All-*trans*/13-*cis* isomerization of retinal is required for phototaxis signaling by sensory rhodopsins in *Halobacterium halobium*

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ABSTRACT An analogue of all-*trans* retinal in which all-*trans*/13-*cis* isomerization is blocked by a carbon bridge from C12 to C14 was incorporated into the apoproteins of sensory rhodopsin I (SR-I) and sensory rhodopsin II (SR-II, also called phoborhodopsin) in retinal-deficient *Halobacterium halobium* membranes. The "all-*trans*-locked" retinal analogue forms SR-I and SR-II analogue pigments with similar absorption spectra as the native pigments. Blocking isomerization prevents the formation of the long-lived intermediate of the SR-I photocycle (S_{373}) and those of the SR-II photocycle (S-II_{360} and S-II_{530}). A computerized cell tracking and motion analysis system capable of detecting 2% of native pigment activity was used for assessing motility behavior. Introduction of the locked analogue into SR-I or SR-II apoprotein in vivo did not restore phototactic responses through any of the three known photosensory systems (SR-I attractant, SR-I repellent, or SR-II repellent). We conclude that unlike the phototaxis receptor of *Chlamydomonas reinhardtii*, which has been reported to mediate physiological responses without specific double-bond isomerization of its retinal chromophore (Foster et al., 1989), all-*trans*/13-*cis* isomerization is essential for SR-I and SR-II phototaxis signaling.

INTRODUCTION

Retinal (vitamin A aldehyde) is widely used as the chromophore of photosensitive proteins (rhodopsins) from archaebacteria (Spudich and Bogomolni, 1988) or eukaryotic algae (Foster et al., 1984) to vertebrate and invertebrate eyes (Wald, 1968; Ottolenghi, 1980; Birge, 1981). In the archaebacterial and visual rhodopsins, a molecule of retinal is covalently bound to an intrinsic membrane apoprotein via a protonated Schiff base. The isomerization of retinal from 11-*cis* to all-*trans* was postulated to be the trigger for signal generation by visual pigments (Hubbard and Kropf, 1958). Spectroscopic studies proved that photoisomerization occurs (Eyring et al., 1980, 1982; Bagley et al., 1985) and produces a highly constrained chromophore within picoseconds (Hayward et al., 1981). The energy stored in this process is sufficient to induce conformational changes in the chromoprotein (Chabre, 1985).

Direct tests that 11-*cis*/all-*trans* isomerization is essential for signal transduction by visual pigment were made possible by retinal analogues (Derguini and Nakanishi, 1986). The 11,12-dihydro retinal (Gawinowicz et al., 1977), which exhibits free rotation and therefore no stable isomers about the 11,12-bond, was incorporated into the retinae of vitamin A deprived rats and did not restore sensitivity (Crouch et al., 1981). Another non-isomerizable rhodopsin was derived from a retinal with a fixed 11-ene structure (cycloheptatrienylidene structure), whose seven-membered ring forces the 9,11,13-triene system to adopt a nonplanar 11-*cis*-12-s-*trans* conformation (Akita et al., 1980). This analogue blocked the native photochemical reaction of bovine rhodopsin as measured by low temperature spectroscopy and flash photolysis (Mao et al., 1981), protein conformational changes monitored by Fourier transform infrared difference spectroscopy and photoactivation of rhodopsin kinase (Zankel, T., H. Ok, R. Johnson, C. W. Chang, N. Sekiya, H. Naoki, K. Yoshihara, and K. Nakanishi, manuscript submitted for publication). A similar rhodopsin analogue generated from cyclopentatrienylidene 11-*cis*-locked retinal was also shown to lack the native photochemical reaction and light-induced cGMP hydrolysis (Fukada et al., 1984).

