All-*trans* retinal constitutes the functional chromophore in *Chlamydomonas* rhodopsin

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ABSTRACT Orientation of the green alga *Chlamydomonas* in light (phototaxis and stop responses) is controlled by a visual system with a rhodopsin as the functional photoreceptor. Here, we present evidence that in *Chlamydomonas* wild-type cells all-*trans* retinal is the predominant isomer and that it is present in amounts similar to that of the rhodopsin itself.

The ability of different retinal isomers and analog compounds to restore photosensitivity in blind *Chlamydomonas* cells (strain CC2359) was tested by means of flash-induced light scattering transients or by measuring phototaxis in a taxigraph. All-*trans* retinal reconstitutes behavioral light responses within one minute, whereas *cis*-isomers require at least 50 × longer incubation times, suggesting that the retinal binding site is specific for all-*trans* retinal. Experiments with 13-demethyl(dm)-retinal and short-chained analogs reveal that only chromophores with a β-methyl group and at least three double bonds in conjugation with the aldehyde mediate function. Because neither 13-dm-retinal, nor 9,12-phenylretinal restores a functional rhodopsin, a *trans*/13-*cis* isomerisation seems to take place in the course of the activation mechanism. We conclude that with respect to its chromophore, *Chlamydomonas* rhodopsin bears a closer resemblence to bacterial rhodopsins than to visual rhodopsins of higher animals.

INTRODUCTION

Rhodopsins not only serve as specialized photoreceptors for vision in higher animals (Boll, 1876, Kühne, 1878), but also as light sensors (sensory rhodopsin, SR I, and phoborhodopsin, SR II) and ion pumps (bacteriorhodopsin, BR, and halorhodopsin, HR) in *Halobacteria* (Oesterhelt und Stoeckenius, 1971, Matsumo-Yagi and Mukohata, 1977, Bogomolni and Spudich, 1982, Takahashi et al., 1985). Recently, a rhodopsin was also found in the green alga *Chlamydomonas* (*Chlamydomonas* rhodopsin, Foster et al., 1984), where it functions as a photoreceptor for behavioral light responses. Most information available on *Chlamydomonas* rhodopsin has been gained from physiological experiments, and only very recently *Chlamydomonas* rhodopsin has been identified spectroscopically in purified membrane preparations (Beckmann and Hegemann, 1991). In previous studies, the retinal binding site of *Chlamydomonas* rhodopsin was probed by supplementation of blind mutant cells (strain FN68) with different retinal isomers and retinal analogs and subsequent testing of phototaxis in a population assay (Foster et al., 1984). For this purpose, cells were placed in a small petridish and the phototactic activity was determined by visual inspection, i.e., by measuring the distance the cells had travelled away from the light within 5 or 10 min of irradiation from one side (Foster et al., 1984; Hegemann et al., 1988). It was reported that several retinal isomers (all-*trans*, 11-*cis* and 9-*cis*) could restore phototaxis when added at high concentrations (10–25 μM). The phototactic sensitivity restored by all-*trans* and 11-*cis* retinal (maxima at 501 and 505 nm) peaked very close to that of wild type cells (maximum at 503 nm), whereas in the presence of 9-*cis* a blue shift was observed (maximum at 488 nm) (Foster et al., 1984). Analog compounds with truncated polyene systems shifted the action spectrum further to the blue. Surprisingly, even compounds with only one (hexenal) or no C=C double bond (hexanal) yielded phototactic responses, and the corresponding action spectra showed opsin shifts as large as 11,000 cm^{-1} (Foster et al., 1988*a*). It was concluded that the length of the conjugated polyene chain in analog compounds has no functional importance other than controlling the position of the absorption spectrum. The observation that locked retinal analogs, unable to isomerize around certain double bonds, could restore phototaxis, suggested that the rhodopsin function does not require isomerisation of a particular C=C double bond (Foster et al., 1989, Nakanishi et al., 1989). Consequently it was proposed that *Chlamydomonas* rhodopsin activation is either a direct consequence of charge redistribution in the excited state, as previously discussed by Salem and Bruckmann for vertebrate rhodopsin (1975), or caused by isomerisation of the C=N bond of the Schiff base (Nakanishi et al., 1989).

Originally, 11-*cis* retinal was favored as the natural

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retinal isomer, because a three times higher final phototactic sensitivity was obtained with 11-*cis* than with all-*trans* retinal (Foster et al., 1984). However, because in the case of retinal analogs, the all-*trans* isomers showed the same or sometimes even higher sensitivity than the corresponding 11-*cis* forms (Nakanishi et al., 1989), the question as to the natural isomer was left open.

