Retinal analog restoration of photophobic responses in a blind *Chlamydomonas reinhardtii* mutant

Evidence for an archaebacterial like chromophore in a eukaryotic rhodopsin

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ABSTRACT The strain CC-2359 of the unicellular eukaryotic alga *Chlamydomonas reinhardtii* originally described as a low pigmentation mutant is found to be devoid of photophobic stop responses to photostimuli over a wide range of light intensities. Photophobic responses of the mutant are restored by exogenous addition of all-*trans* retinal. We have combined computer-based cell-tracking and motion analysis with retinal isomer and retinal analog reconstitution of CC-2359 to investigate properties of the photophobic response receptor. Most rapid and most complete reconstitution is obtained with all-*trans* retinal compared to 13-*cis*, 11-*cis*, and 9-*cis* retinal. An analog locked by a carbon bridge in a 6-s-*trans* conformation reconstitutes whereas the corresponding 6-s-*cis* locked analog does not. Retinal analogs prevented from isomerization around the 13–14 double bond by a five-membered ring in the polyene chain (locked in either the 13-*trans* or 13-*cis* configuration) do not restore the response, but enter the chromophore binding pocket as evidenced by their inhibition of all-*trans* retinal regeneration of the response. Results of competition experiments between all-*trans* and each of the 13-locked analogs fit a model in which each chromophore exhibits reversible binding to the photoreceptor apoprotein. A competitive inhibition scheme closely fits the data and permits calculation of apparent dissociation constants for the in vivo reconstitution process of 2.5×10^{-11} M, 5.2×10^{-10} M, and 5.4×10^{-9} M, for all-*trans*, 13-*trans*-locked and 13-*cis*-locked analogs, respectively. The chromophore requirement for the *trans* configuration and 6-s-*trans* conformation, and the lack of signaling function from analogs locked at the 13 position, are characteristic of archaebacterial rhodopsins, rather than the previously studied eukaryotic rhodopsins (i.e., visual pigments).

INTRODUCTION

Retinal (vitamin A aldehyde, Fig. 1) is used as the chromophore of visual pigments throughout the animal kingdom (Wald, 1968; Ottolenghi, 1980; Birge, 1981) and of prokaryotic photosensory receptors (archaebacterial sensory rhodopsins) (Spudich and Bogomolni, 1988). In both classes, the retinal is linked as a protonated Schiff base to the epsilon amino group of a lysine residue in the receptor apoprotein, but the two groups differ in the isomeric configuration of their retinal polyene chain. In all known cases, visual pigments contain as their chromophore 11-*cis* retinal 4 (Wald, 1968) with a 6-s-*cis* configuration around the 6–7 single bond (Mollevanger et al., 1987; Smith et al., 1990). In contrast, the archaebacterial sensory rhodopsins as well as the archaebacterial ion pumping rhodopsins contain as their chromophore all-*trans* retinal 1 (Oesterhelt and Stoekenius, 1971) with a 6-s-*trans* conformation 2 (Harbison et al., 1985; van der Steen et al., 1986; Baselt et al., 1989; Takahashi et al., 1990; Creuzet et al., 1991). The trigger for signal generation in visual pigments is 11-*cis* to all-*trans* photoisomerization (Akita et al., 1980; Chabre, 1985) and in archaebacterial sensory rhodopsins all-*trans* to 13-*cis* (Yan et al., 1990).

Chlamydomonas reinhardtii exhibits both orientation responses (phototaxis) and photophobic or stop responses to 500 nm flashes of light (Boscov and Feinleib, 1979; Foster et al., 1984; Hegemann and Bruck, 1989). Addition of retinal and retinal analogs restores normal phototactic behavior in a low sensitivity mutant, FN68. The mutant exhibits a small phototaxis response to high intensity light stimuli and this response is strongly enhanced by prolonged preillumination without the addition of exogenous retinal (Foster et al., 1988; Beckmann and Hegemann, 1991). A wide variety of retinal analogs with various polyene configurations and ring chain conformations restore the phototactic responses in FN68 and the wavelength of the maximum sensitivity is dependent on the particular analog added (Foster et al., 1984). No specific double bond isomerization appeared to be necessary for the phototaxis response because retinal analogs prevented from isomerizing anywhere along the polyene chain restored phototaxis (Foster et al., 1988, 1989). All-*trans* retinal (Derguini et al., 1991) and a retinal-binding pigment with maximal absorption at 495 nm have been identified in *C. reinhardtii* cells (Beckmann and Hegemann, 1991).