The unicellular eukaryote *Chlamydomonas reinhardtii* is a phototactic alga. Retinal and retinal analogues restore normal phototactic behavior in a blind mutant, and moreover, the wavelength of maximum sensitivity is dependent on the particular analogue added (Foster et al., 1984). Further studies showed that analogues preventing retinal from isomerization around the 7-ene, 9-ene, 11-ene, 13-ene, or 15-ene ($C = NH^+$) bonds retained full activity. It was therefore concluded that there exists a *Chlamydomonas* rhodopsin and its activation in vivo does not require specific double-bond isomerization of the retinal (Foster et al., 1988, 1989).

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According to the *Chlamydomonas* result, the use of isomerization as a trigger may not be an essential motif of all retinal proteins. It is of interest to determine whether the sensory rhodopsins of archaebacteria use retinal isomerization as a photosignal.

Two retinal-containing photosensory receptors have been characterized in *Halobacterium halobium*. During aerobic growth, SR-II$_{487}$ (also called phoborhodopsin; Takahashi et al., 1985*a*), a repellent receptor, enables cells to avoid potentially damaging solar radiation. When oxygen tension becomes low, two photoenergy converters, bacteriorhodopsin (BR) and halorhodopsin (HR), are synthesized to supplement energy production. Also synthesized is SR-I$_{587}$, an attractant receptor, which guides cells to yellow-red regions optimal for BR and HR (Bogomolni and Spudich, 1982). S$_{373}$, a long-lived intermediate of the SR-I photocycle, is a repellent receptor for avoidance of harmful UV light (Spudich and Bogomolni, 1984).

The four bacterial rhodopsins use all-*trans* retinal as their chromophore and exhibit similar photochemical reaction cycles (Stoeckenius and Bogomolni, 1982; Lanyi, 1986; Spudich and Bogomolni, 1988; Takahashi et al., 1988). Although all-*trans* retinal possesses several isomerizable double bonds, only all-*trans*/13-*cis* isomerization has been detected in bacterial rhodopsin photocycles (Pettei et al., 1977; Braiman and Mathies, 1982; Tsuda et al., 1985; Fodor et al., 1987). A retinal analogue in which the all-*trans*/13-*cis* isomerization is prevented by a five-membered ring blocked the BR photochemical reaction and its proton pumping function (Fang et al., 1983; Chang et al., 1985). Using the same analogue, we have tested whether all-*trans*/13-*cis* photoisomerization is essential for the photochemical reactions of the two sensory rhodopsins and the generation of phototaxis signals by these receptors in *H. halobium*.

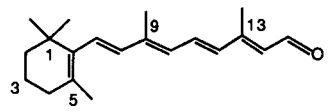
MATERIALS AND METHODS

Retinal and retinal analogue

All-*trans* retinal *1* was purchased from Sigma Chemical Co. (St. Louis, MO) and retinal analogue *2* was synthesized as previously described with minor modifications (Fang et al., 1983) (Fig. 1). Both *1* and *2* were purified by HPLC before use. A Waters Associates (Milford, MA) chromatography pump, model M-6000A, equipped with a YMC-PACK A203 SIL column and UV detector was used with an ether-hexane solvent system.

Strains, culture, and vesicle preparations

H. halobium strain Flx5R which lacks BR and HR apoproteins (BOP$^-$HOP$^-$) and is blocked in retinal synthesis was used for making membrane vesicles (Spudich, E. N., et al., 1986). Flx5R overproduces



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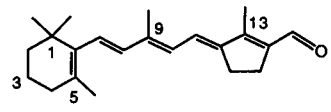

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FIGURE 1 Structural formulae of all-*trans* retinal (*1*) and all-*trans*-locked retinal (*2*).

SR-I apoprotein and SR-II apoprotein was not detectable in our preparations. Flx3R is also BOP$^-$HOP$^-$ and retinal deficient. It was used for behavioral measurements because of its better motility. Flx3b is a strain which produces only SR-II chromoprotein (Takahashi et al., 1988) and was used for both the vesicle preparation and the behavioral measurements.

