Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 402–406, January 1987
Biochemistry

Biochemical and spectroscopic characterization of the blue-green photoreceptor in *Halobacterium halobium*

(halobacteria/rhodopsin/phototaxis/retinal/photosensory receptor)

P. SCHERRER, K. MCGINNIS, AND R. A. BOGOMOLNI

Cardiovascular Research Institute and Department of Biochemistry and Biophysics, San Francisco, CA 94143-0130

Communicated by Walther Stoeckenius, October 6, 1986

ABSTRACT **Spectroscopic evidence indicates the presence of a second sensory receptor sR-II in *Halobacterium halobium*, which causes a repellent response to blue-green light. Reactions with hydroxylamine and $NaCNBH_3$ and reconstitution of the bleached pigment with retinal show that it is very similar to the other retinylidene pigments bacteriorhodopsin, halorhodopsin, and especially the earlier-discovered phototaxis receptor, sensory rhodopsin, renamed $sR\text{-}I_{587}$. The second sensory receptor, $sR\text{-}II_{480}$, has an absorbance maximum at 480 nm and undergoes a cyclic photoreaction with a half-time of ≈200 msec. Its predominant photocycle intermediate absorbs maximally near 360 nm. The receptor can be detected spectroscopically in the presence of $sR\text{-}I_{587}$ and quantitated through its transient response to 450-nm excitation. It is selectively bleached by low hydroxylamine concentrations that are insufficient to bleach $sR\text{-}I_{587}$ significantly. Its photochemical and phototactic activities can be restored by addition of retinal. The mobility of the receptor, on $NaDodSO_4$/polyacrylamide gels, was similar or identical to that of $sR\text{-}I_{587}$ and slightly faster than bacteriorhodopsin, yielding an apparent molecular mass of 23–24 kDa.**

The membranes of *Halobacterium halobium* contain photochemically active retinal pigments. Two of these proteins, bacteriorhodopsin (bR) and halorhodopsin (hR), undergo rapid cyclic photoreactions and are light energy transducers (for review, see refs. 1–3). A third pigment, slow or sensory rhodopsin (sR), is a sensory light transducer with a much slower photocycle (4, 5). *H. halobium* cells are motile and phototactic; they are attracted by long-wavelength light and repelled by near-UV light, which causes the cells to migrate to areas with illumination optimal for their light energy transduction systems (6, 7). The absorbance maximum of sR is at 587 nm, in the spectral region where the attractant response is maximal. The dominant intermediate of its cyclic photoreaction, S_{373}, has a strong absorbance band around 373 nm, a region where the repellent response shows a maximum. S_{373} decays thermally back to the 587-nm absorbing state in ≈600 msec or can be photoconverted to it in 80 msec. Thus, Spudich and Bogomolni proposed (5) that sR_{587} and S_{373} are the photoreceptors for the opposite phototactic responses. If the intermediate S_{373} is allowed to decay thermally, an attractant response results, but if it is photoconverted back to sR_{587}, a repellent response is generated. Though the model is not universally accepted (see ref. 8 for a review), it is supported by strong experimental evidence (5, 9).

However, repellent responses have been observed under conditions where no significant amount of S_{373} should be present, and evidence for the existence of a second repellent receptor, which does not require background light, has been presented (10–12). Its action spectrum, covering the blue-green region, could explain the differences between the observed action spectra of *H. halobium* and that expected from the sR_{587} pigment. This second sensory receptor has an absorbance maximum near 480 nm and has been termed $sR\text{-}II_{480}$ to distinguish it from sR_{587}, which has been renamed $sR\text{-}I_{587}$. We have spectroscopically and biochemically identified this second sensory receptor in our strain Flx3-KM_1 and have shown that it is a retinal protein with similar properties to bR, hR, and $sR\text{-}I_{587}$.

MATERIALS AND METHODS

***H. halobium* Strain Flx3-KM_1.** *H. halobium* Flx3 (14) colonies grown on agar plates were screened for spontaneous mutants forming white colonies and tested for phototactic activity by the blue repellent response as described elsewhere (5) and for chemotactic activity on soft agar plates (7) after passage in liquid medium. Due to the high revertant rate found in the isolated photo- and chemotactic-positive white mutants, the screening was repeated several times after a few passages in liquid medium. Finally, a stable, carotenoid-deficient mutant that was phototactic and chemotactic was isolated and labeled *H. halobium* strain Flx3-KM_1. Like its parent Flx3, it contained no bR or hR detectable by flash spectroscopy.