The photophobic response is especially convenient for

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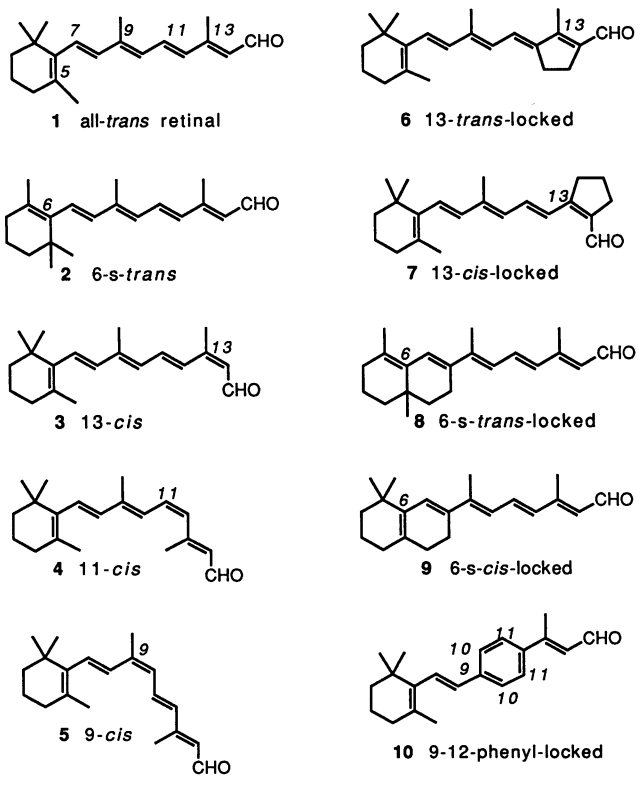

FIGURE 1 Retinal isomers and analogs. All-*trans*, 13-*cis*, 11-*cis*, and 9-*cis* refer only to the bond configuration along the polyene chain. 6-s-*trans* and 6-s-*cis* refer to the conformation of the ring relative to the chain.

the study of chromophore specificity, because the behavior of large numbers of individual cells can be quantitatively assessed within a short time window by established computer-assisted video microscopic cell tracking and analysis methods (Sundberg et al., 1986; McCain et al., 1987; for review see Khan, 1990).These methods have been applied to *C. reinhardtii* photophobic responses by Hegemann and Bruck (1989). In this paper, we have combined computer-assisted motion analysis and both retinal isomer and analog reconstitution to investigate chromophore properties of the photophobic response receptor.

MATERIALS AND METHODS

Retinal isomers and analogs

All-*trans* retinal was purchased from Sigma Chemical Co. (St. Louis, MO). 13-*cis* 3, 11-*cis* 4 and 9-*cis* 5 retinals were prepared according to Denny et al. (1981). 13,14 locked retinals 6 and 7 were synthesized according to Fang et al. (1983). 6-s-*cis*-locked 8 and 6-s-*trans*-locked retinal 9 were prepared according to van der Steen et al. (1989). 9,12-Phenylretinal 10 (3-[4[2-(2,6,6-trimethylcyclohexenyl)vinyl]phenyl-2-butenal) was prepared according to Kolling et al. (1984). All retinals were purified by HPLC on a 10 × 25 mm porasil column eluted with 5% ethyl acetate in hexane at a flow rate of 3.0 ml/min. Retinals were dissolved in methanol and stored in the dark at −70°C under argon.

Strains

Chlamydomonas reinhardtii strain CC-124 (mt⁻ nitrate reductase⁻) was isolated from wild-type strain 137c (Harris, 1989) and exhibits both normal motility and photophobic responses. CC-124 was grown on high salt medium (Sueoka et al., 1960) with trace elements (Hutner et al., 1950), at 25°C and used in the behavioral assay procedure after 2 h of light in a 12 h light, 12 h dark cycle (Spudich and Sager, 1980).

Strain CC-2359 (also called lts-1), a low pigmentation mutant isolated by Iroshnikova et al. (1982), was obtained from the *Chlamydomonas* Genetics Center at Duke University. A single colony isolate with good motility was grown on high salt acetate medium (Suoeka et al., 1960) containing trace elements as above, in the dark at room temperature (23–26°C). CC-2359 is completely deficient in photophobic responses to visible light stimuli even after extended preillumination with white light (data not shown).

Behavioral Assay

Cells were grown for 10–14 days on 1.5% agar plates. Cells from 2–3 plates were resuspended in 50 ml low nitrogen liquid NMM medium (Sears et al., 1980) to a concentration of 2×10^6 cells/ml and incubated without shaking overnight to induce gametogenesis. Retinal and retinal analogs were delivered from a methanolic solution to the cultures to give final concentrations ranging from 5×10^{-12} to 1×10^{-6} M. Methanol added never exceeded 10 μl/10ml cell suspension. Concentrations of retinal greater than 1×10^{-6} M caused reduced motility in strain CC-2359.

Swimming behavior was monitored in dark field by nonactinic infrared illumination (wavelength 730–850 nm) at room temperature. Light from a 200 W mercury arc lamp was passed through a 500 ± 20-nm interference filter (Ditric Optics, Hudson, MA) controlled by means of an electronic shutter (Vincent Associates, Rochester, NY) and combined with the monitoring beam through a beam splitter. The shutter was coupled to a digitizing unit of the automated tracing system (Motion Analysis Corporation, Santa Rosa, CA) via a pulse generator (Grass Instrument Company, Quincy, MA) that controlled the delay between an event marker used to initiate data acquisition and delivery of the stimulus. The stimulus was a pulse of 7.2×10^4 ergs/cm^2 · s at a pulse duration of 5 or 20 ms as indicated in the figure legends. Cells were delivered to a chamber formed by a slide and a bridge mounted coverslip and exposed to six repetitive stimuli delivered at 45 second intervals.

