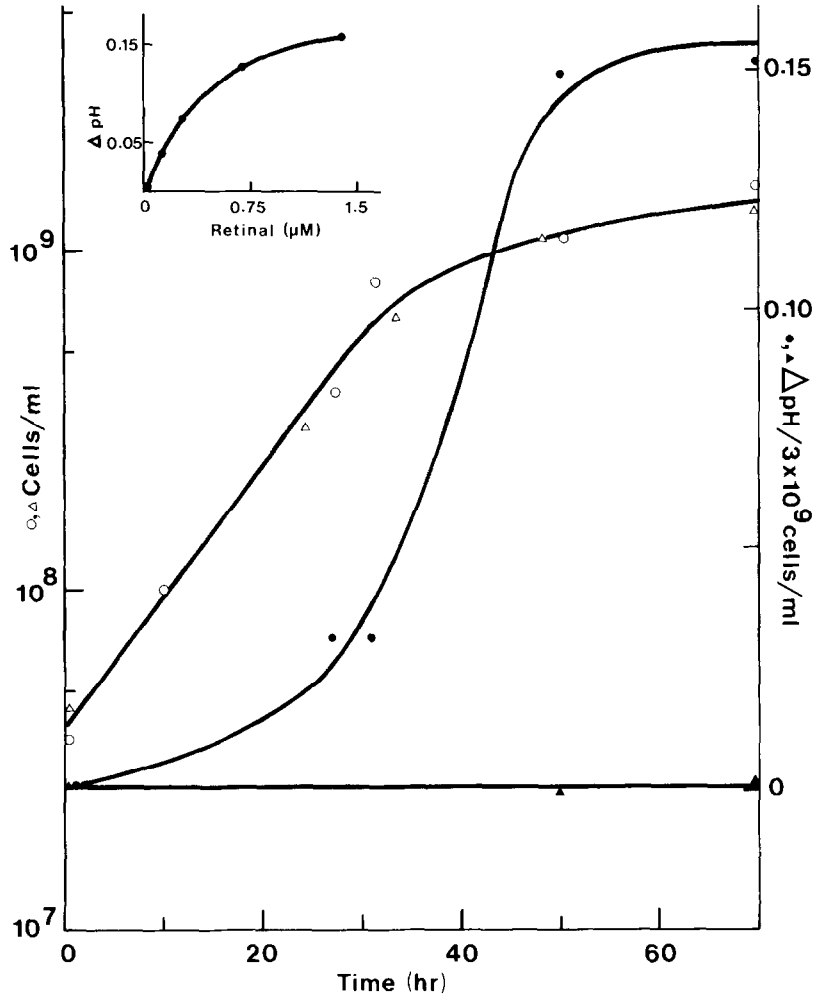

Figure 2. Kinetics of hR induction by retinal. OD2R (o,●) and Flx3R (△,▲) cells inoculated 2 days before time 0 on graph. At time 0, retinal was added to a final concentration of 1.4 μM. hR activity measured as in reference 4 using cells at 3 X 10^9/ml. ΔpH is the change caused by 2 min illumination. INSET: hR activity assayed for OD2R cells grown to stationary phase in various concentrations of retinal.

and the concentration dependence of hR induction by retinal are shown in Fig. 2.

The retinal-binding hR polypeptide can be labeled and identified by comparing mutant membranes. In the visual pigments and bR, retinal is linked to the protein as a protonated Schiff base. Reduction of the pigments with borohydride (BH_4^-) or cyanoborohydride (BH_3CN^-) eliminates the visible absorption band and photoreactions and converts the Schiff base to a stable secondary amine (9,10, 11). The membranes can then be solubilized and the

TABLE 1. Reduction of hR and sR by BH_3CN^-.

	PHOTOCHEMICAL ACTIVITY[a]			
	No BH_3CN^-[b]		+ BH_3CN^-	
	hR	sR	hR	sR
OD2R	1.22	0.17	nil	nil
Flx3R	nil	0.22	nil	0.06

[a] $\Delta A \times 10^{-3}$, actinic wavelength 580nm. Fast (hR) and slow (sR) kinetic component amplitudes of flash-induced absorbance changes measured at 600nm as described in reference 2. In each case 0.3 - 0.4 mg protein was flashed.

[b] Diethylether, but no reducing agent added.

retinyl proteins identified on SDS-polyacrylamide gel electrophoresis. The close similarity of hR and sR to the other rhodopsins suggests that the same approach can be used. The expected disappearance of the photoreactions (Table 1) and visible absorbance (not shown) are observed upon treatment with BH_3CN^-; and Lanyi and Oesterhelt have shown that in membranes from a retinal and bR-deficient H. halobium mutant at least one polypeptide can be labeled with added retinal (7).