Membrane Preparation. The cells were grown in 12-liter fermentors and harvested by standard procedure (15). They were resuspended in 200 ml of 4 M NaCl and, after addition of 10 mg of DNase, lysed by dialysis against 6 liters of 0.1 M NaCl for 18 hr at 4°C. The lysate was centrifuged at 6000 × *g* for 20 min to remove large debris and the membranes in the supernatant were pelleted by centrifugation at 260,000 × *g* for 1 hr. The membranes were resuspended in 3 M NaCl/50 mM Hepes, pH 7.0, and stored at 4°C in the dark at a protein concentration of 15–20 mg/ml. The protein concentration was determined by the Lowry method (16) with bovine serum albumin as standard.

Spectroscopy. Rapid transient absorbance changes were measured on a system essentially as described (17) using a 10-nsec actinic light pulse at 475 nm or 579 nm from a nitrogen-pumped dye laser (Molectron, Sunnyvale, CA) with both light pulses saturating $sR\text{-}I_{587}$. For routine measurements to follow the pigment activity during bleaching, regeneration, and reduction, an electronic flash (1-msec duration) was used as excitation light with either a 450 ± 8 nm interference filter or a 2-58 sharp cut red filter (Corning) providing light ≥620 nm. The 450-nm light pulse excited about 7% $sR\text{-}I_{587}$, as measured at 590 nm, and 28–30% $sR\text{-}II_{480}$. From the absorbance of the two pigments at 500 nm and the fraction cycling, we estimated a 10–15% contribution of $sR\text{-}I_{587}$ to the 500-nm absorbance change.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: bR, bacteriorhodopsin; hR, halorhodopsin; sR, sensory rhodopsin.

UV-visible static absorption spectra were recorded with an Aminco DW-2a spectrophotometer linked to a Nicolet 1180 data acquisition system. Absorption spectra in the presence of an actinic light were obtained on a spectrometer designed and built in our laboratory (18).

Bleaching and Regeneration of Pigments. Membrane suspensions were treated with (*i*) 1 M, (*ii*) 25 mM, or (*iii*) 1 mM hydroxylamine in the dark at 20°C. (*i*) To 0.8 ml of membrane (≈10–15 mg of protein per ml in 3 M NaCl/50 mM Hepes, pH 7.0), 0.2 ml of 5 M hydroxylamine (pH 7.0) was added and the suspension was incubated for 8 hr. (*ii*) To 0.8 ml of membrane (10–15 mg of protein per ml in 3 M NaCl/50 mM Hepes, pH 7.0), 0.15 ml of distilled H_2O and 0.05 ml of 500 mM hydroxylamine (pH 7.0) were added and the suspension was incubated for 1 hr. (*iii*) To 0.8 ml of membrane (10–15 mg of protein per ml in 3 M NaCl/50 mM Hepes, pH 7.0), 0.18 ml of distilled H_2O and 0.02 ml of 50 mM hydroxylamine (pH 7.0) were added and the suspension was incubated for 30 min.

After times indicated, the hydroxylamine-treated membranes were diluted to 10 ml and washed at least five times by centrifugation (30 min, 280,000 × *g*) with 3 M NaCl/50 mM Hepes, pH 7.0, to remove all hydroxylamine. Between centrifugations, the membrane suspensions were incubated at room temperature for ≈2 hr.

To regenerate the bleached pigments, washed membranes were resuspended in 1 ml of 3 M NaCl/50 mM Hepes, pH 7.0, and 1 μl of all-*trans*-[^{3}H]retinal in ethanol [430 mCi/mmol (1 Ci = 37 GBq); 1.5 mM in ethanol] was added under dim red light. The samples were kept overnight at 20°C in the dark and the excess retinal was removed by washing three times with 3 M NaCl/50 mM Hepes, pH 7.0, by centrifugation (30 min, 280,000 × *g*). In some cases 1% bovine serum albumin was included in the first wash. Bleaching and regeneration of the pigments were followed by monitoring the flash-induced absorbance change.

Electrophoresis and Fluorography of the Regenerated Pigments. The regenerated, washed membrane preparations were resuspended in 1 ml of 3 M NaCl and 100 μl of 20% cyanoborohydride was added, followed by addition of 25 μl of 2 M sodium acetate buffer (pH 4.5) and 5 μl of 100 mM γ-myristoyl L-α-lysolecithin (Calbiochem-Behring). The mixture was kept at 20°C for 24 hr prior to gel electrophoresis to reduce the labeled pigment.