Recently, retinoids from *Chlamydomonas* were analyzed by high pressure liquid chromatography (HPLC). Only all-*trans* retinal was found in noticable amounts (Beckmann and Hegemann, 1991). However, this analysis was carried out with cells from the white strain FN68, which are blind if grown in darkness but are sensitized by irradiation with bright green light (Foster et al., 1988*b*). Sensitized FN68 cells were chosen for retinal analysis because they lack the large amounts of chlorophyll and carotenoids present in wild type cells (Krinski and Litvin, 1964). However, the retinal content after irradiation of the cells with bright light may not reflect the natural retinal isomer composition in wild type cells.

Further evidence for all-*trans* retinal being the native chromophore of *Chlamydomonas* rhodopsin was derived from the rhodopsin absorption in purified membrane preparations. The absorption is fine structured which, according to current interpretation, is caused by a rigid and planar all-*trans,* 6-s-*trans* chromophore conformation (Beckmann and Hegemann, 1991; Hegemann, 1991).

Here we present data that identify all-*trans* retinal as the functional chromophore in wild type cells and demonstrate the in vivo reconstitution of *Chlamydomonas* rhodopsin in white strain CC2359 cells with various retinal isomers and analog compounds. Reconstitution was assayed by detection of flash-induced light scattering changes, reflecting alteration of the flagellar activity, or by observation of the negative phototaxis in a taxigraph. Controls were performed in a computer supported motion analysis system. Our results allow a more detailed description of retinal-protein interactions in *Chlamydomonas* rhodopsin and lead to the identification of the minimal chromophoric unit required for physiological function.

MATERIAL AND METHODS

Strain CC2359 cells (Iroshnikova and Kvitko, 1986) were originally provided by the *Chlamydomonas* culture collection (E. Harris, Duke University, Durham), but in most experiments cells were used that had been grown from a clone, which was selected for optimal motility by Moara Lawson in J.L. Spudich's laboratory.

Cells were grown for 8–14 d on high salt acetate (HSA) agar plates (trace elements according to Hutner et al., 1950) and differentiated into gametes for 18 or 36 h in nitrogen deficient minimal medium (NMM pH 6.8, Foster et al., 1984). They were diluted to the appropriate concentration in the same medium. Green strain 806 cells were irradiated during growth and gametogenesis with 2 W m^{-2} "cool fluorescent white light," whereas strain CC2359 cells were always kept in the dark.

