PROTEINS: Structure, Function, and Genetics 1:239–246 (1986)

Properties of a Second Sensory Receptor Protein in *Halobacterium halobium* Phototaxis

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ABSTRACT **A second slow-cycling retinylidene protein, in addition to slow-cycling (sensory) rhodopsin (SR), can be bleached with hydroxylamine and regenerated with all-*trans* retinal in photosensory signaling *Halobacterium halobium* membranes. Flash photolysis shows this protein undergoes a photochemical reaction cycle characterized by photoconversion of its ground state (λ_{max} 480 nm) to a species with $\lambda_{max} \leqslant 360$ nm, which thermally regenerates the 480-nm species with a $t_{1/2}$ of 260 msec at 25°C, under conditions in which SR photocycles at 650 msec in the same membranes. Mutants characterized with respect to their phototaxis behavior are identified which contain SR and the 480-nm pigment, the latter ranging from undetectable to a concentration equal to that of SR. Receptor mutants lacking all phototaxis sensitivity lack both of the photochemically reactive proteins. The mutant properties contribute to an accumulation of behavioral and spectroscopic evidence that the 480-nm pigment is a second sensory photoreceptor in *H. halobium*. NaDodSO$_4$-polyacrylamide gel electrophoresis of [^{3}H]retinal-labeled membrane proteins from the mutants indicates SR and the 480-nm pigment contain distinct chromophoric polypeptides differing in their migration rates. The data implicate polypeptides of 25,000 M_r and 23,000 M_r as retinal-binding polypeptides of SR and the 480-nm protein, respectively.**

Key words: halobacteria, photosensory receptor, retinal, slow-cycling rhodopsins, sensory rhodopsins

INTRODUCTION

Phototaxis reception in *Halobacterium halobium* is mediated through the chromophore vitamin A-aldehyde (retinal) (for review, see references 1 and 2). In the membranes of *H. halobium* two retinal-containing pigments, bacteriorhodopsin (BR) and halorhodopsin (HR), function as light-driven ion pumps.[3,4] Mutant strains lacking both BR and HR have been isolated[5] and a sensory rhodopsin has been revealed ("slow-cycling" rhodopsin (SR)[6]). The BR^-HR^- strains (ion flux mutants, e.g., Flx3, Flx15) lack spectroscopic and functional interference by the retinal-dependent ion transporters and therefore have simplified the analysis of sensory reception. In particular, studies of Flx3 and its retinal-deficient derivative Flx3R have clarified many of the properties of SR.[7] SR is a retinal-containing integral membrane chromoprotein, which undergoes nonelectrogenic photochemical reactions.[7,8] Its dark-adapted form (λ_{max} 587 nm) mediates attractant responses to orange light and its long-lived photointermediate (λ_{max} 373 nm) mediates repellent responses to light in the near-UV.[9,10]

Repellent responses to 450–550-nm light were reported in wild-type cells, and an accessory role of carotenoids in photoreception was suggested to explain these responses.[11] The known photoreactions of SR cannot account for such responses directly because the repellent form of SR, S_{373}, does not absorb in this region.[6] A number of behavioral and spectroscopic observations support the proposal of Takahashi et al.[12] for a second repellent system in *H. halobium*. Phototaxis action spectra of Flx3 derivatives have shown a peak repellent sensitivity at 480 nm[12,13] in addition to the 370-nm peak due to SR. The 480-nm response, unlike that at 370 nm, does not depend on orange background light, and the two repellent systems can be further distinguished on the basis of differences in their signal-processing times.[14]

Photoexcitation of membranes from a Flx3 derivative with 486-nm light induces an absorption change not due to the known photoreactions of SR.[12] This is likely to result from photochemical reaction of a second repellent receptor since it is in the spectral range of the second repellent system.[12] This reaction and SR photoreactions are missing in a mutant lacking both repellent sensitivities (Pho81), whereas its parent (Flx15) exhibits both photosystems by behavioral and flash photolysis measurements[16] (and our unpublished observations).

In this report we compare photochemical reactions and [^{3}H]retinal labeling of membrane proteins from selected Flx mutants with varying amounts of the two sensory photosystems. The results confirm the existence of a second distinct repellent receptor in *H.*

Received June 6, 1986; accepted August 1, 1986.

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halobium and show the receptor is a retinal-dependent pigment which is present in the membrane with SR and differs from it in several of its properties.