H. halobium strains Flx5R and Flx3R were grown in complex medium in the dark and Flx3b was grown in complex medium plus 2 mM nicotine to inhibit retinal synthesis (Howes and Batra, 1970). Even in the absence of nicotine, retinal synthesis in Flx3b is negligible in exponential phase growth under vigorous aeration conditions (Takahashi et al., 1988). Vesicles were prepared by sonication as described (Manor et al., 1988).

Absorption spectra

Ethanolic solutions of *2* were added to Flx5R and nicotine-grown Flx3b vesicle suspensions in 4 M NaCl, 20 mM Tris-HCl, at pH 6.8 and 23–25°C. A maximum of 5 μl ethanolic solution was added to 2 ml vesicles. Absorption spectra were determined on a Hitachi 110B double-beam spectrophotometer equipped with an integrating sphere. Unreconstituted vesicles were used as reference. Path length was 1 cm.

Flash spectroscopy

(*A*) In vitro measurements. Flash-induced absorbance changes were measured in vesicles as described previously (Spudich, E. N., et al., 1986). A Vivitar (model-283; Santa Monica, CA) electronic flash was used to provide actinic light which was passed through a wide-band interference filter (flash wavelength ±20 nm). The monitoring wavelength was selected by a monochromater and the photomultiplier tube was further protected by a narrow band filter transmitting the monitoring wavelength ±5 nm. Optical filters were from Ditric Optics (Hudson, MA). All measurements were at 25°C. Data points from 15–20 flashes were averaged for each measurement. (*B*) In vivo measurement. A 14-ml suspension of late exponential-phase cells incubated with various amounts of all-*trans* retinal for 12 h were pelleted by centrifugation (4500 *g*, 5°C, 20 min), suspended in basal salt containing 1% arginine (pH 7.0) (Stoeckenius et al., 1988; Manor et al., 1988), repelleted and resuspended in 4 M NaCl, 20 mM Tris-HCl at pH 6.8, and at 1.4×10^{10} cells/ml. For flash spectroscopy 2 ml of this cell suspension was used in a 1-cm path-length cuvette.

Behavioral assay

Three cultures of Flx3R cells were grown to late exponential phase in complex medium and 5 μl of ethanol, or 5 μl of ethanolic solutions of *1* or *2* (final concentration, 2×10^{-6} M) were added. The cultures were maintained in exponential phase by diluting 1:10 each day with fresh medium and addition of 5 μl of ethanol, *1* or *2* to maintain a chromophore concentration of 2×10^{-6} M. After 15 d, cells were diluted 1:67 into 2 ml fresh medium and the suspension was incubated for 2–4 h before measurements.

Flx3b cells were grown to late exponential phase in complex medium containing 2 mM nicotine to inhibit retinal synthesis. Aliquots were incubated for 4–5 h after addition of 5 μl of ethanol, *1* or *2* (final chromophore concentration 5×10^{-7} M).

Swimming behavior was monitored in dark field at 37°C with nonactinic infrared illumination ($\lambda > 700$ nm). Light from a 200-W mercury arc lamp beam was passed through a 600 ± 20-nm (for SR-I_{587} stimuli) or 500 ± 20-nm (for SR-II_{487} stimuli) interference filter to produce intensities of 5.0×10^4 and 7.0×10^3 erg · cm^{-2} · s^{-1}, respectively, at the sampler chamber. The 390 stimulus was from the mercury lamp beam passed through a 400 ± 20-nm bandpass filter and a 7-59 short-pass filter, which produced 6.0×10^4 erg · cm^{-2} · s^{-1}. All optical filters were from Ditric Optics, Inc. except the 400-nm bandpass, which was from Corion Corp (Holliston, MA). The swimming response video data were acquired as described (Sundberg et al., 1986) with the EV1000 motion analysis computerized cell tracking system (Motion Analysis Systems, Inc., Santa Rosa, CA) coupled to electronic shutters and analyzed by using a previously described reversal frequency program (Sundberg et al., 1986) and a modified program (Takahashi, T., B. Yan, K. Nakanishi, and J. L. Spudich, manuscript in preparation). Reversal frequency is calculated as the number of reversals occurring at a particular frame divided by the number of paths present at that frame. The cycling time for consecutive delivery of stimuli was 90 s for 600-nm stimuli and 20 s for 500-nm and 390-nm stimuli.