Fifteen percent $NaDodSO_4$/polyacrylamide slab gels were run according to the method of Laemmli (19). The samples were desalted by dialysis against distilled H_2O at 20°C prior to 1 hr of solubilization at room temperature and run in duplicates. Half of the gel was exposed to Kodak X-Omat AR film for 4–6 weeks at −80°C after soaking in autoradiography enhancer (Amplify; Amersham) according to manufacturer's instructions and drying, and the other half was stained with Coomassie brilliant blue. The stained tracks were cut and sliced into ≈2-mm slices, the pieces were dissolved in 0.7 ml of 30% H_2O_2 at 75°C for 2 hr, and radioactivity was counted with Aquasol II (New England Nuclear).

RESULTS

Spectroscopic Assay for the sR-II_{480} Pigment in the Presence of sR-I_{587}. Illumination of Flx3-KM_1 membranes at wavelength ≥620 nm yields the photosteady state difference spectrum expected for sR-I_{587} with a depletion maximum at 590 nm, an absorbance increase at 375 nm, and the isosbestic point near 420 nm (4). Illumination at shorter wavelength yields an additional depletion below 500 nm that becomes predominant under illumination at 450 nm. The new depletion maximum is at 480 nm and a form absorbing maximally near 360 nm is generated (data not shown). Although this demonstrates the presence of substantial amounts of sR-II_{480}, the spectra are noisy and also suffer from contribution by the Soret band of cytochromes. Time-resolved spectroscopy gives better results.

The membranes were exposed to 450 ± 8 nm or ≥620-nm flashes and the absorbance changes were recorded at 500 and 580 nm (Fig. 1). The amplitude of the absorbance decrease induced by ≥620-nm excitation was about four times smaller at 500 nm than at 580 nm (ΔA_{500}:ΔA_{580} = 0.22), as expected from the absorbance spectrum of sR-I_{587}, and decayed with $t_{1/2}$ ≃ 500 msec. The 450-nm excitation yielded an absorbance decrease with a ratio of ΔA_{500}:ΔA_{580} = 1.9, which was apparently caused by an additional kinetic component decaying with $t_{1/2}$ ≃ 200 msec. The decay kinetics at 580 nm were the same with 450-nm or ≥620-nm excitation pulses. The additional blue-green light-induced absorbance change is obviously due to the pigment sR-II_{480}.

To obtain the flash-induced difference spectra for the two pigments, we used actinic flashes at 475 nm and 580 nm, which both saturated sR-I_{587}. Since a retinal pigment with an absorbance maximum at 480 nm is expected to show very little remaining absorbance at 580 nm, a flash-induced absorbance spectrum with 580-nm excitation will result in a different spectrum of only sR-I_{587} (Fig. 2*a*). It shows the depletion maximum at 590 nm and an absorbance increase at 375 nm with the crossover point at 420 nm. The actinic flash

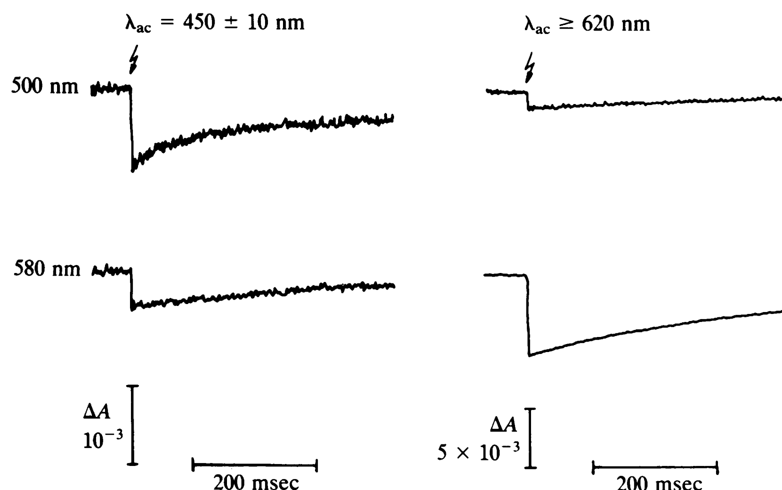

FIG. 1. Flash-induced absorbance changes measured at 500 and 580 nm upon excitation with 450 ± 8 nm and ≥620-nm light, respectively, in a membrane suspension (6 mg of protein per ml; 3 M NaCl/50 mM Hepes, pH 7.0) at 20°C. The signals represent the accumulation of 16 flash-induced changes for 450-nm and 8 for ≥620-nm excitation of 1-msec duration with a repetition rate of 0.1 Hz.