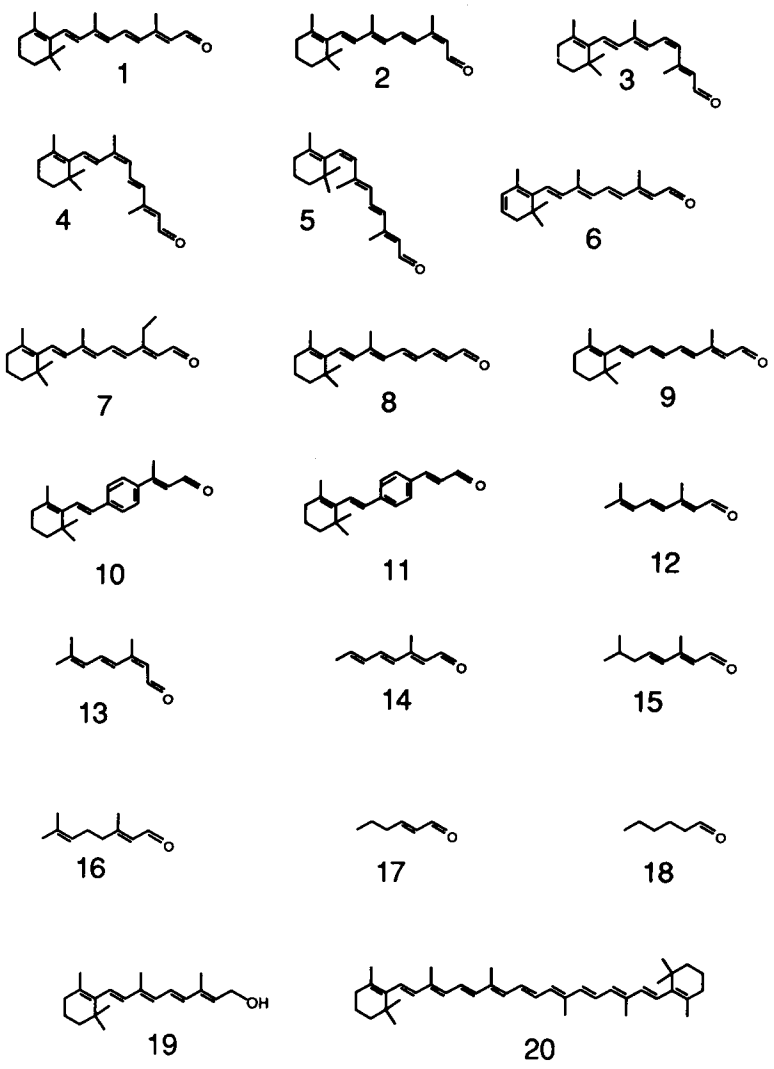
Retinal and retinol (1 and 19, see Table I) were purchased from Fluka Chemical Corp. (Ronkonkoma, NY) citral (16), hexenal (17) and hexanal (18) were from Aldrich Chemical Co. (Milwuakee, WI), and β-carotene (20) from Sigma Chemical Co. (St. Louis, MO). Isomers of retinal (2–5) were produced by irradiating a 40-μM solution of all-*trans* retinal in acetonitrile for 2 min with a 250 W slide projector from 10-cm distance (345 nm cut off filter, and a heat protection filter, 10 W m^{-2}). Retinal isomers were isolated on an HPLC column (Si 60, 5 μm) using 5% ethylacetate in hexane as solvent (v/v). They were always kept in darkness, except when handled under safe red light. Isomers with a purity better than 96% were stored in dry form at −70°C under argon. They were used for up to three weeks and thawed only once. Extinction coefficients were taken from Groenendijk et al. (1980). A_2-retinal (3,4-dehydroretinal, 6) was synthesized from retinal by oxidation with MnO_2 according to protocols from the literature. 13-Ethylretinal (7) was synthesized after standard procedures by an aldol condensation between butanone and "C_{15}"-aldehyde (β-Ionylidene acetaldehyde). The resulting ketone was chain-extended with a C_2-phosphonate via a Wittig-type reaction. Demethylretinals (8 and 9) were synthesized as described (Gärtner et al., 1983). 9,12-phenylretinal (10) was prepared from the corresponding acid (BASF, Ludwigshafen) by DIBAL-reduction and subsequent oxidation of the produced alcohol (Kölling et al., 1984). The 13-demethyl-derivative (11) was provided by the BASF in its final form. Trienal (12) was kindly provided by Dr. E. Kölling (Kölling, 1987). Compound 14 (3-methyl-2,4,6-octatrienal) was synthesized by condensing crotonaldehyde with a C_5-phosphonate (Wittig-type reaction), which yielded the nitrile of the full length compound. This derivative was converted into the aldehyde by DIBAL reduction. 3,7-dimethyl-octadienal (dienal 15) was prepared identically to compound 14 except of using 3-methyl-butyraldehyde as starting compound. All compounds were checked for purity prior to use. All retinal compounds were added to the cells from 1,000-fold concentrated stocks in methanol (HPLC grade).

Pigments were extracted by the methods of Groenendijk, et al. (1980) or Susuki et al. (1986) from ~1 × 10^8 strain-806 cells. Aliquots were applied to a LiChrosorb Si-60 (5 μm) HPLC column and eluted according to Susuki and Makino-Tasaka (1983). The absorbance of the eluate was recorded continuously between 300 and 500 nm with 2-nm resolution by using a multichannel detector (Waters Chromatography Division, Milford, MA) which allowed the generation of spectra during chromatography.

Phototaxis was measured in a taxigraph (Uhl and Hegemann, 1990*b*) at a cell density of 5 × 10^5 cells/ml. Actinic light was selected by single cavity interference filters (8–12 nm half band-width, Schott Mainz, Germany).

Light scattering transients were measured in a multiangle flash photolysis apparatus (Uhl et al., 1985) which detects changes in scattered light intensity at eight different angles simultaneously. Because the light transients were largest at 16°, all measurements presented here were recorded at this angle (Uhl and Hegemann, 1990*a*). Unless indicated otherwise, cells were stimulated with green flashes (499 nm, 8 nm half band-width, single cavity interference filter, Schott, Mainz). The flash duration was 200–400 μs, depending on the photon exposure. Stimulating light reached the cells from the same side as the measuring light (LED, 840 nm). The light scattering amplitude was ~30% of that observed with strain 806 cells, which is probably due to the smaller size, the shorter flagella, and to the presence of ~10% immotile cells, which cannot be removed by photoselection as usually done with wild type cells.