RESULTS AND DISCUSSION

Reconstitution

The stepwise formation of SR-I analogue pigment from *2* is shown in Fig. 2. It is interesting to analyze this process in terms of previous study on binding of all-*trans* retinal to bacterioopsin, which was resolved into three steps at 0°C (Schreckenbach et al., 1977*a*, *b*). Steps 1 and 2 correspond to the formation of two reconstitution intermediates absorbing at ~400 and ~440 nm with structured absorption spectra. Step 3 is the formation of BR (Schreckenbach et al., 1977*a*, 1977*b*, 1978). The first step was suggested to be coplanarization of the β-ionone ring with the polyene chain (Schreckenbach et al., 1977*a*, 1977*b*, 1978) and step 3 to be Schiff base formation (Gärtner et al., 1983). However, recent resonance Raman studies suggest the 440-nm intermediate is already a protonated Schiff base (Callender, R., personal communication).

Reconstitution intermediates can be seen at room temperature if the reaction in step 3 is slowed down. When *2* was reacted with bacterioopsin, step 3 was remarkably retarded and 15 d were needed to convert the intermediate into BR analogue (Fang et al., 1983). The slow step

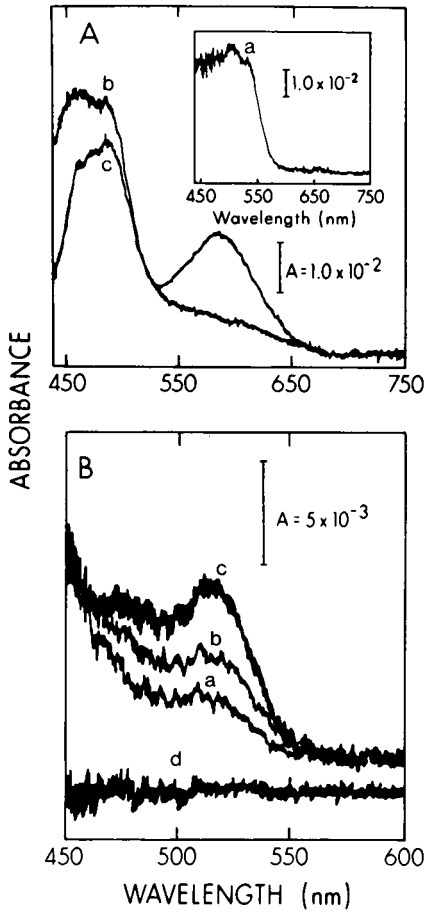

FIGURE 2 Absorption spectra of all-*trans*-locked SR-I (*A*) and SR-II (*B*) analogues. (*A*) Immediately after addition of *2* to Flx5R vesicle suspension at room temperature (analogue/SR-I apoprotein = 1:1), an early intermediate absorbing at 470–530 nm with a structured absorption spectrum was observed (*a*, *inset*). After 1 h, it was converted to the second intermediate at 435–490 nm also with a structured absorption spectrum (*b*). After 15 d of reconstitution the second intermediate was partially converted to a pigment with an absorption band maximum at 585 nm (*c*). (*B*) *2* was added to a nicotine-grown Flx3b vesicle suspension at room temperature (analogue/SR-II apoprotein = 3:1) and the spectra were recorded at (*a*) 20 min, (*b*) 35 min, and (*c*) 100 min. A baseline was recorded before the retinal analogue addition (*d*).