The analysis of *Chlamydomonas* swimming behavior was carried out with an EV1000 motion analysis cell tracking system (Motion Analysis Corporation, Santa Rosa, CA) using combinations of the operators defined in the Motion Analysis Corp. software package. The first step of the analysis was to generate a two dimensional projection of the cells' swimming paths from the video data. Video images were digitized at 15 frames/second and transferred to the computer, where a search operator tracked cells from one frame to the next for 3 s, defining a path for each cell. The program carries out path calculations on six 3-s segments of digitized data, and merges the individual path files to form a single file containing 500–900 paths.

The second stage of the analysis made use of two parameters to identify the stop response: the rate-of-change-of-direction (RCD), which reaches a maximum value when a cell undergoes a directional change, and the swimming speed (SPD) whose value drops as the cell stops. The quotient RCD/SPD generated a distinct peak corresponding to the stop response (see Results). Cells whose average speed was < 20 microns/s were discarded to eliminate immotile cells, as were

cells that remain in the field for less than five frames (1/3 s). After these selection steps, 300–600 paths remained in the analysis. The average RCD/SPD was calculated on a frame-by-frame basis. The area under the generated peak (10 frames) was calculated minus the area under the baseline for an equal time period. The percent response was calculated defining the response of strain CC-2359 reconstituted with 1×10^{-6} M retinal as the maximum response.

Curve fitting was performed with Asystant 3.0 software (Asyst Software Technologies Incorporated, Rochester, NY). Goodness of fit was assessed as the average sum of the squared residuals.

RESULTS

Computerized motion analysis assay of the stop response

Two parameters of *Chlamydomonas* swimming behavior that are affected during the photophobic response are speed (SPD) and rate of change of direction (RCD) (Fig. 2). For the path shown, the SPD decreases, and the RCD increases within the 67 ms video frame in which the flash was delivered. The SPD for that cell is a continuous trough while the RCD plot contains a double peak. The function RCD/SPD shows a greater signal-to-noise ratio than either RCD or SPD alone (Fig. 2 *A*). Hegemann and Bruck (1989) have characterized the swimming behavior of a cell during a photophobic response as a stop within 50 ms after the stimulus, a short period of backward swimming, another stop, and finally a resumption of forward swimming in a random direction. Our data is consistent with their observations. The double peak in the RCD/SPD is expected from the two stops (Fig. 2 *A*).

The mean RCD/SPD of 560 cells selected by the motion analysis program from a population of 724 cells was calculated for each video frame for 3 s (Fig. 2 *B*). The small dip in the plot immediately before the stimulus is seen repeatedly in our data as well as other published work (Hegemann and Bruck, 1989). We have determined that this dip is an artifact caused by the path selection in the motion analysis program. The stimulus recruits rapidly swimming cells into the data set which would have otherwise left the field of view prior to satisfying the five-frame-residency requirement, but now remain in the field due to their stop response. The relatively higher SPD of these cells causes a decrease in the average RCD/SPD in the four frames preceding the response.

Cells can be divided into three categories based on visual assessment of their swimming behavior following a light stimulus: those cells not affected by the stimulus, those exhibiting brief directional changes, and those stopping for prolonged (0.5 to 1.0 s) periods. As the intensity of the light stimulus increases, more cells undergo a stop response, whereas less show no apparent response (Fig. 3). At intermediate light stimulus intensities there is an increase in the number of cells exhibiting a brief directional change. The computer generated RCD/SPD correlates closely to the photophobic stop response assessed visually, and can be applied to a much larger number of cells than can be practically tracked visually.

Retinal reconstitution of a mutant nonresponsive to photophobic stimuli

Chlamydomonas strain CC-2359 was originally selected as a pale (i.e., chlorophyll and carotenoid-deficient) mutant (Iroshnikova et al., 1982). CC-2359 cells show no photophobic response to a flash stimulus (Fig. 4), and are unresponsive to stimuli even at fluences 6,000-fold greater than that which produces a saturating wild-type response (Fig. 2). Exogenously added all-*trans* retinal restores photophobic sensitivity to CC-2359 cells (Fig. 4). The maximum effective wavelength for the reconstituted response is 500 nm, (*Inset,* Fig. 4), matching the action spectrum maxima reported for wild type flash-induced movement responses (Uhl and Hegemann, 1990).