TABLE 1 **Structures of retinoid compounds used in reconstitution experiments**



Control experiments were carried out with a motion analysis system as described in Hegemann and Bruck (1989).

RESULTS

Identification of natural retinoids in green cells

To reach maximal phototactic sensitivity, green cells (strain 806) were grown in white light of low photon irradiance (Hegemann et al., 1988). After over night differentiation of vegetative cells into gametes, organic compounds were extracted and analyzed by HPLC (Fig. 1). The results are in agreement with previously reported data on the retinal content of white strain FN68 cells, which had been resensitized by exposure to green light (Beckmann and Hegemann, 1991). The new results allow to extend previous conclusions now to *Chlamydomonas* wild type cells: (*a*) All-*trans* retinal is the dominant retinal isomer. 13-*cis* retinal, not found in white cells, is present in small amounts in green cells. (*b*) The total number of retinal molecules per cell is ~30,000, close to the estimated number of rhodopsin (Melkonian and Robenek, 1984, Smyth et al., 1988). (*c*) Retinol is present to a variable extent, which strongly depends on the light conditions during gametogenesis. Its concentration shows no direct correlation with the phototactic sensitivity of the cells (data not shown). Excess retinoids are apparently stored as retinol.

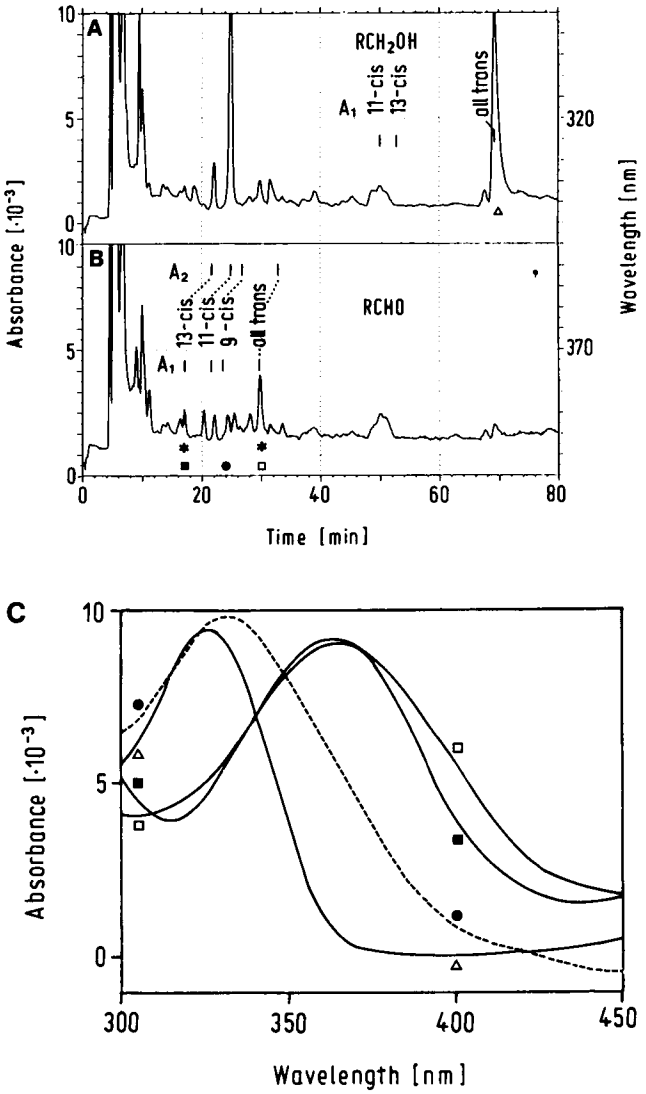


FIGURE 1 HPLC analysis of a methanolic extract of strain 806 cells. Retinol compounds were measured at 320 nm (*A*) whereas retinal was detected at 370 nm (*B*). The retention time of retinal (A1) and 3,4-dehydroretinal (A2) isomers are indicated. Only compounds marked with * showed a retinal absorption spectrum. Spectra of the compounds eluting at the position of 13-*cis* retinal (■), all-*trans*-retinal (□) and of all-*trans*-retinol (△) are shown in (*C*).