MATERIALS AND METHODS

Strains

Flx3, Flx5, and Flx15 are independent BR^-HR^- isolates derived from *H. halobium* OD2 by the ion flux mutant selection procedure.[5] Flx3R and Fx5R are blocked in retinal synthesis (ret^-) and were obtained by isolating white (i.e., carotenoid deficient) colonies, which are often ret^-. Our screening criterion for retinal deficiency was that the strains lack phototaxis responses and phototaxis is restored by addition of all-*trans* retinal.[15] Flx3-12 is a variant isolated from Flx3 in Kobatake's laboratory[12] and was kindly provided by Tetsuo Takahashi. Flx3-12W has a reduced carotenoid content more suitable for spectroscopy and was selected by screening single colonies for diminished red coloration. Flx3-12 and Flx3-12W have greatly diminished sensitivities to both phototactic and chemotactic stimuli compared to Flx3, indicating a defect in the sensory machinery beyond the receptors (our unpublished observations). Flx3-KM1 is a variant with reduced carotenoid content[13] isolated from Flx3 and was kindly provided by Walther Stoeckenius. Pho81 is a phototaxis-deficient (pho^-) mutant selected from Flx15.[16] Pho81 was classified as a photoreception mutant on the basis of its pho^- phenotype, which cannot be restored by addition of all-*trans* retinal, normal chemotaxis (che^+), and the lack of photochemically reactive pigments according to flash photolysis[16] (and our unpublished observations).

Flash Photolysis

The flash instrument used was constructed based on a design by Roberto Bogomolni. The monitoring light was provided by a 12-V 50-W tungsten-halogen lamp (Osram HLX 64610 or equivalent) powered by a 12-V DieHard battery and passed through a Bausch and Lomb model 33-86-76 monochromator. The monitoring beam at the intensities used (e.g., 1.3×10^3 ergs·cm^{-2}·sec^{-1} at 590 nm) was nonactinic. After passing through the sample, temperature-controlled with a water-jacketed cuvette holder, and appropriate interference filters, the beam was focused onto a Hamamatsu (Middlesex, NJ) R928 photomultiplier powered by a Pacific Instruments (Concord, CA) model 204 high-voltage power supply. The R928 photocurrent was terminated with a 220-kΩ load resistor to ground. The dark current level (monitoring beam blocked) of the photomultiplier was set to +0.10 V by using a DC offset; the photocurrent level prior to the actinic flash was then set to 0 V by adjusting the photomultiplier gain. The photomultiplier output was relayed via a Tektronix (Beaverton, OR) AM502 differential amplifier to a Nicolet (Madison, WI) digital oscilloscope (4094A mainframe with 4562 A-to-D converter), set at 2ms per point, 3,968 points per sweep. The actinic flash was delivered at 90° to the monitoring beam with a Vivitar (Santa Monica, CA) model 283 electronic flash (approximately 1-msec duration) passed through 600 ± 20 nm, 610 ± 5 nm, 450 ± 20 nm, or 500 ± 5 nm interference filters (Ditric Optics, Hudson, MA). The photomultiplier was protected from the actinic flash by narrow-band interference filters transmitting at the wavelength being monitored. The flash was triggered 800 msec after initiation of the oscilloscope sweep by a Grass (Quincy, MA) S44 stimulator. Signals were collected and averaged over 20–100 sweeps at 10-second intervals. Membrane vesicle suspensions were prepared as described[5] and flash photolysis was performed at 25°C.

[^{3}H] Retinal-Labeling

Membrane vesicles prepared as described[5] were incubated at 5 mg protein per ml in the presence of 0.5 M NH_2OH (pH9.0) at 37°C under orange light (Corning 3-69 filter) illumination at 2×10^6 ergs·cm^{-2}·sec^{-1} for 1 hour, pelleted by ultracentrifugation at 4°C, suspended in 4 M NaCl, 25 mM Tris hydrochloride (pH 6.8), and washed two more times with this buffer. The washed membranes were then incubated with [^{3}H]retinal (2.9 μM; 400 μCi/μmole) for 2 hours, reduced with $NaCNBH_3$, and processed for autofluorography as described previously.[17,18]

RESULTS AND DISCUSSION

Flash Photolysis of Membrane Vesicles

Membranes prepared from Flx3R contain the SR-apoprotein and addition of all-*trans* retinal generates the active pigment.[7,8] Flash-induced absorbance changes for such membranes (Fig. 1) show a depletion at 570 nm after a 600-nm flash, as expected from the SR absorbance in this region (λ_{max} 587 nm). The absorbance change at 500 nm is 26% of that at 570 nm, and both decay with the same first-order half-time of 650 msec (25°C) (Fig. 4A); 450-nm light is less effective than 600 nm in inducing SR absorbance changes. A flash at 450 nm induces a greater absorbance change at 570 nm than at 500 nm (Fig. 1) and both decay at a first-order rate characteristic of SR (Fig. 4B). A similar pattern of traces is obtained from Flx5R membranes, which contain 4–5× the amount of retinal-reconstitutable SR apoprotein as Flx3R (Fig. 2).