Evidence the native chromophore polyene configuration for the photophobic response is all-*trans*

We have compared the rate of restoration of the response by the retinal isomers 13-*cis* 3, 11-*cis* 4, and 9-*cis* 5 to that of all-*trans* retinal. Isomers were purified by HPLC and added from a methanolic solution (maximum 10 microliter to 20 ml cell suspensions) at 1.5×10^8 retinal molecules/cell. All-*trans* retinal restored the response most rapidly and to a greater extent than 13-*cis,* and 11-*cis* and 9-*cis* show no restoration of the response in 60 min (Fig. 5). Although reconstitution rates do not necessarily define the configuration of the native chromophore, the data strongly suggest all-*trans* retinal to be the native polyene configuration of the chromophore for the photophobic response. Additionally, overnight incubation with 1 micromolar of an analog locked in a 6-s-*trans* 8 conformation (Fig. 1) reconstitutes >90% maximum sensitivity, whereas incubation with the corresponding 6-s-*cis*-locked 9 analog does not reconstitute a photophobic response detectable by our assay.

Inhibition of the reconstituted photophobic response by isomer-locked analogs

Analogs of retinal unable to isomerize around the 13–14 double bond do not restore the photophobic stop re-

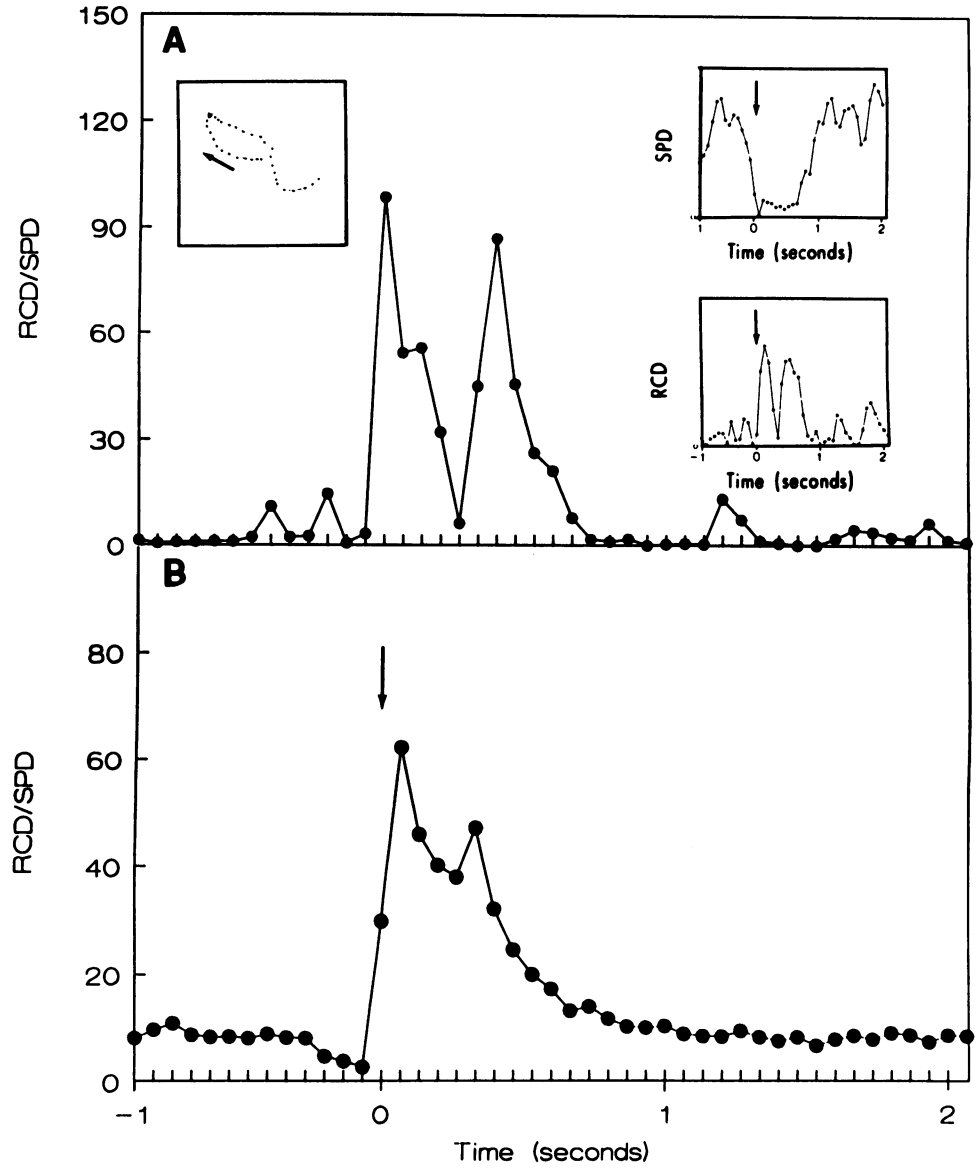

FIGURE 2 (*A*) Computer assessment of the motion of a single *Chlamydomonas* cell undergoing a photophobic response. (*Inset left*) A 3-s path (45 frames) of a cell travelling in the direction of the arrow in which a 20 ms pulse of 500-nm light was delivered after 1 s. From this path, the speed of the cell (SPD, ordinate maximum = 150 microns/s) (*inset top right*) and the rate of change of direction in degrees/s (RCD) (*inset bottom right*) are calculated. (*Main panel*) The ratio of the RCD/SPD for the path. (*B*) Computer assessment of a *Chlamydomonas* population photophobic response. Wild type strain CC-124 was subjected to a 20 ms pulse of 500 nm light (*at arrow*). The average RCD/SPD of 560 cells was plotted for a 3 s time period.