Flash Induced responses in pigment deficient cells

Reconstitution with retinal isomers

Strain CC2359 cells are phenotypically similar to FN68 cells, i.e., they are almost completely blind if grown in darkness. They lack retinal because the biosynthesis of the precursor molecule, β-carotene, is blocked (Iroshnikova and Kvitko, 1986). Visual sensitivity is restored by exogenous retinal compounds. Reconstitution of functional rhodopsin can be assayed either by observing flash-induced stop responses and direction changes, or by detection of phototaxis in continuous light. Light responses to a pulsed stimulus yield a better time resolution than phototaxis experiments and are thus preferable in reconstitution experiments. They can either be studied under the microscope, most conveniently with a computer supported motion analysis system (Hegemann and Bruck, 1989), or by detection of the altered flagellar beating in a cell population (Uhl and Hegemann, 1990*a*), which gives rise to transient light scattering changes (LS-signals) in a multiangle flash photolysis apparatus (Uhl et al., 1985). For reasons of better time resolution, higher reproducibility, and easier data analysis, light-scattering experiments were preferably used. Whenever controls were carried out using motion analysis, similar results were obtained.

Dark grown CC2359 cells exhibit no behavioral light responses to green flashes of light (499 nm), even at exposures as high as 10^{20} photons m^{-2}. Because the threshold for light responses in green strain 806 cells lies below 10^{15} photons m^{-2} (Uhl and Hegemann, 1990*a*), the light sensitivity of these blind cells is more than 10^5-fold lower than in green cells. When cells are incubated with all-*trans* retinal, within 1 min light responses appear, which are very similar to those of green strain 806 cells (Fig. 2). The light scattering transients reflecting these light responses are graded with photon exposure over more than three orders of magnitude, i.e., their shape, sign and amplitude is altered with increasing flash brightness. Negative LS signals (b-wave), observed at lower photon irradiances, reflect direction changes caused by a transient inactivation of only one flagellum (Kamiya and Witman, 1984), whereas the positive LS-signal (c-wave), appearing at higher exposure, reflects the stop response, during which the cells switch from forward to slow backward swimming for some hundred ms (Schmidt and Eckert, 1976). The light responses of white cells are apparently well synchronized, thus allowing to determine both the response time (t_R), i.e., the time between flash and first reversal, and of t_{RS}, the time of slow backward swimming, from the time difference between the two c-wave maxima (Fig. 2). Their values are t_R = 150 ms and t_{RS} = 250 ms, respectively.

With all-*trans* retinal the reconstitution kinetics were almost independent of the retinal concentration as long as it did not drop below 1 nM. Assuming a number of 30,000 rhodopsin molecules per cell, a cell density of 2×10^6 cells/ml, and a retinal concentration of 1 nM implies a 10:1 stochiometry. Remarkably, even at this low concentration full sensitivity was restored within 1 min, suggesting that the rhodopsin is readily accessible from the extracellular medium. This favors the eyespot overlaying part of the plasmamembrane over the outer