We prepared vesicles from OD2R and Flx3R cells grown with (^3H)retinal and reduced them with BH_3CN^-, which eliminates their photoreactions (Table 1). SDS-polyacrylamide gel electrophoresis of the OD2R vesicles shows heavy labeling of a band with apparent molecular weight 25K (Fig. 3). Three other bands are labeled less heavily: one just above the heavily labeled 25K band and additionally faint 94K and 19K bands (Fig. 3). Flx3R vesicles lack the heavily labeled lower 25K band but show the other 3 bands. There is also less Coomassie blue staining in the 25K region of Flx3R (data not shown), but the difference is less pronounced probably due to the presence of other polypeptides. Since both strains have sR activity but Flx3R lacks hR, the simplest interpretation of these results is that the lower 25K band represents the retinal-binding polypeptide of hR. This is further confirmed by preparations from OD2R cells grown without retinal, where the vesicles were

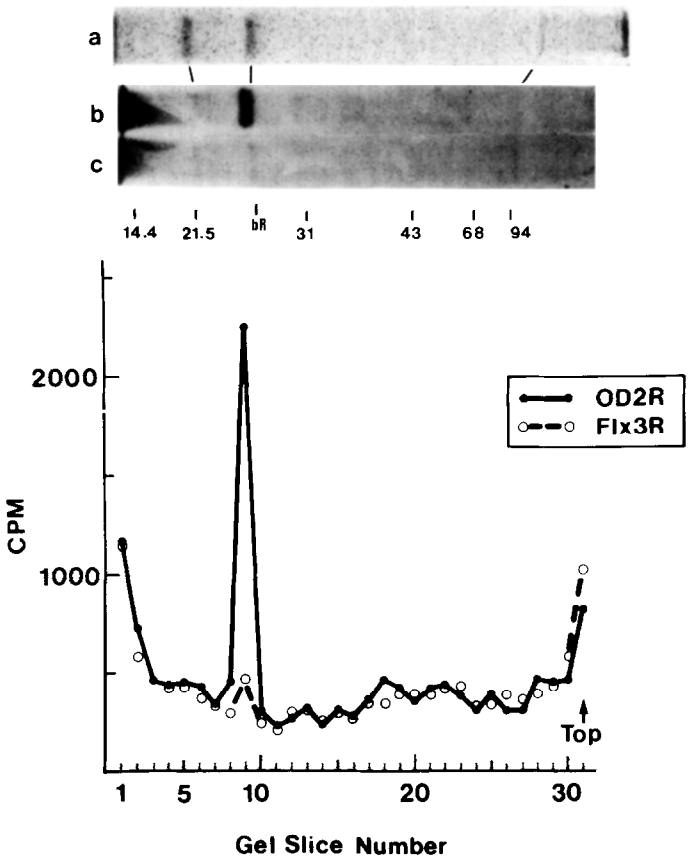

Figure 3. Polypeptides labeled with (^{3}H)retinal. Lanes b and c: vesicles from cells grown in (^{3}H)retinal (identical preparations used in Fig. 1 and Table 1) reduced and processed as described in Materials and Methods. Gels were treated with ENHANCE (NEN, Boston) and autofluorographed on X-ray film for 2 weeks. Duplicate gels were sliced into 4 mm pieces, extracted with PROTOSOL (NEN, Boston) overnight at 37°C, and counted as described (6). 0.050mg protein loaded/lane of a 12% polyacrylamide gel. Lane a: from a separate experiment, 1 month exposure autofluorogram of Flx3R vesicles incubated with (^{3}H)retinal, reduced and processed. The 3 lines between lanes a and b connect positions of equivalent M_r. In lane a the 19K, upper 25K, and 94K bands, which are also present in lanes b and c, are particularly evident.

incubated with (^{3}H)retinal. This generates the sR but not the hR photocycle or ion transport activity (Fig. 1). In the gel the heavily labeled lower 25K band is again missing, while the 19K, upper 25K, and 94K bands are present. As shown in Fig. 3, Flx3R vesicles labeled with the same procedure give the same 3-band pattern.

DISCUSSION

We identify the lower of the 2 bands at 25K M_r as the retinal-binding polypeptide of hR because it only appears in OD2R membranes when the hR

photocycle and transport activity are also present, i.e., after induction of hR synthesis with retinal, and because it cannot be induced in its hR⁻ derivative, Flx3R, under the same conditions. The other 3 bands are present in the membranes of both strains.

We cannot decide at present to what extent the other 3 bands represent different pigments or are artifacts. One known retinal pigment, sR, is present in all of the membranes examined in this study and is reduced by BH_3CN^- (Table 1). Therefore radiolabeled sR is likely to be present in the protein pattern. Preliminary experiments show that a loss of spectroscopic sR signal after partial reduction roughly correlates with label in the upper 25K band, indicating it may contain the retinal-binding polypeptide of sR.

In their previous attempt to identify the hR retinal-binding polypeptide Lanyi and Oesterhelt (7) report labeling one band near 25K. In our hands, their procedure of adding retinal to membranes from ret⁻ mutants does not generate significant amounts of hR. Therefore our data indicate they must have labeled the upper band at 25K, which may represent the sR but not the hR retinal-binding polypeptide.

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