J. Membrane Biol. 82, 89–94 (1984)

The Journal of
Membrane Biology

Bacterial Rhodopsins Monitored with Fluorescent Dyes in Vesicles and *in Vivo*

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Summary. Three retinal-containing pigments have been detected in *Halobacterium halobium* membranes: bacteriorhodopsin (bR), halorhodopsin (hR), and slow-cycling rhodopsin (sR). The first two hyperpolarize the cell membrane by electrogenic transport of H^+ and Cl^-, respectively. The third pigment, sR, may be a photosensory receptor since mutants lacking bR and hR retain their retinal-dependent phototaxis responses. We monitored light-induced changes in fluorescence of several voltage-sensitive dyes in cells and membrane vesicles. Red light-induced potential changes generated by bR and hR were similar to signals described previously. Signals generated by hR could be identified using four criteria: wavelength dependence, Cl^- dependence, shunting by valinomycin and K^+, and the absence of these signals in hR-deficient mutants. The absence (detection limit ~0.5 mV) of hyperpolarization signals in $bR^-hR^-sR^+$ vesicles and cells shows that sR photochemical reactions are nonelectrogenic. Two signals independent of bR and hR were measured: blue light caused a decrease and red light an increase in dye fluorescence. Both signals appear to derive from sR on the basis of their retinal-dependence and action spectra. In a retinal-deficient mutant strain (Flx3R), both sR signals appeared after addition of all-*trans* retinal. In this strain retinal also restores phototaxis sensitivity within the same time scale. The retinal concentration dependence for all four parameters monitored—the attractant (red) and repellent (blue) phototaxis, and the red light and blue light-induced fluorescence signals—is the same. This correlation is consistent with the hypothesis that both attractant and repellent responses are mediated by sR, as suggested by Bogomolni and Spudich (*Proc. Natl. Acad. Sci. USA.* **79**:6250–6254 (1982)).

Key Words *Halobacterium halobium* · voltage-sensitive dye · bacteriorhodopsin · halorhodopsin · slow-cycling rhodopsin

Introduction

Halobacterium halobium are salt-loving flagellated bacteria. Their membranes contain pigments including a family of retinal-containing proteins, which are responsible for light-induced membrane hyperpolarization and phototaxis. Two retinal-containing proteins are known to hyperpolarize the cell: bacteriorhodopsin (bR), which is a light-driven proton pump (for review *see* [20]), and halorhodopsin (hR) [10, 12], which is a light-driven Cl^- pump [14]. This hyperpolarization drives ATP synthesis and other energy-requiring processes [20]. The phototaxis behavior of the cells consists of attraction to red light and repulsion by blue light [3, 7, 19]. Since *H. halobium* cells that are lacking bR and hR still exhibit retinal-dependent phototaxis responses, neither of these pigments is required for sensory reception [16].

A third retinal protein, slow-cycling rhodopsin (sR), was recently detected [2, 18]. Its photocycle takes seconds to complete in contrast with the msec photocycling of bR and hR. SR is photochromic and in natural light exists as two species: a form absorbing maximally in the red (λ_{max} 587 nm) and a form absorbing maximally in the blue (λ_{max} 373 nm). Both the presence of sR in bR^-hR^- mutant strains which show normal phototaxis and sR's spectroscopic properties suggest that sR is the sensory receptor for the attractant and repellent phototaxis responses [2] (for reviews *see* [1, 17]). Proton flux measurements suggest that sR does not hyperpolarize the membrane and, therefore, is not an electrogenic pump [2, 16]. However, these measurements may have missed transient changes and small movements of ions. Voltage-dependent dyes have been used in vesicles to monitor the hyperpolarization by bR and hR photoexcitation [13, 14]. We confirmed these earlier results both in vesicles and whole cells. In addition, we detected dye fluorescence signals arising from sR photocycling. These signals are not generated by transmembrane potential changes.