sponse when added to strain CC-2359 (Fig. 6), although they have previously been reported to restore phototaxis in the mutant FN-68 (Foster et al., 1989). Additionally, a retinal analog with a phenyl ring 10 which hinders all-*trans* to 13-*cis* isomerization (Kolling et al., 1984), also does not reconstitute responses in our assay (data not shown). Both 13-*trans*-locked 6 and 13-*cis*-locked 7 retinal inhibit the all-*trans* restoration of the mutant CC-2359 when added prior to the all-*trans* addition (Fig. 7). Moreover, addition of the locked analog after all-*trans* restoration reduces the response to near the same level as inhibitor pre-incubation. All-*trans* retinal was added to a cell suspension of CC-2359 after an overnight incubation with 1 nM 13-*trans*-locked analog, and the percent photophobic response generated is 60% of the noninhibited value (Fig. 7*A*). This inhibition is

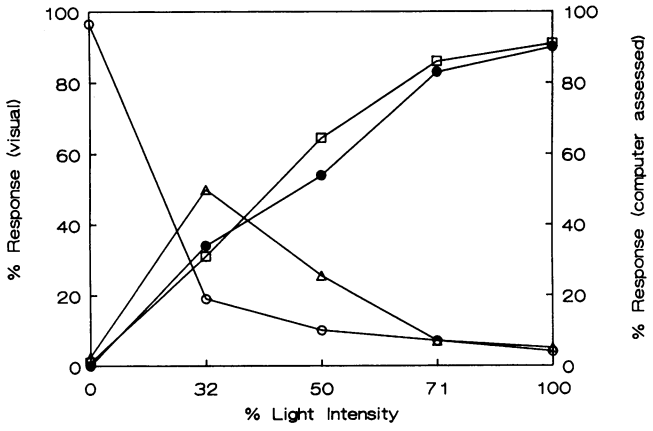

FIGURE 3 Comparison of computer assessed and visually assessed swimming behavior. (○)—smoothly swimming cells, (△)—cells which undergo brief reorientations of swimming direction, (□)—cells exhibiting a stop, the computer generated average RCD/SPD (●).

dose dependent, a 10-fold increase in analog concentration shows greater inhibition (Fig. 7 *B*). The 13-*cis* locked analog is less efficient in inhibiting the all-*trans* reconstituted response, requiring 10 nM concentration to produce a similar extent of inhibition as 1 nM *trans*-locked (Fig. 7 *C* and *D*).

The native photophobic response receptor in wild-type cells also is inhibited in a similar type of measurement as performed with the mutant gametes. 1 h incubation of wild-type gametes with 10 nM 13-*trans*-locked isomer inhibits the photophobic response by ~15%, whereas incubation with 10 nM all-*trans* retinal had no effect on the response (data not shown).

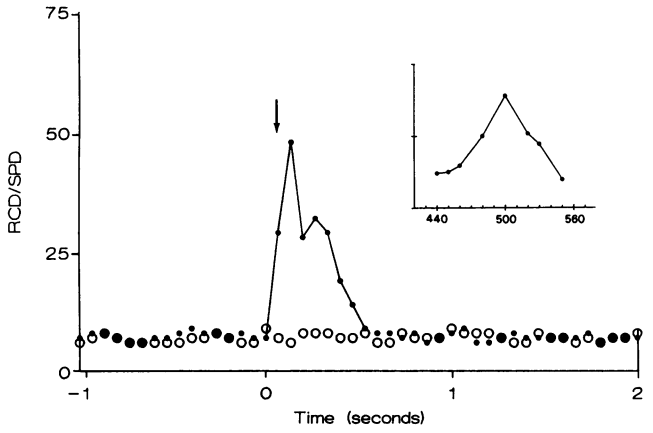

FIGURE 4 Retinal restoration of stop responses in mutant CC-2359. A suspension of the mutant cells was incubated for 2 h with 1 μM all-*trans* retinal in methanol (*filled circles*) or with methanol alone (*open circles*) and exposed to a 20 ms pulse of 500-nm light (*at arrow*). The RCD/SPD data from 300–400 cells in each case were processed and their mean RCD/SPD plotted for a period of 3 s. The area between the two traces (the stimulus induced integrated change in the population mean RCD/SPD) is used as a measure of the response. (*Inset*) Ordinate: the reciprocal of the relative light intensity required to give a criterion response (50% of the maximum). Abscissa: stimulus wavelength.