is probably due to steric restriction in the retinal binding cleft of the apoprotein around the retinal C13-C14 region. Additional support for such steric restriction is that all-*trans* 14-methyl retinal does not produce a red-shifted pigment with BR apoprotein (Fang et al., 1983), nor with SR-I apoprotein (Yan, B., R. Johnson, K. Nakanishi, and J. L. Spudich, unpublished observations). The final reconstitution products were structured absorbing species at ~440 nm. The formation of *trans*-locked SR-I pigment (Fig. 2) was similar to BR and step 3 was also slowed down to the same degree as that of BR. After 15 d of

generation ~50% of pigment was formed as estimated by the amount of native pigment generated from the same concentration of apoprotein. An interesting finding is that the two early intermediates absorb at ~500 and ~470 nm (Fig. 2, *A*, spectrum *a* and *b*) which are red-shifted from those of BR (~400 nm and ~440 nm) and that the spectra exhibit fine structure. The structured bands may be due to multiple species or vibrational fine structure. The retinal analogues 13-desmethyl and 9,13-didesmethyl retinal exhibit similar rapid formation of reconstitution intermediates with structured absorption at ~450 nm and a slow formation of red-shifted SR-I pigments. The structured bands in these cases appear not to be due to multiple pigments because isosbestic points are observed in the process of their formation and in their conversion to the SR-I analogue pigments absorbing near 575 nm (B. Yan, R. Johnson, K. Nakanishi, and J. L. Spudich, unpublished observations). In contrast to the slow binding of *2* with SR-I apoprotein, the binding to SR-II apoprotein is only slightly slower than that of *1*, implying a lenient binding site near the Schiff base linkage. The resulting pigment showed a structured absorption spectrum (Fig. 2 *B*), which is probably due to vibrational structure as has been demonstrated in native SR-II (Takahashi, T., B. Yan, P. Mazur, F. Derguini, K. Nakanishi, and J. L. Spudich, manuscript submitted for publication). Both the opsin shift and the extinction coefficient of the SR-II analogue are similar to those of native SR-II. In several SR-II reconstitutions a species was observed at 437 nm which was formed immediately after addition of *2* (data not shown).

Photochemical reactions

SR-I and SR-II are photochemically reactive. Upon absorption of a photon, red-shifted primary photoproducts are formed which thermally decay through several intermediates and form long-lived intermediates which take hundreds of milliseconds to seconds to return to $SR\text{-}I_{587}$ and $SR\text{-}II_{487}$. This photocyclic process is monitored by the flash-induced absorbance changes as in Fig. 3 (trace 1 in *a* and *b*). The single exponential recovery of trace 1 in *a* results from the return of S_{373} to $SR\text{-}I_{587}$ (Bogomolni and Spudich, 1982) and the biphasic return of trace 1 in *b* is due to the return of $S\text{-}II_{360}$ to $SR\text{-}II_{487}$ through another intermediate—$S\text{-}II_{530}$. Both SR-I and SR-II pigment analogues reconstituted with *2* are photochemically inactive as determined by flash spectroscopy (2-ms time resolution for SR-I analogue and 1 ms for SR-II analogue, see trace 2 in *a* and *b*). Absence of normal photochemical reactivity has also been reported in seven- and five-membered ring locked visual pigments (Mao et al., 1981; Fukada et al., 1984) and five-membered ring locked BR analogue (Chang et al., 1985) providing direct evidence that the isomerization process is essential for photochemical reactions of all retinal-containing pigments so far tested.