Materials and Methods

Strains

The Table shows the distribution of bacterial rhodopsins in the *H. halobium* strains used in this study. OD2W was recently iso-

Table. Bacterial rhodopsins in *H. halobium* strains

	Strain			
	S9	OD2W	Flx3	Flx3R
Bacteriorhodopsin (bR)	+	−	−	−
Halorhodopsin (hR)	+	+	−	−
Slow-cycling rhodopsin (sR)	+	+	+	sR-opsin only

lated as a carotenoid-deficient derivative of OD2. The isolation and characterization of OD2, S9, and the Flx mutants have been described [16, 17]. S9 has also been referred to as S9-P, to indicate the visibly purple color of the cells. Cell growth conditions and preparation of membrane vesicles also have been described [16].

Fluorescence Measurements

Two tungsten halogen lamps (6V50W) set 180° apart were used as dye excitation and actinic light sources. Both beams were passed through heat and interference filters and focused on the sample cuvette. Interference filters passed the emitted fluorescence and prevented scattered incident light from reaching the photodetector (PV-444, EG and G Electro Optics), which was positioned 90° from the excitation light source. Photodetector signals were amplified and then recorded on an oscilloscope and chart recorder.

The carbocyanine dyes $diSC_3(5)$, $diOC_6(3)$, and $diIC_3(7)$ were a gift of A. Waggoner. The styryl dye RH160 was a gift of R. Hildesheim and A. Grinvald. Excitation and emission wavelengths were selected to maximize the fluorescent signal generated by each dye [6, 15]. Wavelengths are indicated in the figure legends and text. Dyes were dissolved in ethanol and 7.5 μl were mixed into 3 ml of a cell or vesicle suspension. After incubation for 10 min, fluorescence measurements were begun. During measurements, the suspension was not stirred.

The optimum dye and vesicle concentrations were chosen from calibration curves. We used vesicles at 0.2 mg protein/ml because this concentration produced the largest signal in response to blue light stimulation. The carbocyanine dyes were used at 2.5 μM and RH160 at 1.0 μM. With the carbocyanine dyes we could detect 0.5 mV of transmembrane potential change, based on the signal-to-noise ratio of our apparatus and the calibration curve of Renthal and Lanyi (*see* Fig. 9 of ref. [13]). Since the carbocyanine dyes respond slowly, changes occurring faster than 100 msec could not be detected. Therefore, we also used RH160, which in mouse neuroblastoma permits detection of very rapid potential changes (i.e., <0.5 msec [5]), but with the filtering of our apparatus (~100 Hz) only signals in the msec range could be detected.

Results

Fluorescence Signals in *H. halobium* Membranes

Changes in steady-state dye fluorescence are induced by illuminating vesicles from *H. halobium*

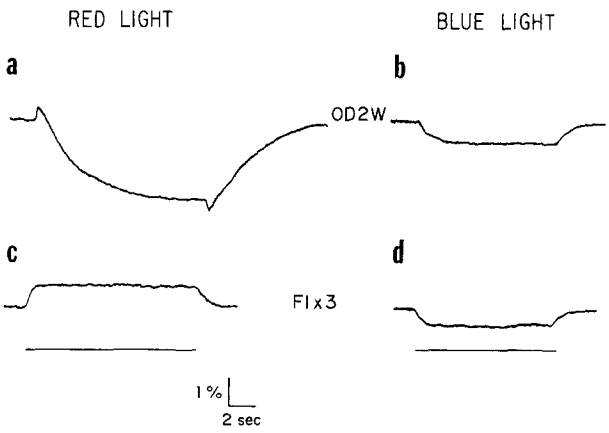

Fig. 1. Fluorescence signals from OD2W and Flx3 vesicles. In all panels 2.5 μM $diOC_6(3)$ and 0.2 mg protein/ml vesicles were used. Dye was excited at 450 nm (3.6×10^4 ergs cm^{-2} sec^{-1}); emission was monitored at 510 nm. Vesicles were photostimulated with 600 nm light (red light; 1.2×10^5 ergs cm^{-2} sec^{-1}) in *a* and *c* and 390 nm light (blue light; 5.6×10^3 ergs cm^{-2} sec^{-1}) in *b* and *d*. All wavelengths are ±15 nm. Bar indicates duration of photostimulation