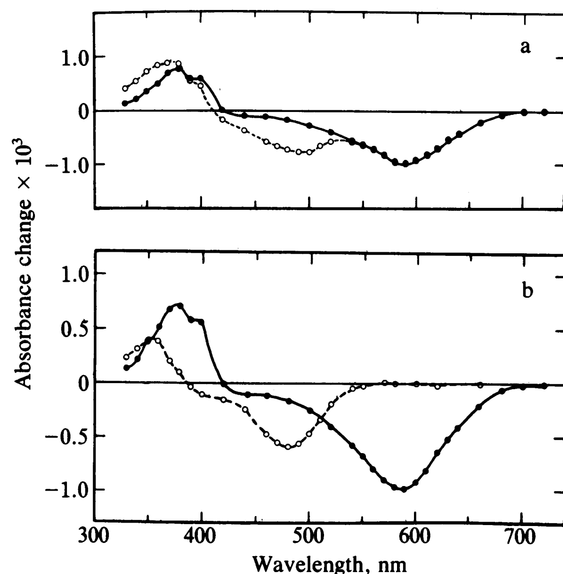

FIG. 2. Flash-induced difference spectra 2 msec after excitation of a membrane pellet mounted in a 0.5-mm pathlength split quartz cuvette in 3 M NaCl/50 mM Hepes, pH 7.0, at 20°C. ●, In *a* and *b*, absorbance changes after excitation with a saturating 580-nm laser pulse. ○, In *a*, absorbance changes 2 msec after excitation with saturating 480-nm laser pulse. ○, In *b*, the difference between the amplitudes of absorbance changes induced by 480-nm and 580-nm laser pulses. Laser flash, 10-nsec duration; 1 millijoule per pulse.

at 475 nm, where sR-I_{587} still shows a considerable absorbance, activates both pigments and the difference spectra show the sum of their absorbance changes (Fig. 2*a*). Since the actinic flash at 475 nm was saturating sR-I_{587} and sR-II_{480}, the flash-induced difference spectrum for sR-II_{480} could be obtained by subtracting the sR-I_{587} spectrum obtained with saturating 580-nm excitation. The resulting difference spectrum has a depletion centered at 480 nm and a maximum near 360 nm; the crossover point is at 385 nm (Fig. 2*b*).

This procedure for obtaining the flash difference spectra for the pigments requires not only negligible absorption of sR-II_{480} at the long actinic wavelength but also the fraction of sR-I_{587} photoconverted at both actinic wavelengths must be the same. During the saturating 10-nsec flash, the first photocycle intermediate S_{680} does not decay significantly and thus is expected to photoconvert back to sR-I_{587}, generating a photostationary state. The fraction of sR-I_{587} photoconverted is a function of the quantum efficiencies for the forward and back reactions and the ratio of extinction coefficients between the two species (20). Because the quantum efficiencies are not expected to vary significantly within the absorption band, this technique only requires a choice of actinic wavelengths at which the extinction ratio between sR-I_{587} and its S_{680} intermediate is also the same. As shown in Fig. 2, this condition is satisfactorily fulfilled at 475 and 580 nm; because the difference spectra in the 550- to 720-nm region are identical for both actinic wavelengths.

The light-saturating conditions were not suitable for routine measurements of pigment activity. Based on the flash-induced and steady-state difference spectra, we chose spectral parameters for routine measurement of the two pigments as follows: for sR-I_{587} the absorbance change at 580 nm upon ≥620-nm flash excitation and for sR-II_{480} the absorbance change at 500 nm upon 450-nm excitation. Under the nonsaturating light conditions used for the 450-nm excitation, we corrected for a 10–15% contribution of sR-I_{587} to the 500-nm absorbance change. Since these preparations scatter light strongly, we took care to choose protein concentrations and light intensities so that responses were linear with respect to pigment concentration and fraction cycling.

Bleaching and Reconstitution of the Retinal Pigments. When the membranes were exposed to 0.4 M hydroxylamine, at pH 7.0 in the dark, a rapid decrease of the 500-nm signal with 450-nm excitation (sR-II_{480}) was observed, which was followed by a much slower decrease of the 580-nm signal upon ≥620-nm excitation (sR-I_{587}). Therefore, the effect of various hydroxylamine concentrations (1 mM–1 M) on sR-II_{480} and sR-I_{587} was tested and the results are summarized in Table 1. We found that 25 mM hydroxylamine at pH 7 bleaches most of sR-II_{480} within 60 min in the dark without significant loss of sR-I_{587} photochemical activity (Fig. 3). The static difference spectrum between 5 and 60 min after addition of 25 mM hydroxylamine yielded the absorption spectrum of sR-II_{480} (Fig. 4).