Flashing Flx3-12W membranes at 600 nm (Fig. 3) produces the expected SR changes at 570 nm and 500 nm with first-order decays (Fig. 4A) similar to those of Flx3R except that the absorbance changes (a relative measure of SR content for these samples) are approximately 50% the Flx3R values. The 450-nm flash produces a smaller change at 570 nm (Fig. 3) than in retinal-reconstituted Flx3R (Fig. 1), as expected from the lesser amount of SR. However, in this case the 450-nm flash generates a larger change at

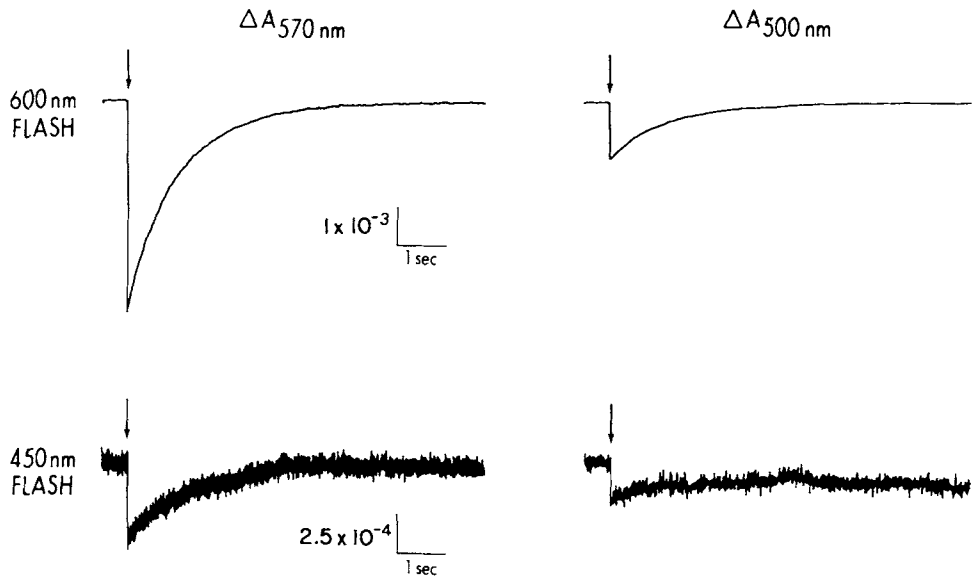

Fig. 1. Flash-induced absorbance changes in reconstituted Flx3R membrane vesicles. Sonicated cell membranes[5] at 5 mg protein/ml, reconstituted with 2.2 nmoles/ml all-*trans* retinal[7], subjected to a 1-msec flash (arrows) at 600 ± 20 nm (upper two traces, upper scale) or 450 nm ± 20 nm (lower two traces, lower scale). Absorbance monitored at 570 nm (leftmost two traces) or 500 nm (rightmost two traces).

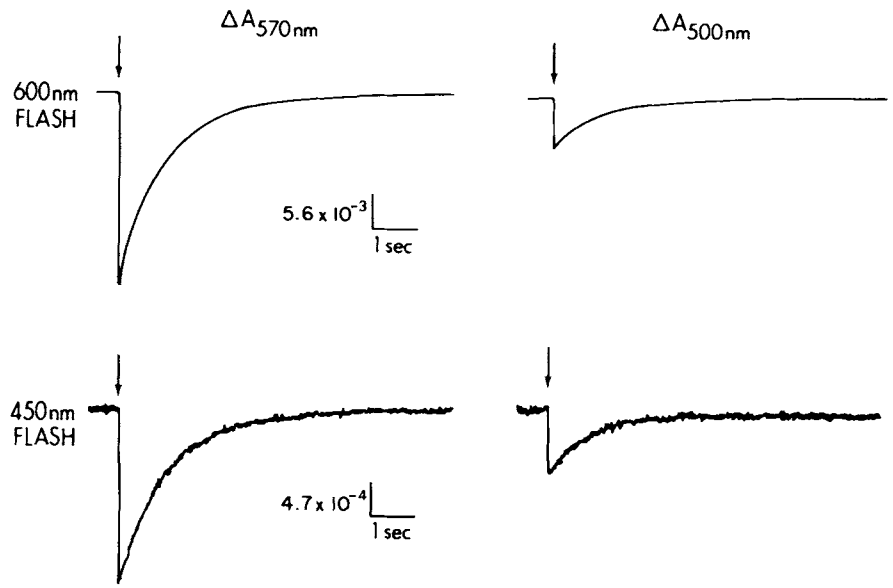

Fig. 2. Flash-induced absorbance changes in reconstituted Flx5R membrane vesicles. Membrane vesicles at 5 mg protein/ml, reconstituted, flashed, and monitored as in Figure 1.

500 nm than at 570 nm (Fig. 3), unlike SR. These last two transient absorbance changes indicate the existence of another photochemically reactive pigment excited by the 450-nm flash. The return of absorption on this time scale is first order with a $t_{1/2}$ of 260 msec, indicating that this pigment cycles at more than twice the rate of SR_{587} in the same membranes (Fig. 4B).