strains OD2W and Flx3 with either red (600 ± 15 nm) or blue (390 ± 15 nm) light (Fig. 1). Blue light causes a decrease in fluorescence in both strains (Fig. 1*b* and *d*). Red light causes a decrease in steady-state fluorescence in OD2W (Fig. 1*a*) and an increase in Flx3 vesicles (Fig. 1*c*). Similar changes in fluorescence were detected in OD2W and Flx3 cells (2×10^9 cells/ml; same dye concentration). When cells of strain S9, which contains bR, were stimulated with red light, the responses were similar to those reported earlier [13]. The time course of the red light-induced decrease in fluorescence in S9 was similar to that measured in OD2W cells, but the magnitude was 6.5 times greater in S9.

Since carbocyanine dye molecules are cations that distribute passively across the membrane, a change in transmembrane potential will alter the intracellular dye concentration. As the concentration of the dye increases, aggregates form which show a reduced fluorescence [15]. A red light-induced decrease in fluorescence was expected in S9 and OD2W because both strains contain light-driven electrogenic ion pumps. When illuminated with red light, the cell membrane hyperpolarizes due to the pumping of H^+ out of the cell by the pigment bR and/or the pumping of Cl^- into the cell by the pigment hR (for review *see* [20]). The signals shown in Fig. 1*b-d* are not due to transmembrane potential changes, as discussed below.

The data shown in Fig. 2 confirm that the red light-induced signal measured with OD2W vesicles and cells results from hyperpolarization of the membrane. We treated the vesicles with the K^+

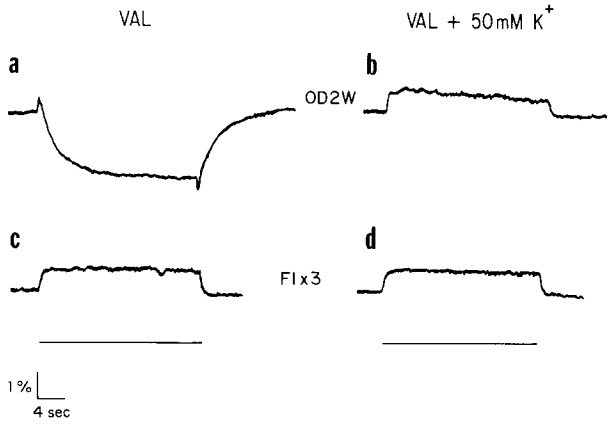

Fig. 2. Effect of valinomycin and K^+ on fluorescence signals. Dye and vesicle concentrations as in Fig. 1. Vesicles were suspended in 3 M NaCl and 2 μM valinomycin. The actinic light was 600 nm. Bar indicates duration of photostimulation. (*a*) OD2W vesicles. (*b*) 50 mM KCl was added to the same suspension as in *a*. This signal now looks like the Flx3 signal shown in Figs. 1*c* and 2*c*. (*c*) Flx3 vesicles. (*d*) 50 mM KCl added to the same suspension as in *c*. Note that valinomycin and K^+ had no effect on the Flx3 signal

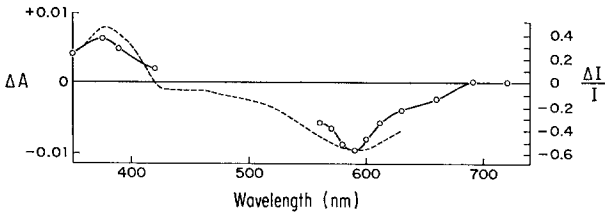

Fig. 3. Comparison of fluorescent dye signals with the spectral properties of sR. Flx3R vesicles plus 1 μM retinal (0.2 mg protein/ml) stained with 2.5 μM $diOC_6(3)$ were photostimulated with constant intensity light of different wavelengths (1×10^5 ergs cm^{-2} sec^{-1}). The points are fluorescent signals ($\Delta I/I$) from photostimulation at the indicated wavelengths. The broken line is the flash-induced difference spectrum (ΔA) and was taken from Bogomolni and Spudich [2]. The scales were adjusted so the absorption and fluorescence signals at 590 nm coincided