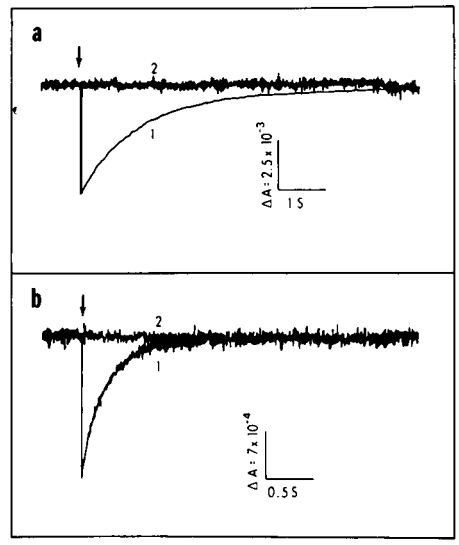

FIGURE 3 Flash-induced absorbance changes of SR-I pigments (*a*) and SR-II pigments (*b*). (*a*) Absorbance changes of the sample at 570 nm were monitored after an actinic flash (1 ms) of 600 nm. Samples were Flx5R vesicles reconstituted with native retinal *1* (1) or analogue *2* (2) with maximum absorbance of 2.0×10^{-2} in both cases. Vesicle suspension were 1.8 and 3.6 mg protein/ml for 1 and 2, respectively. The lower S/N ratio is due to the higher vesicle concentration of the latter. (*b*) Actinic flash (1 ms) was at 500 nm and the absorbance change of the sample at 480 nm was monitored for nicotine-grown Flx3b vesicles reconstituted with native retinal *1* (1) or analogue *2* (2) as in Fig. 2, *B*. Both suspensions contained pigments with maximum absorbance of 1.0×10^{-2} and 2.0 mg protein/ml.

At this point, two results would be anticipated. First, if the photochemical reactions of SR-I and -II are required for their photosignal transduction function, phototaxis responses of cells equipped with locked pigment analogue should not occur. Second, the near UV repellent light receptor in *H. halobium* has been proposed to be the S_{373} intermediate of the SR-I photocycle (Spudich and Bogomolni, 1984). If this is correct, blocking the formation of S_{373} with all *trans*-locked analogue would block the near UV repellent response of the cells.

Phototaxis behavior

H. halobium are propelled by rotary motors and their smooth swimming is interrupted by spontaneous motor switching events which cause the cell to reverse its direction of swimming (for review, see Spudich and Bogomolni, 1988). Repellent and attractant light stimuli induce or suppress reversals, respectively (Hildebrand and Dencher, 1975; Spudich and Stockenius, 1979), as

seen for Flx3R and Flx3b cells reconstituted with native all-*trans* retinal (Fig. 4, *middle column*).

Flx3R incubated with an excess of *2* for 15 d generated neither the SR-I attractant (one photon) nor the SR-I repellent (two photon) responses (Fig. 4, *right panels* of rows 1 and 2). *H. halobium* Flx3b produces only the SR-II chromoprotein. When it was grown under conditions (see Materials and Methods) which suppress retinal synthesis, the SR-II repellent response was not detected (Fig. 4, *bottom left panel*). Addition of all-*trans* retinal restored the full responses within 2 h (Fig. 4, *bottom middle panel*). However, cells incubated with *2* for 5 h did not exhibit the response (Fig. 4, *bottom right panel*).

The rapid and complete binding of *2* to SR-II apoprotein (Fig. 2, *B*) and the inhibition of the SR-II mediated response when 1 was added to cells preincubated with *2* (data not shown) indicate the entry of *2* into the native SR-II binding site. In the case of SR-I, the slow regeneration of the pigment with *2* raises the question of whether SR-I pigment analogue was formed in vivo. Accordingly, we analyzed the effect of prior incubation with *2* on the reconstitution rate of native pigment in vitro and in vivo. The in vitro rate was assayed from the absorption increase at 587 nm and the time course of pigment generation as measured by flash-induced absorbance changes at 570 nm after retinal addition in membrane vesicles (as in Fig. 5, *filled symbol*). The in vivo rate was evaluated by monitoring flash-induced absorbances changes at 570 nm in Flx3R cells. After addition of *1* to the Flx3R cells at the same retinal/apoprotein ratio as in the vesicles, 84% pigment was formed within 1 h and reconstitution was completed in <2 h. This rate is comparable to the in vitro