When the membranes were treated with 1 M hydroxylamine at pH 7.0 in the dark, flash-induced absorbance changes for sR-II_{480} and sR-I_{587} were reduced by a factor of at least 10 after 8 hr. The static absorption difference spectra recorded at various times after addition of 1 M hydroxylamine minus the spectrum 2 min after the reaction started showed the 480-nm depletion to be nearly complete after about 20 min, whereas complete depletion at 590 nm required at least 8 hr. The difference spectra of 20-min- and 5-hr-bleached membranes corresponded to the absorption spectrum of sR-I_{587} (Fig. 4).

When the unreacted hydroxylamine was removed from the membrane suspension by extensive washing and all-*trans*-retinal was added, the absorbance around 480 nm increased in the 25 mM NH_2OH-treated membranes and sR-II_{480} photochemical activity was regenerated (Table 1 and Fig. 3, lower traces). In the 1 M NH_2OH-treated membranes, sR-II_{480} and sR-I_{587} absorbances were restored and the corresponding transient responses were regenerated.

In membranes treated with 1 mM hydroxylamine at pH 7.0 in the dark, a depletion of 590-nm absorbance was observed within a few minutes (Fig. 4) but no loss of any flash-induced absorbance change at 590 nm could be detected (Table 1). Addition of all-*trans*-retinal to these membranes after removal of hydroxylamine resulted in the regeneration of 590-nm absorbance, again without any detectable change in the flash-induced absorbance changes. The amount of this absorbance decrease at 590 nm varied greatly in different membrane preparations of Flx3-KM_1 and Flx3 and was maximally of the order of 0.010. Our flash spectrometer would have allowed us to detect a photoactive pigment with this absorbance if it showed ≥30-nm absorbance shifts in the microsecond-to-second time range.

Identification of the Retinal-Binding Proteins. The retinal–protein linkage in sR-II_{480} and sR-I_{587} is probably a Schiff's base as in visual pigments, bR, and hR and will be cleaved during detergent solubilization for $NaDodSO_4$/poly-

Table 1. Effect of various hydroxylamine concentrations and subsequent retinal addition on sR-II_{480} and sR-I_{587}

NH_2OH, mM	% activity after NH_2OH*		% activity after retinal addition	
	sR-II_{480}	sR-I_{587}	sR-II_{480}	sR-I_{587}
0	100	100	97	104
1	98	103	96	102
25	21	103	98	102
1000	9†	11	81	78

*Relative amplitude of the flash-induced absorbance change at 500 nm with 450-nm excitation for sR-II_{480} and at 580 nm with ≥620-nm excitation for sR-I_{587} measured after incubation for 30 min in 1 mM, 60 min in 25 mM, and 8 hr in 1 M hydroxylamine. The estimated error for the activities is ±5%.

†Not corrected for sR-I_{587} contribution.

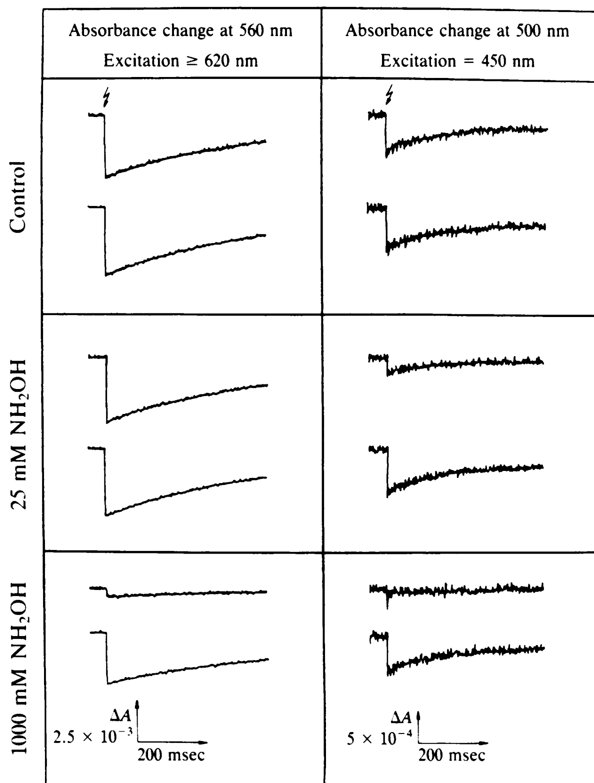

FIG. 3. Flash-induced absorbance change at 560 nm upon ≥620-nm excitation and at 500 nm upon 450-nm excitation in a membrane suspension (3 M NaCl/50 mM Hepes, pH 7.0, at 20°C; 4 mg of protein per ml). Upper traces, after treatment with hydroxylamine; lower traces, after subsequent addition of all-*trans*-[^{3}H]retinal.