ionophore valinomycin. Valinomycin itself had no effect on the signals when the vesicles were suspended in 3 M NaCl (Fig. 2*a*). Additions of small amounts of K^+ (i.e., 1–5 mM) to OD2W vesicles reduced the size of the fluorescence signal. When 50 mM K^+ is added to the extravesicular medium the fluorescence signal was completely abolished (Fig. 2*b*). This result shows that the red light-induced decrease in fluorescence can be dissipated by a passive K^+ flux, as expected for a fluorescence change due to hyperpolarization of the membrane. The hyperpolarization can be assigned to hR since this red light-induced decrease in fluorescence does not occur in the hR-deficient Flx3 strain (Fig. 1*c*). Additional evidence that this signal is generated by hR is that the fluorescence change is maximal in the spectral range of maximal hR absorption and that the signals are Cl^--dependent (data not shown), as previously shown for hR-dependent signals [14].

In contrast to the hR signal of OD2W, the red light-induced Flx3 signal is unaffected by treatment with valinomycin and K^+, indicating that this fluorescence change is not due to a transmembrane potential change (*compare* Fig. 2*c* and *d*). Furthermore, when the red light-induced signal in OD2W is eliminated by valinomycin and K^+, a signal is revealed which is similar to that of Flx3 (*compare* Fig. 2*b* and *c*). The blue light-induced fluorescence changes shown in Fig. 1 also are not affected by valinomycin and K^+ in either strain. The basis for the signals unaffected by valinomycin and K^+ will be discussed below.

Correlation of Fluorescence Signals with sR

Several criteria correlate the Flx3 signals with photoexcitation of the pigment sR. The fluorescent dye signals peak at an actinic wavelength of 585 ± 5 nm (Fig. 3, solid line) which corresponds to the sR absorption maximum at 587 nm [18]. Furthermore, the action spectrum for the dye signal corresponds closely to the flash-induced difference spectrum generated by sR (Fig. 3, broken line, from [2]) and the pH dependence of the signal corresponds to the pH dependence of the flash yields of sR [9].

The dependence of the Flx3 fluorescent dye signals on retinal was assessed. The photoactive sR molecule consists of a protein moiety (sR-opsin) and retinal. In Fig. 4, the mutant Flx3R was used because this strain is blocked in its retinal synthesis pathway, but it still synthesizes sR-opsin. These cells do not respond phototactically to light, nor do they have photoactive sR. Photoactive sR can be generated by addition of retinal to Flx3R membrane vesicles or cells [18]. In retinal-free Flx3R vesicles we could not detect any signal regardless of the wavelength used for the actinic light (shown for red and blue illumination of membrane vesicles in Fig. 4*a* and *b*). With the addition of all-*trans* retinal (0.5 μM) to the Flx3R vesicle suspension, both blue and red light induced signals appeared (Fig. 4*c* and *d*). In all of these experiments, both the red light-induced increase and the blue light-induced decrease vary together, consistent with the suggestion that both the red and blue light signals derive from a single pigment, sR.

The opposing effects of red and blue light on swimming behavior (phototaxis) have been suggested to be mediated by the two forms of sR [2]. To test this hypothesis, we compared the retinal con-

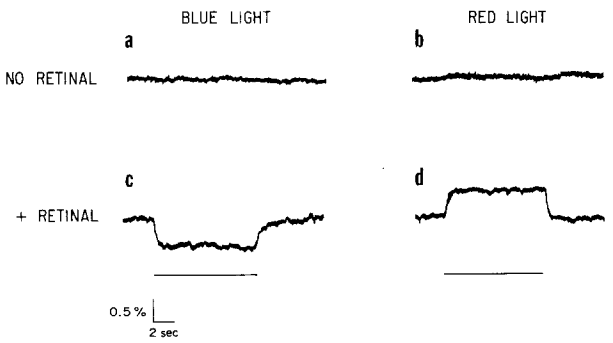

Fig. 4. Retinal dependence of fluorescence signals. Flx3R vesicles (0.2 mg protein/ml) stained with 2.5 μM $diOC_6(3)$ did not generate fluorescent signals at any wavelength. Records are shown for actinic lights of (*a*) 390 nm and (*b*) 600 nm. The same vesicles were incubated for >10 min and all-*trans* 0.5 μM retinal, which was added from a 3 mM ethanolic stock. Then fluorescence signals were measured with actinic light of (*c*) 390 nm and (*d*) 600 nm. These signals are similar to those shown in Fig. 1*c* and *d*