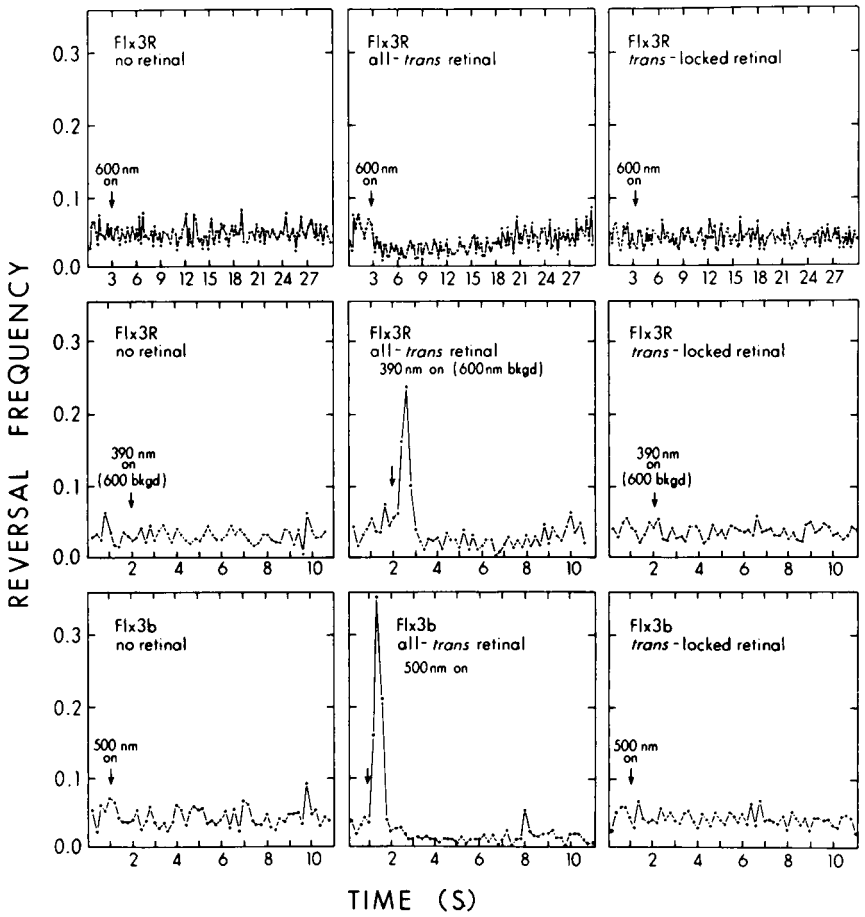

FIGURE 4 SR-I and SR-II mediated reversal frequency responses to photostimuli. Video data acquired with infrared illumination were processed with the computerized motion analysis system (Sundberg et al., 1986) at 5 frame/s for 30 s (row 1) and 11 s (row 2 and row 3) and the population reversal frequencies determined as in Materials and Methods. At the time indicated by the arrow, a 600-nm light (row 1) was delivered for 30 s, a 390-nm light for 3 s with a constant 600-nm background light (row 2), or a 500-nm light for 600 ms (row 3). Flx3R cells were incubated with 5 μl of ethanol (*left*, rows 1 and 2), 5 μl ethanolic solution of *1* (*middle panels* of rows 1 and 2) or *2* (*right*, rows 1 and 2). Nicotine-grown Flx3b cells were incubated with 5 μl of ethanol (*left*, row 3), *1* (*middle*, row 3), and *2* (*right*, row 3), as described in Materials and Methods.

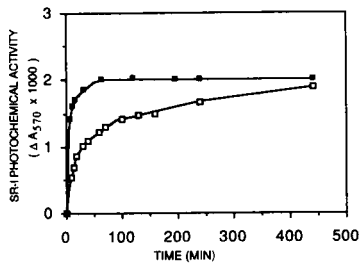

FIGURE 5 Inhibition of SR-I generation by preincubated analogue *2*. Vesicles were prepared from 100 ml late exponential-phase Flx3R cells incubated with ethanol or *2* for 15 d as for the behavioral measurements (Fig. 4). To these vesicle suspensions, 5 μl ethanolic solution of *1* (final concentration, 6×10^{-8} M) was added and the development of SR-I up to 440 min assessed by monitoring 600-nm flash-induced absorbance changes (3,968 points, 2 ms/point) at 570 nm. Values of ΔA_{570} 4 ms after the flash are plotted. The open symbols are for vesicles preincubated with *2* in ethanol and the solid symbols are those with ethanol alone.