acrylamide gel electrophoresis. In bR (21) and hR (22, 24) the retinal can be firmly attached to the protein by cyanoborohydride reduction of the Schiff's base with concomitant loss of photochemical activity. When we followed the photochemical activity of sR-II_{480} and sR-I_{587} in Flx3-KM_1 membranes after addition of $NaCNBH_3$ at pH 4.5, we observed only a very slow loss of activity. Addition of lysolecithin (0.44 mM) accelerated the reduction, which was complete after about 8 hr, leaving no detectable transient absorbance changes of sR-II_{480} and <6% of sR-I_{587}. (Addition of lysolecithin without $NaCNBH_3$ did not reduce the photochemical activity.)

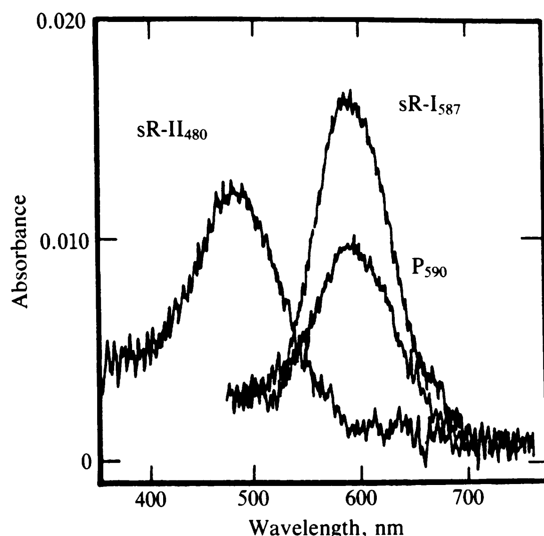

FIG. 4. Absorbance difference spectra of membrane suspensions (4 mg of protein per ml; 3 M NaCl/50 mM Hepes, pH 7.0, at 20°C) treated with hydroxylamine. P_{590}, the spectrum 10 min after incubation in 1 mM hydroxylamine was subtracted from the spectrum obtained before hydroxylamine addition. sR-II_{480}, the membranes were incubated in 25 mM hydroxylamine and the spectrum after 60 min was subtracted from the 5-min spectrum. sR-I_{587}, the membranes were incubated in 1000 mM hydroxylamine and the spectrum obtained after 300 min was subtracted from the one after 20 min.

The bleached pigments were reconstituted with all-*trans*-[^{3}H]retinal and reduced, and the proteins were separated by $NaDodSO_4$ gel electrophoresis. The positions of the bands containing [^{3}H]retinal were slightly below the bR band, indicating proteins with a molecular mass of 23–24 kDa. In preparations in which only sR-II_{480} was labeled and in preparations in which both sR-II_{480} and sR-I_{587} were labeled, the label was found in the same position, but with much more label in the latter preparation (Fig. 5). No distinctly labeled protein band was found in the [^{3}H]retinal reconstituted membranes of the 1 mM hydroxylamine-bleached preparation.

The fluorogram of the $NaDodSO_4$ gels showed a double band when only sR-II_{480} was bleached and reconstituted with higher intensity in the upper band (Fig. 5 *Inset*). When both sR-II_{480} and sR-I_{587} were reconstituted, the upper band appeared clearly broader and much more pronounced while the lower band remained virtually unchanged.

Retinal bound to a protein by a Schiff's base linkage in bR and hR can slowly exchange with excess free retinal. To estimate the retinal exchange in our preparations, membranes without prior hydroxylamine bleaching were incubated with [^{3}H]retinal and reduced under the same conditions as the bleached preparations. A weakly labeled band was found