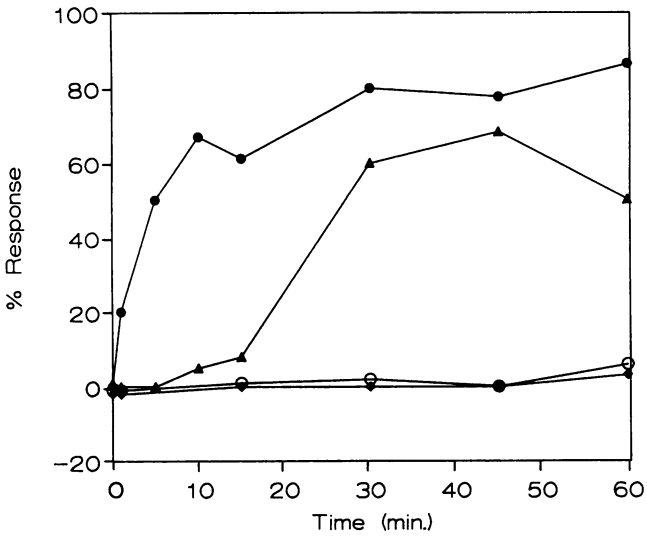

FIGURE 5 Time course of restoration of CC-2359 stop responses. Isomers of retinal (●—all-*trans*, ○—11-*cis*, ▲—13-*cis*, ◆—9-*cis*) were added (each at 1 nM) to strain CC-2359 at time 0 and responses to 20 ms flashes of 500-nm light assessed as in Fig. 4.

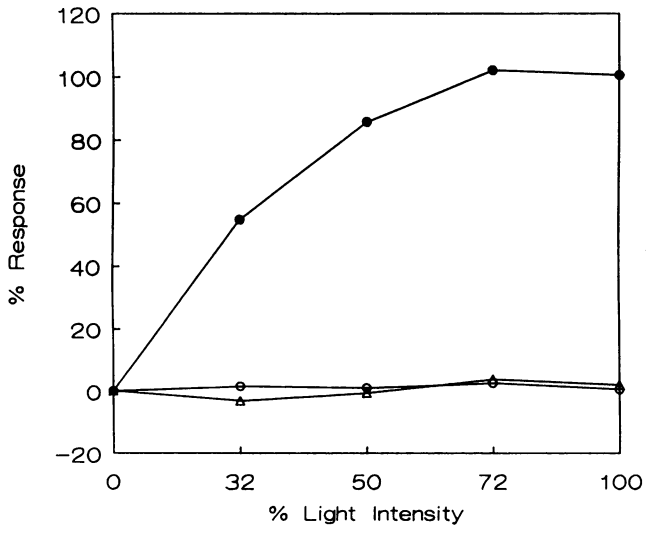

FIGURE 6 Fluence response curves. All-*trans* retinal (●), 13-*trans* locked (○) and 13-*cis* locked (△) analogs were added (each at 1 μM) to CC-2359 and incubated for 18 h. Stimulus was a 20 ms flash of 500-nm light at 7.2×10^4 ergs/cm^2 · s. Stimulus intensity was varied with neutral density filters.

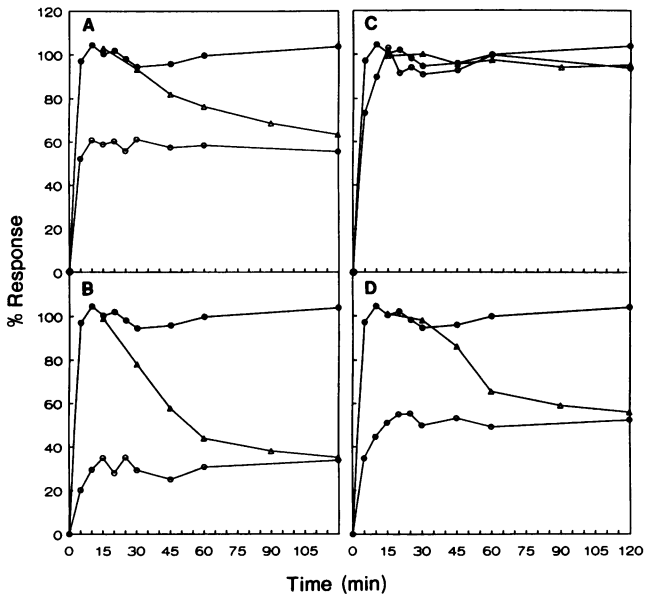

FIGURE 7 Inhibition of retinal restoration of the response by 13-ene-locked analogs. At time 0, all-*trans* retinal (1 nM) was added to each of two cell suspensions of CC-2359 and the development of photophobic sensitivity was monitored. One suspension (○) was preincubated 18 h with a locked analog, and the other with methanol as a control (●). After 120 min, the locked analog was added to the control suspension (△), and times shown are the time after analog addition. (*A*) 1 nM 13-*trans*-locked; (*B*) 10 nM 13-*trans*-locked; (*C*) 1 nM 13-*cis*-locked; (*D*) 10 nM 13-*cis*-locked.