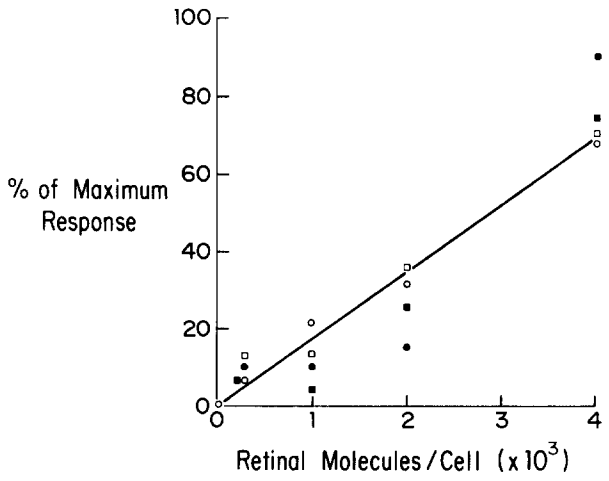

Fig. 5. Retinal dependence of fluorescent dye signals *in vivo* and of phototaxis responses. A late log phase culture of Flx3R cells was pelleted in a microfuge (2 min spin) and resuspended in fresh medium at 3.0×10^9 cells/ml. 10 μl of ethanolic solutions of all-*trans* retinal at various concentrations were added to 2 ml aliquots of the cell suspension. After incubation for 2 hr at 37°C in the dark 100 μl of the suspension was diluted 1 : 10 with fresh medium. After a further 15-min incubation at room temperature, reversal induction to attractant (■) and repellent (●) light stimuli was assayed with the double beam method described [19]. The remaining 1.9 ml of each cell suspension was pelleted in a microfuge and resuspended in 2 ml of 3 M NaCl, 10 mM HEPES, pH 7.0 and 2.5% growth medium. These cells were then stained with 2.5 μM $diOC_6(3)$ and assayed as the previous figures at two actinic wavelengths, 390 nm (○) and 600 nm (□). The line was drawn by eye through the 390-nm dye fluorescence data. Note that all four responses show a similar concentration dependence

centration dependence for generation of phototaxis and for sR dye fluorescence signals in Flx3R cells. We monitored the size of red light- and blue light-induced dye signals after adding various concentrations of retinal to the cells, and in the same cell suspensions we measured red and blue phototaxis sensitivities. As shown in Fig. 5, all four parameters show a similar concentration dependence. By extrapolating the phototaxis data (Fig. 5, filled symbols) to 100% response, we estimate that 5.5×10^3 molecules of retinal are needed to generate maximal phototaxis sensitivity. This number is close to the 6.5×10^3 copies of sR per cell estimated by flash photolysis [2]. The close correspondence of the retinal dependence of phototaxis and of the sR fluorescence signals is consistent with the hypothesis [2] that sR is the sensory receptor for phototaxis.

What are the sR Signals?

Although it was possible to shunt the hR hyperpolarization signal by adding 1 μM valinomycin and 50 mM KCl to the vesicle preparation (Fig. 3*a* and *b*), the sR signals monitored *from the same vesicles* were unchanged by this treatment. We conclude that the sR signals do not derive from transmembrane potential changes.

In some systems a change in membrane surface charge can influence dye distribution and fluorescence [8]. With halobacteria this possibility seems unlikely since our measurements were made in 3 M NaCl, which would be expected to screen surface charges. Addition of up to 50 mM La^{3+}, Cd^{2+}, or Ca^{2+} to insure even more effective charge screening did not measurably influence the sR signals, further indicating that they do not derive from membrane surface potential changes.