There may be a small amount of the second photocycling pigment in reconstituted Flx3R and Flx5R as well. Following the 450-nm flash of Flx3R and Flx5R membranes, the initial absorbance change at 500 nm is 37% of that at 570 nm, instead of 26% as in the case of the 600-nm flash. This difference may be explained by a small contribution in the Flx3R and

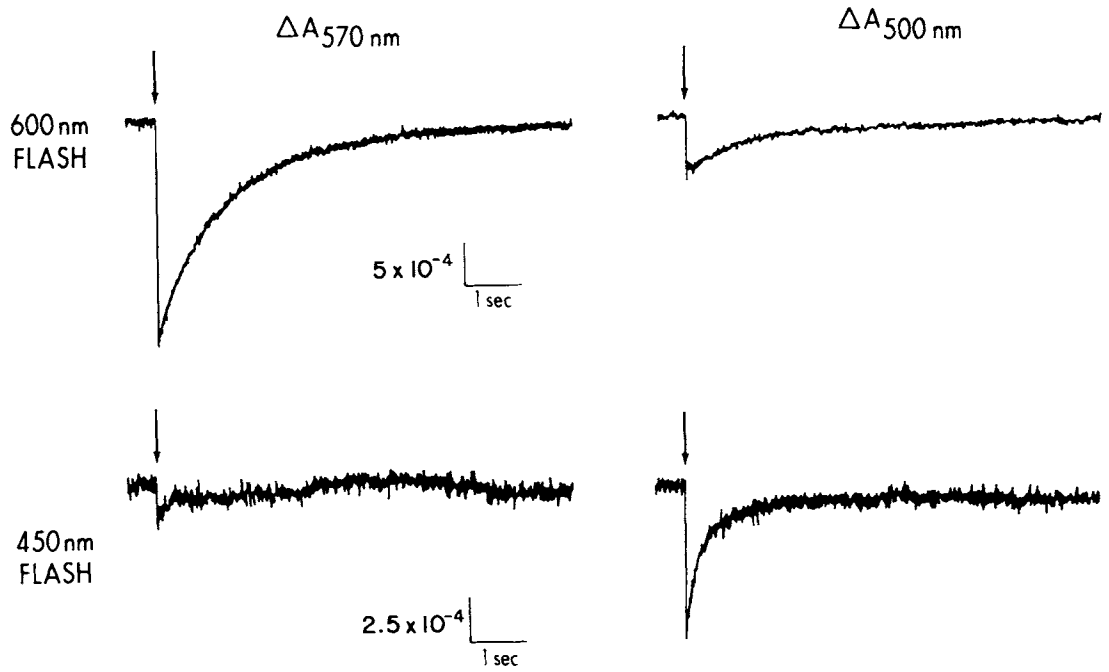

Fig. 3. Flash-induced absorbance changes in Flx3–12W membrane vesicles. Membrane vesicles at 5 mg protein/ml flashed and monitored as in Figure 1.