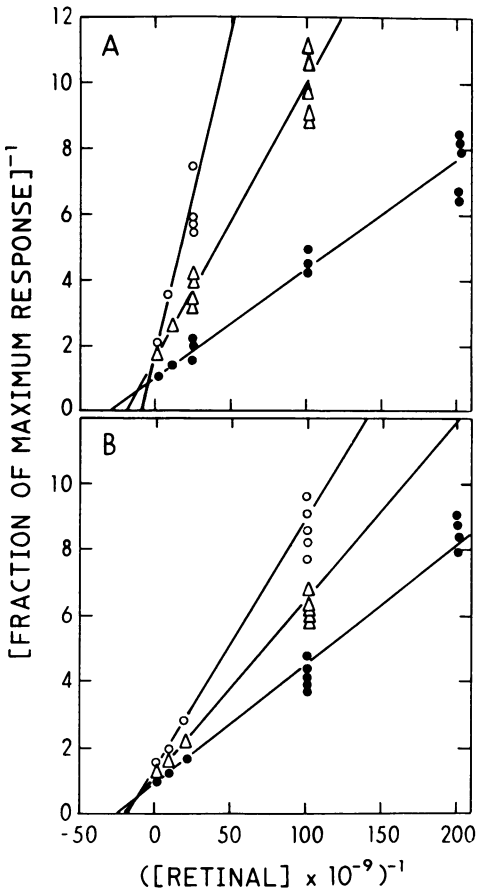

FIGURE 8 (*A*) Double reciprocal plot of response vs retinal concentration. Suspensions of CC-2359 cells were incubated overnight with all-*trans* retinal ranging from 5×10^{-2}–1 nM and 1 nM (△), 2 nM (○) 13-*trans*-locked analog or methanol (●) as a control. Stimulus was a 5 ms flash of 3.6×10^4 ergs/cm^2 s of 500-nm light. Points below 5×10^{-2} nM retinal consist of 4–5 nearly superimposable values for which the average is plotted. (*B*) Same as in 8*A* with inhibitor concentrations of 3 nM (△) or 6 nM (○) 13-*cis* locked analog or methanol as a control (●). Points below 1×10^{-3} nM consist of 4–5 nearly superimposable values for which the average is plotted.

Determination of apparent dissociation constants for all-*trans* retinal and retinal analog binding to the photoreceptor apoprotein

The extent of photophobic response restoration depends on the concentration of retinal and the response saturation curves are shifted by addition of nonisomerizable inhibitors. Saturation curves in the presence or absence of inhibitors can be well fit to straight lines on a double reciprocal plot (Fig. 8, *A* and *B*). Considering first the data in the absence of inhibitors, the linear fits suggest modeling this system as a simple one-ligand/one-receptor binding process, so that:

$$1/R = 1/R_{max} + (K_{app}/R_{max})(1/[\text{ret}]), \qquad (1)$$

where R = the fraction of maximum response, R_{max} = the response generated by incubation with 1 μM retinal in the absence of inhibitor, [ret] = the exogenous concentration of retinal, and K_{app} = the apparent dissociation constant for all-*trans* retinal binding to the receptor apoprotein.

The assumptions implicit in applying equation (1) to our measurements are: (*a*) The retinal and the apoprotein exist in a simple binding equilibrium with a single noncooperative binding constant, (*b*) the response is directly proportional to the concentration of chromophore-bound aproprotein, and (*c*) the local concentration of retinal near the receptor is proportional to that in the medium.

The results show this formulation provides at least a good first approximation, because deviation of the average values from the fit are well within the measurement error of these values in independent runs. The K_{app} for the binding of retinal to the apoprotein, calculated using Eq. 1 is 2.5×10^{-11} M.

To investigate the nature of the inhibition, various concentrations of all-*trans* retinal were added to samples

of CC-2359 containing 1 nM or 2 nM *trans*-locked analog or 3 nM or 6 nM *cis*-locked analog. Three possible competition schemes can be distinguished by the following criteria: (*a*) Competitive inhibition: all lines intersect on the *Y*-axis, and the *Y*-intercept equals the measured $1/R_{max}$. This will occur if the inhibitor competes with all-*trans* retinal for binding with the apoprotein. (*b*) Noncompetitive inhibition: all lines intersect on the *X*-axis. This will occur if the inhibitor binds to the receptor independently of all-*trans* retinal binding. (*c*) Uncompetitive inhibition: all lines are parallel. This will occur if the inhibitor binds only to the retinal-apoprotein complex.

Each of the five data sets (Table 1) was fit without constraint, with a fixed *Y*-intercept, a fixed *X*-intercept, and a fixed slope to determine which type of inhibition best describes the data. The mean sum of the squared residuals was calculated for each case and was used to determine the goodness of fit. For each data set, competition inhibition gave a better fit than did noncompetitive or uncompetitive inhibition (Table 1). Calculated from the mean goodness of fit (Table 1), the competitive inhibition model fits 7-fold and 13-fold better than noncompetitive and uncompetitive inhibition models, respectively.