Is the sR signal peculiar to carbocyanine dyes? The carbocyanine dyes usually work by autoquenching after redistribution of the dye molecules across the membrane. We also used the styryl dye, RH160, which has been shown to produce a signal faster than could be generated by redistribution and therefore is thought to function by another mechanism, possibly an electrochromic effect [11]. Vesicles stained with RH160 generate sR signals in response to both red and blue light, similar to those generated with carbocyanine dyes. The sR signals therefore apparently do not depend on dye redistribution across the membrane nor on the particular class of dye used.

In contrast, using a dye with a different wavelength dependence did give different results. The fluorescence excitation and emission wavelengths used for $diOC_6(3)$, $diSC_3(5)$, and RH160 (excitation $\lambda \pm 15$ nm: 450, 630, and 510 nm; emission $\lambda \pm 15$ nm: 510, 690, 690 nm, respectively) all overlap with

the absorption spectrum of sR. All three dyes give similar fluorescence signals. When we used $diIC_3(7)$, which is photoexcited (720 ± 15 nm) and emits (780 ± 15 nm) outside the spectral range of sR absorption, we could measure an hR signal (from OD2W vesicles), but we could not detect an sR signal. The ability to detect an hR signal shows that $diIC_3(7)$ was effective in our system. The inability to detect an sR signal suggests that the dye signal requires spectral overlap between sR and the dye (e.g., energy transfer or internal filtering). However, this interaction is not due to internal filtering where the sR itself absorbs excitation photons before they reach the dye or dye-emitted photons before they reach the photodetector as has been seen for bR [4]. Filtering can be ruled out based on calculations of the maximum absorption change sR could generate. Using the previous estimates of sR content and extinction [2, 18], we calculate a maximum effect of 0.02% on the resting dye fluorescence at the wavelengths used in our experiments. Absorbance measurements in our apparatus confirm that the actual absorbance change is less than 0.02%.

Discussion

Light-induced changes in fluorescence of several voltage-sensitive dyes can be detected in *H. halobium* cells and membrane vesicles. Red light-induced hyperpolarization of the membrane by bR and hR generated signals similar to those described previously in vesicles [13, 14]. In the measurements reported here, two signals were detected and shown to be independent of bR and hR by their occurrence in bR^-hR^- mutants: a blue light-induced decrease and red light-induced increase in dye fluorescence. Both signals appear to derive from sR on the basis of their retinal dependence, action spectra, and pH dependence. Since addition of valinomycin and K^+ did not shunt the sR signals, we concluded that these signals are not caused by changes in membrane polarization. The absence of hyperpolarization signals in $bR^-hR^-sR^+$ vesicles and cells confirms that sR photochemical reactions are nonelectrogenic, as was suggested from proton flux measurements [2, 16].

The bR and hR dye signals represent the decrease in fluorescence caused by dye accumulation following a transmembrane hyperpolarization [15]. Since the sR signals do not derive from transmembrane potential changes, dye accumulation cannot be used to explain the fluorescence changes. The nature of the effect of sR photochemical reactions on the dye fluorescence is not clear. From absorbance measurements we can rule out the possibility that sR is internally filtering the dye signals, yet spectral overlap of the fluorescence excitation or emission with sR absorption seems to be required for the signal.

Even though the chemical basis of the sR dye signals is unknown, we have confirmed that these signals are generated by sR and, therefore, we could exploit these signals as an additional measure of sR function. In particular, we compared the sR dye signals in the cell with the cell's phototaxis responses. We found that the same retinal concentration was needed to generate attractant and repellent phototaxis and sR fluorescent dye signals. This result is consistent with the suggestion of Bogomolni and Spudich [2] that sR is the phototaxis receptor.

It is a pleasure to acknowledge the assistance and comments of L.B. Cohen, A. Finkelstein, A. Grinvald, B.M. Salzberg, E. Spudich, S. Sundberg, and A. Waggoner. This work was supported by NIH grant GM27750, Jane Coffin Childs Memorial Fund Project Grant No. 360, and an Irma T. Hirschl Trust Career Scientist Award to JLS, NIH Short Term Training Grant HD 07220 to CRS, and a Grant-in-Aid from the NY Heart Association to BEE. BEE is a NY Heart Young Investigator.

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Received 26 March 1984