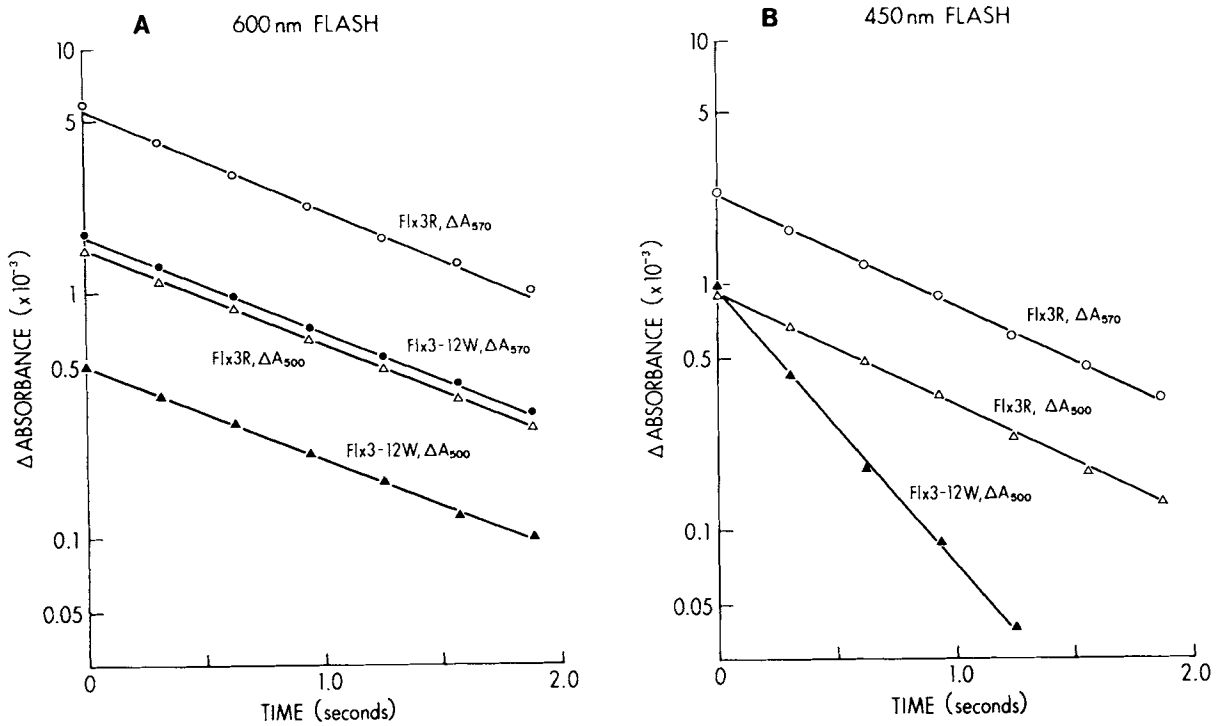

Fig. 4. Kinetics of recovery after flash-induced depletion. Plotted from traces similar to those in Figure 1 and Figure 3 for 600-nm flashes (A) or 450-nm flashes (B).

Flx5R membranes of the same pigment which dominates the 450-nm flash-induced Flx3–12W membrane absorbance changes at 500 nm. If we subtract the SR contributions from the total absorbance changes (maximum depletion minus final value of the trace) and attribute all the difference to the second pigment, then reconstituted Flx3R and Flx5R contain maximally 4% and 15%, respectively, of the second pigment content of Flx3–12W.

Flash-Induced Absorption-Difference Spectra of SR and the Second Pigment

Flx3–12W membranes subjected to a 450-nm actinic flash exhibit a relatively small absorbance change due to SR, as one can see at 570 nm, which is near the SR absorption maximum (Fig. 3). Absorption changes were monitored at various wavelengths after 450-nm flashes and after 500-nm flashes (for the 440-nm and 460-nm points, to avoid spectral overlap of the actinic and monitoring beams). The resulting absorption difference spectrum shows a depletion maximum at 480 nm and concurrent appearance of absorption in the near-UV with a peak value $\leqslant$ 360 nm (Fig. 5). Taking into consideration the small SR contribution, the data indicate the photoconversion of a pigment with 480-nm absorption maximum to a species with maximum $\leqslant$ 360 nm, which thermally regenerates the 480–nm species with a $t_{1/2}$ of 260 msec. Other unresolved intermediates may contribute to the absorption difference spectrum.

The difference spectrum obtained from the 600-nm flash of Flx3–12W vesicles (plotted as squares in Fig. 5) has the same shape as that of SR in retinal-reconstituted Flx3R (continuous line, Fig. 5).

Amounts of SR and the Second Pigment

Since for both pigments we are in the linear range of photocycling with our subsaturating 450-nm actinic flash, we can estimate the amount of the second pigment present in membrane vesicle samples by comparing the change in absorbance at 480 nm (ΔA_{480}) to that at 590 nm (ΔA_{590}), following the 450-nm flash. The flash-induced absorbance change due to SR is given by

$$\Delta A^{SR}_{590} = \epsilon^{SR}_{590} \cdot \Delta C_{SR} \cdot \ell \qquad (1)$$

where ϵ^{SR}_{590} is the molar extinction coefficient, ℓ is the pathlength, and ΔC_{SR} is the transient change in SR_{587} concentration. The flash yield (Y_{SR}), the fraction of SR molecules undergoing photochemical reaction as a result of the actinic flash, is