To determine the apparent inhibition constants (K_i), which can be thought of as apparent dissociation constants for the binding of inhibitor to the receptor, we refit the data of Fig. 8 to a competitive inhibition scheme using the average *Y*-intercept (1.3) from the unconstrained fits in the following equation:

$$1/R = 1.3 + 2.1 \times 10^{-11}\,(1.3)(1 + [\mathrm{I}]/K_i)(1/[\mathrm{ret}]), \quad (2)$$

where *R* equals the fraction of maximum response; [ret] equals the exogenous concentration of retinal; and [I] equals the exogenous concentration of inhibitor.

From Eq. 2, we calculate K_i for the 13-*trans*-locked and 13-*cis*-locked analogs to be 5.21×10^{-10} M and 5.43×10^{-9} M, respectively.

TABLE 1 Goodness of fit of response restoration data with retinal and 13-locked analogs to various inhibition models

	Mean sum of squared residuals			
Inhibitor	Unconstrained	Competitive	Noncompetitive	Uncompetitive
None	3.7×10^{-2}	1.6×10^{-1}	1.3	10.0
1 nM *trans*-locked	1.9×10^{-2}	5.1×10^{-2}	4.0×10^{-1}	6.3×10^{-2}
2 nM *trans*-locked	6.4×10^{-2}	2.3×10^{-1}	1.4	7.3×10^{-1}
3 nM *cis*-locked	3.8×10^{-3}	8.6×10^{-3}	2.4	9.0×10^{-1}
6 nM *cis*-locked	9.4×10^{-3}	9.4×10^{-3}	2.9×10^{-1}	1.2×10^{-2}
Mean	(2.6×10^{-2})	(1.8×10^{-1})	(1.2)	(2.3)

Summary and conclusions

The restoration data presented above indicate the structure of the native chromophore for the *Chlamydomonas* photophobic response receptor is all-*trans* retinal with a 6-s-*trans* geometry 2. Moreover, the lack of restoration by analogs prevented from isomerizing around the 13–14 double bond but demonstrated to enter the receptor binding site, argues for a requirement for all-*trans*/13-*cis* isomerization for the photophobic response. This chromophore structure and isomerization requirement are characteristic of archaebacterial rhodopsins, but have not been observed in any other eukaryotic rhodopsin (namely, invertebrate or vertebrate visual pigments). The extraction of all-*trans* retinal from *Chlamydomonas* (Derguini et al., 1991) and the apparent similarity of the absorption spectrum of a *Chlamydomonas* retinal-binding chromoprotein (Beckmann and Hegemann, 1991) to archaebacterial sensory rhodopsin II (Takahashi et al., 1990) are consistent with an archaebacterial-like chromophore in the receptor(s) of this unicellular eukaryotic alga. It is too early to tell whether these properties of the *Chlamydomonas* photoreceptor derive from evolutionary relatedness to the archaebacterial receptors or from a convergent evolutionary process. An argument favoring the latter is that all-*trans* is the most thermodynamically stable isomer of retinal and its greater availability may have favored its use as a chromophore by microorganisms.

The results reported here extend to the photophobic response the basic finding of Foster et al. (1984, 1988, 1989) that retinal and retinal analogs restore phototaxis in a pigment deficient *Chlamydomonas* mutant. However, the analogs which indicate a requirement for 13-ene isomerization, 13-*trans*-locked 6, 13 *cis*-locked 7, and phenyl-locked retinal 10, restored phototaxis in the measurements of Foster and coworkers but did not restore the photophobic response in our assay. The apparent difference between these results may be possibly explained as follows: (*a*) Different behavioral phenomena were assayed. Foster and coworkers examined the restoration of phototaxis migration in response to continuous directional light stimuli, whereas in this report we measure the restoration of the photophobic reaction to nondirectional light flashes. These two behaviors may be mediated by different photoreceptors and/or by different second messenger systems. (*b*) The assays are applied in different time windows. Phototaxis of cell populations was measured after 10 min of illumination. In contrast, the assay used here monitors the photophobic responses of individual cells over a 2 s period

following 5 or 20 ms light pulses. (*c*) The mutants used were different. For example, the residual sensitivity in the strain FN68 used by Foster and coworkers (1984) is not observed in strain CC-2359 used in this study. The phototaxis sensitivity of FN68 has been demonstrated to increase upon sustained illumination without the addition of exogenous retinal (Foster et al., 1988; Beckmann and Hegemann, 1991). Perhaps some of the analogs enhance this photoregulation system providing a second mechanism by which they could enhance phototaxis sensitivity.

Because our reconstitution analysis is based on an in vivo measurement of responses, the K_{app} and K_i cannot be taken as true microscopic binding constants for the apoprotein and chromophore. Many nonspecific binding events may occur between retinal and different components of the cell, but presumably the response can only be restored upon binding of the chromophore to the photoreceptor. Nonspecific interactions would scale the concentrations required for response restoration. Assuming analogs exhibit the same nonspecific interactions, as expected on the basis of their chemical similarity to retinal, inhibition of reconstitution should occur only at the level of the chromophore binding the photoreceptor. Therefore, the different apparent binding affinities of the various analogs, measured in vivo, can be assumed to reflect proportional differences in analog/apoprotein interactions.

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