reconstitution rate. The formation of the SR-I pigment analogue and the occupancy of the native retinal binding site by *2* was established by (*a*) formation of a red-shifted pigment (Fig. 2, *A*) and the comparable in vitro and in vivo binding rates of *1*, (*b*) inhibition of SR-I generation

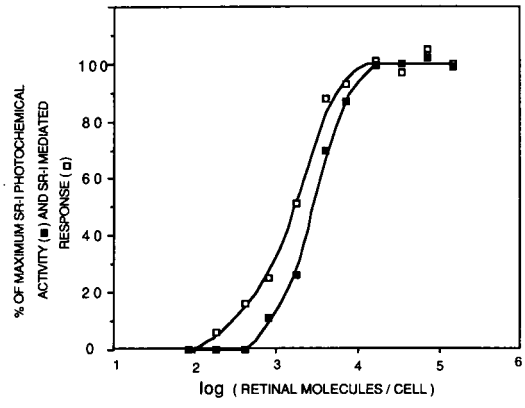

FIGURE 6 Detection sensitivities of behavior assay and flash photolysis assay of SR-I. Suspensions of late exponential phase Flx3R cells (2×10^9 cells/ml) were incubated with various amounts of *1* for 12 h. 30 μl was diluted 1:67 for motion analysis and the remainder was concentrated for in vivo flash spectroscopic measurements (as in Materials and Methods). Reversal suppressions by a 600-nm stimulus (30 s duration) as in Fig. 4 was determined by integrating the reversal frequency over 25 s after photostimulation and subtracting the integral of 25 s of spontaneous reversal frequency. Maximum reversal suppression is shown in Fig. 4 (*top middle* panel) and the spontaneous reversal frequency of the cells was 0.036 s^{-1}. The photochemical activity of SR-I in cells was evaluated by ΔA_{570} 4 ms after the flash. Open symbols are percentages of the maximum behavioral response mediated by SR-I and the filled symbols are percentages of the maximum photochemical activity of SR-I.

when all-*trans* retinal was added to vesicles made from cells reconstituted with *2* for 15 d (Fig. 5, results in this figure show that chromophore *2* is slowly replaced by *1*), and (*c*) inhibition of SR-I response generation when all-*trans* retinal was added to those cells (data not shown).

A simultaneous measurement of phototaxis responses and SR-I content (measured by flash spectroscopy in vivo) showed that the behavioral assay is more sensitive than flash photolysis under our assay conditions (Fig. 6). From the flash photolysis data in Fig. 6, we estimate 9,600 retinal molecules/cell are required to reconstitute full pigment photochemical activity in this cell preparation. A behavioral response is evident at 200 retinal molecules/cell. Therefore, there is at most 2% of the total pigment photochemical activity at the lowest retinal concentration at which we detect a response. In view of the high sensitivity of the behavioral assay, we interpret the lack of response generation by 2 as demonstrating that all *trans*-locked SR-I and SR-II do not produce phototaxis signals.

The SR-I photointermediate S_{373} was proposed to be the near-UV light repellent receptor based on the correlation of behavioral responses with SR-I photoreactions (Spudich and Bogomolni, 1984; Takahashi et al., 1985*b*, *c*). In this work, we show that all *trans*-locked analogue reconstituted cells do not exhibit responses to near UV light (Fig. 4, *right panel* of row 2), as expected from the inability of the all-*trans*-locked SR-I analogue pigment to form the S_{373} intermediate.

From the results above we conclude that all-*trans*/13-cis isomerization of the retinal is required for activation of the sensory rhodopsins in *H. halobium*. Therefore, unlike the proposed mechanism for *Chlamydomonas*, photoactivation of these primitive archaebacterial photoreceptors resembles that of evolutionarily distant visual pigments from higher organisms.

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