$$Y_{SR} = (1-T) \cdot f_{SR} \cdot \phi^{SR}_{c},$$

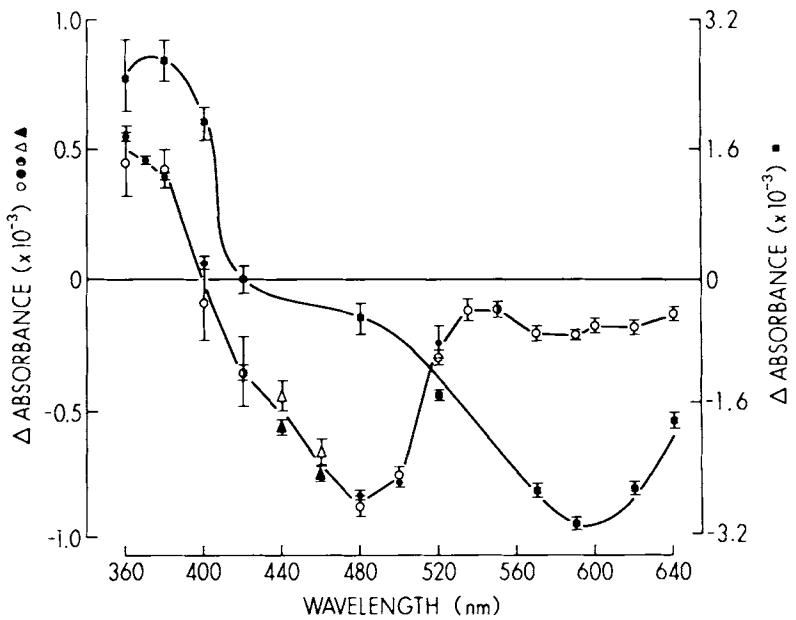

Fig. 5. Absorption difference spectra for Flx3–12W membrane vesicles. Absorbance changes 15 msec after the flash assessed at various wavelengths for 450-nm flashes (leftmost scale) and 600-nm flashes (rightmost scale). △, 440-nm and 460-nm points obtained from vesicles flashed at 500 nm and normalized to the 450-nm flash absorbance changes (○) at 480 nm; ●, ▲, as above except suspension clarified with 10 mM CHAPS detergent (Sigma, St. Louis, MO); ◑, ○ and ● points of the same value. ■, vesicles; point at 590 nm obtained from a 610 ± 5-nm flash normalized to the 600-nm flash-induced absorbance change at 570 nm. Continuous line is for retinal-reconstituted Flx3R 600-nm flash data scaled to the Flx3–12W maximum absorbance change at 590 nm. Length of error bars corresponds to the noise envelope from the traces.

where T is the transmittance of the sample for the actinic flash, f_{SR} is the fraction of absorbed actinic radiation captured by SR, and ϕ_c^{SR} is the quantum efficiency for photochemical reaction. Then

$$\Delta C_{SR} = Y_{SR} \cdot C_{SR} = (1-T) \cdot f_{SR} \cdot \phi_c^{SR} \cdot C_{SR}, \quad (2)$$

where C_{SR} is the total concentration of SR. Combining equations (1) and (2) and rearranging terms yields

$$C_{SR} = \Delta A_{590}^{SR}/\epsilon_{590}^{SR} \cdot (1-T) \cdot f_{SR} \cdot \phi_c^{SR} \cdot \ell.$$

A similar expression obtained for the relation between the concentration of the second pigment (designated P) and the flash-induced absorbance change at 480 nm is

$$C_P = \Delta A_{480}^{P}/\epsilon_{480}^{P} \cdot (1-T) \cdot f_P \cdot \phi_c^{P} \cdot \ell.$$

To obtain an estimate of the relative amounts of the two photocycling pigments, we have to assume values for ϕ_c^{SR}/ϕ_c^{P} and $\epsilon_{590}^{SR}/\epsilon_{480}^{P}$. We will assume $\phi_c^{SR} = \phi_c^{P}$, likely to be accurate at least within a factor of 2, since quantum efficiencies are in the range 0.5 ± 0.2 for known retinylidene proteins, and that the molar extinction coefficients of SR and the second pigment at their respective absorption maxima are the same. This yields

$$C_P = \frac{\Delta A_{480}^{P} \cdot f_{SR} \cdot C_{SR}}{\Delta A_{590}^{SR} \cdot f_P} \quad (3)$$

We estimate the ratio f_P/f_{SR} graphically by comparing the overlap of the known SR absorption spectrum[7] and that of the second pigment (estimated from the depletion spectrum of Figure 5 and corrected for the small SR contribution) over the wavelength range of the actinic flash. Assuming uniform screening by other membrane components in the actinic wavelength region, we estimate $f_{SR}/f_P = 1/3$. We measured $\Delta A_{590}^{SR} = 2.8 \times 10^{-3}$ following the 450-nm flash for retinal-reconstituted Flx5R membranes at 5 mg protein/ml, which contained 0.52 μM SR, determined by monitoring absorption generated by adding all-*trans* retinal.[7] In Flx3–12W membranes at 5 mg protein/ml the same flash produced $\Delta A_{480} = 8 \times 10^{-4}$ attributable to the second pigment after subtraction of the small SR contribution. From equation (3), C_P = 49 nM. The concentration of SR in the same Flx3–12W membrane suspension is 44 nM (determined by comparing Flx3–12W flash data with that of reconstituted Flx5R.)

There is considerable uncertainty in the estimate of second pigment content necessitated by the assumptions regarding its extinction and quantum efficiency, but this first approximation is useful in evaluating the [^{3}H] retinal labeling data shown below.