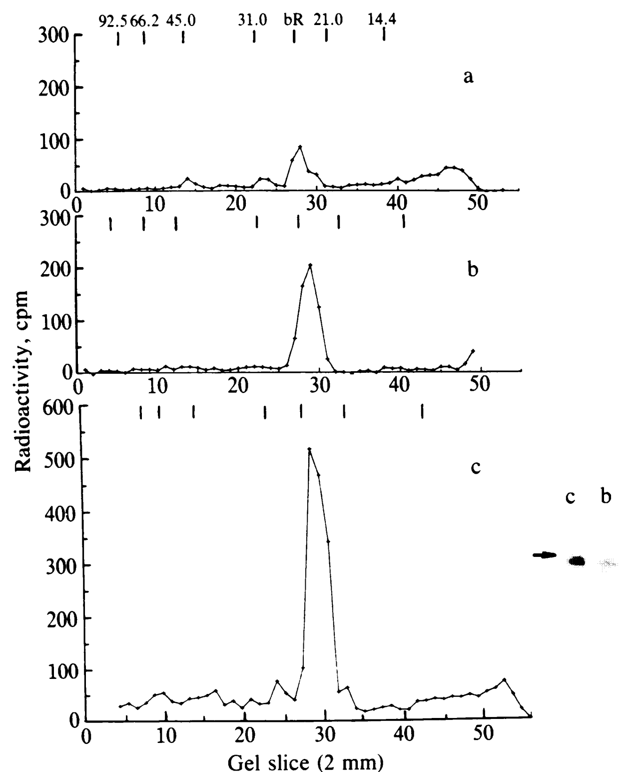

FIG. 5. Radioactivity profile in the $NaDodSO_4$/polyacrylamide gels after [^{3}H]retinal reconstitution of unbleached (*a*), 25 mM hydroxylamine-bleached (*b*), and 1000 mM hydroxylamine-bleached (*c*) membranes. The traces are aligned for the bR marker. Molecular mass standards are shown in kDa. (*Inset*) Corresponding fluorogram for the 14- to 45-kDa region of the gels. The arrow indicates the position of bR in the marker gel. The tracks, labeled b and c, correspond to the traces in *b* and *c*.

in the same position as in the bleached samples. This exchange could account for <30% of the total radioactivity in the 25 mM and <12% in the 1 M hydroxylamine-treated sample.

When membranes of Pho 81, a chemotaxis-positive but phototaxis-negative mutant, which also lacks detectable photochemical activity even after retinal addition (9), were treated identically, no proteins in the 20- to 30-kDa region were labeled.

DISCUSSION

The absorbance change kinetics and flash-induced difference spectra described support the interpretation that in addition to sR-I_{587} a second photoactive receptor absorbing in the blue-green region is present in *H. halobium* cells (10–12). We have further confirmed its presence by selective bleaching with hydroxylamine and reconstitution with retinal. Assuming a similar extinction, we estimate from the loss of absorbance at 590 and 480 nm with 1 M and 25 mM hydroxylamine, respectively, that the two pigments in Flx3-KM_1 membranes are present in about equal amounts. The close correspondence between the ratio of absolute absorbances (Fig. 4) and flash-induced absorbance difference under saturating conditions (Fig. 2) suggests that the two pigments also have similar photochemical quantum efficiencies. The bleaching and reconstitution experiments show that sR-II_{480} is a retinal pigment similar to sR-I_{587}, which has been identified in the retinal-minus mutant Flx3R (23). The two pigments must have a distinctly different chromophore environment because of their different absorbance maxima, photoreaction cycles, and reactivity to hydroxylamine. However, at present we cannot be certain that they also have different polypeptide chains. It is still possible that the spectral and kinetic differences between sR-I_{587} and sR-II_{480} are due to the same pigment in a different local environment. Differences in molecular mass, if not artifacts, could be due to posttranslational modification of the protein. In our hands, this difference amounts to not more than 500 Da and is not resolved in the sliced gels. A larger molecular mass difference was obtained by Spudich *et al.* (13). Their labeling technique and membrane preparation differ from ours and that may explain small differences in electrophoretic mobility. We found a double band when only sR-II_{480} was reconstituted and we note that double bands are also often observed when bR or presumably pure hR preparations were subjected to $NaDodSO_4$ gel electrophoresis. It has not been established whether these are artifacts or are due to *in vivo* modification of the proteins (24).

The nature of the 590-nm absorbing species that bleaches in 1 mM hydroxylamine and shows no detectable photochemical activity in the $\geq$1-μsec time range remains unknown. Reconstitution with retinal shows an absorbance gain, but the amount of [^{3}H]retinal incorporated was of the same order as in the unbleached control membranes, reflecting either retinal exchange with sR-II_{480} and sR-I_{587} or unspecific labeling. The retinal in this pigment may not form a Schiff's base linkage to the protein that can be reduced or the pigment is too labile and loses the retinal under the reduction conditions.

The isolation of Flx3-KM_1, a carotenoid-deficient daughter strain of Flx3, greatly facilitated the detection of sR-II_{480}. It is also present in Flx3 but was difficult to estimate due to the large absorbance of carotenoids in the blue-green region. sR-II_{480} is already formed in early logarithmic growth phase and is found to precede sR-I_{587} formation as seen by the changing ratios of the two pigments during growth (10, 11). It is not necessary to add exogenous retinal to Flx3-KM_1 cells during early growth to detect the phototactic response and sR-II_{480} photochemical activity, contrary to the observation, in a different Flx3 mutant, Flx3-12, by Takahashi *et al.* (10).

Pho 81, the only mutant isolated so far (from Flx15) that lacks phototaxis and contains no sR-I_{587}, also lacks sR-II_{480}. It may be a double mutant, which would be expected under the selection conditions used and would explain the relatively low frequency at which it occurred (9).

Note. After this manuscript had been submitted for publication, we received a manuscript from N. Kamo, which describes the transient absorbance changes in *H. halobium* Flx3-12 and another Flx3 mutant, which apparently contains even higher concentrations of sR-II_{480} and less sR-I_{587} (25). Their results agree well with ours and they detect evidence of an additional late intermediate in the sR-II_{480} photocycle with a maximum at 530 nm in the difference spectrum. They also note the higher susceptibility of sR-II_{480} to bleaching by NH_2OH and propose the name phoborhodopsin for the blue-green receptor.

This work is dedicated to Dr. Luis F. Leloir in celebration of his 80th birthday. We thank John Spudich for communication of his unpublished results and are grateful to Walther Stoeckenius for helpful discussions and critical reading of the manuscript. This work was supported by National Institutes of Health Grants GM-27057 and GM-34219 and National Science Foundation Grant DMB-8444103.

1. Stoeckenius, W. & Bogomolni, R. A. (1982) *Annu. Rev. Biochem.* **52**, 587–615.
2. Stoeckenius, W. (1985) *Trends Biochem. Sci.* **10**, 483–489.
3. Lanyi, J. K. (1986) *Annu. Rev. Biophys. Biophys. Chem.* **15**, 11–28.
4. Bogomolni, R. A. & Spudich, J. L. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 6250–6254.
5. Spudich, J. L. & Bogomolni, R. A. (1984) *Nature (London)* **312**, 509–513.
6. Hildebrand, E. & Dencher, N. (1975) *Nature (London)* **257**, 46–48.
7. Spudich, J. L. & Stoeckenius, W. (1979) *Photobiochem. Photobiophys.* **1**, 43–53.
8. Dencher, N. A. (1983) *Photochem. Photobiol.* **38**, 753–767.
9. Sundberg, S. A., Bogomolni, R. A. & Spudich, J. L. (1985) *J. Bacteriol.* **164**, 282–287.
10. Takahashi, T., Tomioka, H., Kamo, N. & Kobatake, Y. (1985) *FEMS Microbiol. Lett.* **28**, 161–164.
11. Wolff, E. K., Bogomolni, R. A., Scherrer, P., Hess, B. & Stoeckenius, W. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 7272–7276.
12. Sundberg, S. A., Alam, M. & Spudich, J. L. (1986) *Biophys. J.* **50**, 895–900.
13. Spudich, E. N., Sundberg, S. A., Manor, D. & Spudich, J. L. (1986) *Proteins* **1**, 239–246.
14. Spudich, E. N. & Spudich, J. L. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 4308–4312.
15. Oesterhelt, D. & Stoeckenius, W. (1974) *Methods Enzymol.* **31**, 667–678.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
17. Lozier, R. H. (1982) *Methods Enzymol.* **88**, 133–162.
18. Kouyama, T., Bogomolni, R. A. & Stoeckenius, W. (1985) *Biophys. J.* **48**, 201–208.
19. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
20. Lozier, R. H. & Niederberger, W. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 1805–1809.
21. Katre, N. V., Wolber, P. K., Stoeckenius, W. & Stroud, R. M. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4068–4072.
22. Lanyi, J. K. & Oesterhelt, D. (1982) *J. Biol. Chem.* **257**, 2674–2677.
23. Spudich, J. L. & Bogomolni, R. A. (1983) *Biophys. J.* **43**, 243–246.
24. Taylor, M. E., Bogomolni, R. A. & Weber, H. J. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 6172–6176.
25. Tomioka, H., Takahashi, T., Kamo, N. & Kobatake, Y., (1986) *Biochem. Biophys. Res. Commun.* **139**, 389–395.