Bleaching of SR and the Second Pigment and Regeneration of Both With [^{3}H] Retinal

Treatment of membranes with hydroxylamine has been used to remove retinal from BR, HR, and SR, and the photochemical properties of all three pigments are readily regenerated by incubation of the bleached membranes with all-*trans* retinal.[7,19,20] Incubation of reconstituted Flx3R and Flx3–12W membranes with 0.5 M hydroxylamine at pH 9.0 for 1 hour diminishes SR photochemical activity, monitored as the absorbance change at 570 nm after a 600-nm flash (Fig. 6). These bleaching conditions also eliminate photochemical activity of the second pigment, as monitored by the absorbance change at 500 nm after a 450-nm flash (Fig. 6). After neutralization

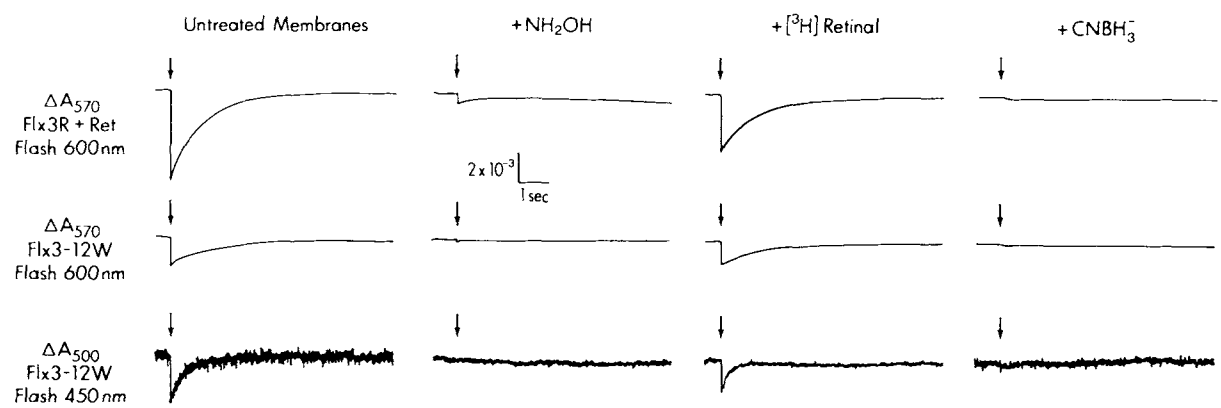

Fig. 6. Hydroxylamine bleaching, [^{3}H]retinal regeneration, and cyanoborohydride reduction of slow-cycling rhodopsin and the second slow-cycling pigment. Absorbance changes corresponding to SR (600-nm flash) for retinal-reconstituted Flx3R and to SR and the second photoreactive pigment (450-nm flash) for Flx3–12W membrane vesicles. Scale for ΔA_{570} traces are indicated. ΔA_{500} traces are plotted at 4x the indicated scale. Details of bleaching, regeneration, and reduction described in text.

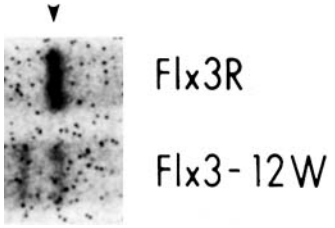

Fig. 7. Autofluorogram of the 20,000–30,000-M_r region of [^{3}H]retinal-labeled proteins electrophoretically separated on a $NaDodSO_4$-12%-polyacrylamide gel; 50 μg protein/lane from the vesicle membranes for which flash data are shown in Figure 6. Arrowhead indicates the migration position of bacterioopsin in this gel system.

TABLE I. Relative Amounts of Slow-Cycling Rhodopsins* in *H. halobium* Mutants†

	SR-I	SR-II
Flx5R + retinal	100	⩽2
Flx3R + retinal	24	⩽0.5
Flx3–12W	12	13
Flx3–KM1	28	9
Pho81	NS‡	NS

*SR and the 480-nm pigment are provisionally referred to as SR-I and SR-II, respectively, since both are slow-cycling rhodopsinlike pigments as originally defined.[6]
†Expressed as percentage of the SR-I value in Flx5R membranes reconstituted with all-*trans* retinal. The ratio of SR-II to SR-I was determined spectroscopically from equation (3).
‡No signal detected.

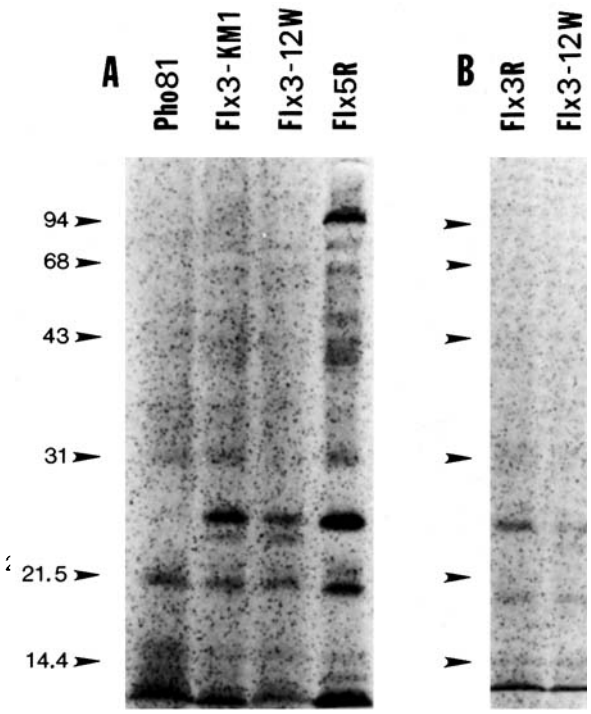

Fig. 8. Autofluorogram of [^{3}H]retinal-labeled proteins electrophoretically separated on an $NaDodSO_4$-12%-polyacrylamide gel; A and B: 50 μg protein loaded per lane from the strains indicated. The two lanes on the right (B) are from a separate gel in which Flx3–12W and Flx3R are compared. Molecular weight standards (BioRad Laboratories, Richmond, CA) from higher to lower M_r ($\times 10^3$): phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme.

and removal of hydroxylamine both SR and the second pigment can be regenerated by addition of [^{3}H]all-*trans* retinal, as seen by the traces in the third column (Fig. 6). Therefore this step has introduced [^{3}H]retinal into the chromophore of both pigments in the Flx3–12W and Flx3R membranes. This provides direct evidence that the second photoactive pigment also uses retinal in its chromophore.

$CNBH_3^-$ Reduction and $NaDodSO_4$-Polyacrylamide Gel Electrophoresis of [^{3}H]Retinal-Labeled Membrane Proteins

Known retinal/opsin linkages (e.g., rhodopsin, BR, and HR) are Schiff bases, which are labile to $NaDodSO_4$ solubilization, but they can be visualized on an $NaDodSO_4$-polyacrylamide gel after reduction to a stable secondary amine. Accordingly, the [^{3}H]retinal-reconstituted membrane vesicles were treated with the reducing agent $CNBH_3^-$ under conditions which effectively reduced HR and SR in similar vesicle preparations.[17] Photoactivity of both SR and the second pigment are eliminated by this treatment, as is shown by the absence of light-induced absorbance changes (last column of Fig. 6). This procedure establishes a method for visualization of the chromophoric polypeptide of the second pigment by $NaDodSO_4$-polyacrylamide gel electrophoresis.

Previously a 25,000-M_r polypeptide was found to correlate with SR in this type of radiolabeling procedure.[17,18] The bands in the 20,000–30,000-M_r region of the gel are shown (Fig. 7) for the reduced [^{3}H]retinal-labeled Flx3R and Flx3-12W preparations for which flash data are shown in Figure 6. The 25,000-M_r band previously observed in Flx3R is present in reduced amounts in Flx3–12W. Additionally, a 23,000-M_r band is present in Flx3–12W membranes. In these two strains the relative intensity of the two bands are in good agreement with the relative amounts of SR and second pigment (referred to as SR-I and SR-II, respectively, in Table I). Note that Flx3R membranes do not show the 23,000-M_r band whether the reconstituted vesicles are bleached with hydroxylamine (Fig. 7) or the vesicles are directly reconstituted with [^{3}H]retinal,[18] indicating the 23,000-M_r band is not a hydroxylamine-generated fragmentation product of the 25,000-M_r protein, a possibility suggested previously.[16]

In another radiolabeling experiment (Fig. 8) we compared the banding patterns for several strains that have been characterized with respect to content

of both SR-I and SR-II by flash photolysis (Table I). The contents of SR-I and SR-II correlate well with the amount of incorporation in the 25,000-Mr band and 23,000-M_r band, respectively. In Pho81, a mutant which is lacking both SR and the second pigment, neither the 25,000- nor the 23,000-M_r band is labeled.

In addition to the bands correlated with SR-I and SR-II, two sharp bands at 19,000 M_r and 94,000 M_r and several diffuse bands labeled less intensely are evident in some of the lanes. Nonspecific binding of [^{3}H]retinal to proteins might account for some of these bands; additional criteria would be needed to correlate any of these bands with functional retinal binding. The 19,000-M_r band, present in all of the strains, does not correspond to known photochemical activity. The 94,000-M_r band, especially evident in this Flx5R preparation, may derive from the 25,000-M_r band through either specific or nonspecific aggregation (unpublished observations).

ACKNOWLEDGMENTS

The laboratories of Walther Stoeckenius and Roberto Bogomolni have independently obtained results similar to those we report here. We appreciate their open discussions of their results prior to publication. We thank Tetsuo Takahashi for strain Flx3–12, and Walther Stoeckenius for strain Flx3–KM1.

This research was supported by National Institutes of Health Public Health Service grants GM27750 and GM34283 (J.L.S.) and fellowship DRG-839 of the Damon Runyon-Walter Winchell Cancer Fund (S.A.S.).

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