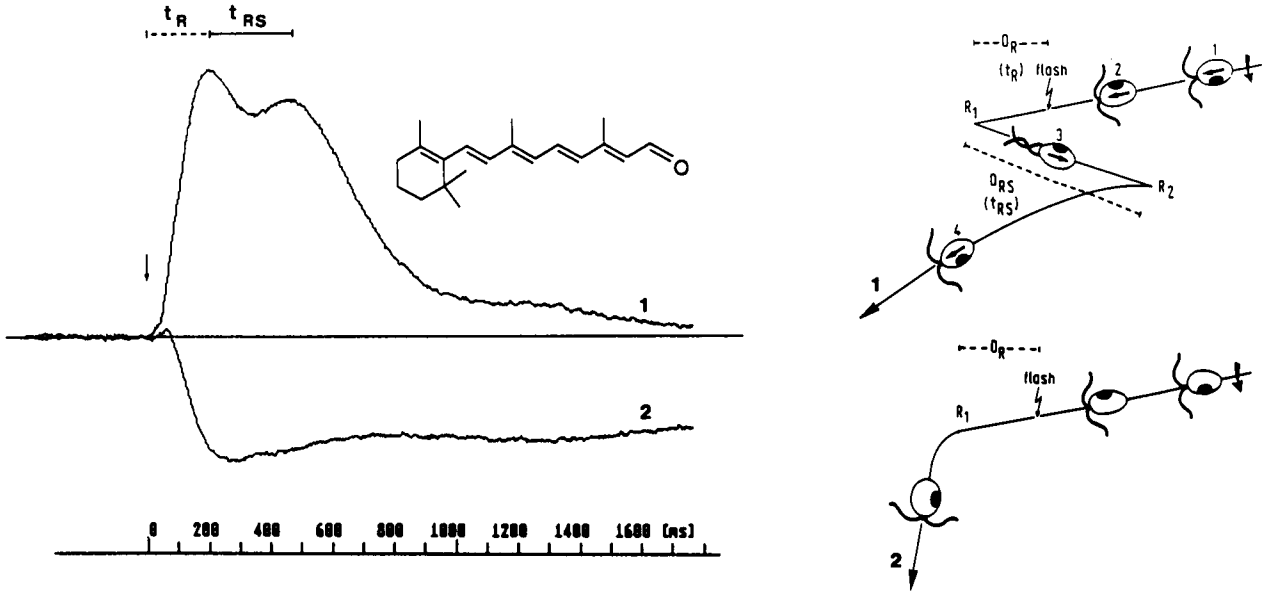


FIGURE 2 Transient light scattering changes from CC2359 cells reconstituted with 100-nm retinal in response to green flashes of two characteristic photon exposures (2.8×10^{16} for trace 1 and 5.6×10^{17} photons m^{-2} for trace 2, 499 nm). Positive LS-signals reflect a stop response whereas a negative signal reflects direction changes. The cell movement during these two responses is explained on the right. The time between flash and reversal or direction change is the response time, t_R, and D_R is the distance the cells travelled during this time. D_{RS} is the distance between the two reversal points R_1 and R_2. The time between the two reversals is the reverse swimming time, t_{RS}.

chloroplast membrane as the location of the rhodopsin in the cell (for discussion see Foster and Smyth, 1980, and Melkonian and Robenek, 1984).

When retinal concentrations <1 nM were used, both reconstitution kinetics and final sensitivity declined. The half saturating concentration was near 0.4 nM for the all-*trans* isomer at a cell density of 2×10^6 cells/ml. At low cell concentrations the half saturating concentration was even lower. To keep the concentration above the critical level, concentrations of 10–100 nM were used in most experiments with unmodified retinal. This is still three orders of magnitude lower than what has been used in former phototaxis experiments (Foster et al., 1984, 1988*a*, 1989).

Mono-*cis* retinal isomers are also able to reconstitute light responses, but reconstitution kinetics are more than 50 × slower than with all-*trans,* taking several hours for 9-*cis* or 7-*cis* retinal (Fig. 3 *a* and Table 2). The relative reconstitution rates were all-*trans* ≫ 13-*cis* > 11-*cis* > 9-*cis* > 7-*cis*. Note that these kinetic differences were only observed at low retinal concentrations (1–10 nM). With all *cis* isomers a small activity (<5% of the maximum at 10 nM), appearing as a small negative LS-signal, was observed immediately after addition (Fig. 3). Its fraction increased with the total concentration of added isomer, probably reflecting the presence of increasing amounts of all-*trans* retinal impurities. When during the remaining slow phase of reconstitution all-*trans* retinal was added, maximal activity was attained instantaneously (Fig. 3 *a*). This suggests that the interaction between *cis*-isomers and the opsin is either very slow or rapidly reversible. Another way to accellerate the slow reconstitution process in the presence of *cis*-compounds is by irradiation of the cells with blue light (380 nm), producing a mixture of several isomers, predominantly all-*trans*. Green light of 520 nm, which is only absorbed by rhodopsin, is inefficient (data not shown).

The final light sensitivity of the cells, reached after an over night incubation, was within a factor of two the same for all isomers. Likewise, the shape of the light scattering responses was the same. The action spectra for the stop response (light scattering c-wave) were clearly rhodopsin-shaped and very similar to that of green strain 806 cells (Uhl and Hegemann, 1990*a*). Variations in λ_{max} between 480 and 500 nm are most likely due to experimental variations.

Precursors of all-*trans*-retinal, i.e., all-*trans*-retinol (19) or β-carotene (20), reconstitute behavioral responses, but only with slower kinetics than all-*trans* retinal itself. Reconstitution in the presence of these two compounds seems to require enzymatic processes like the oxydation by a dihydrogenase or oxidative cleavage by a dioxygenase.

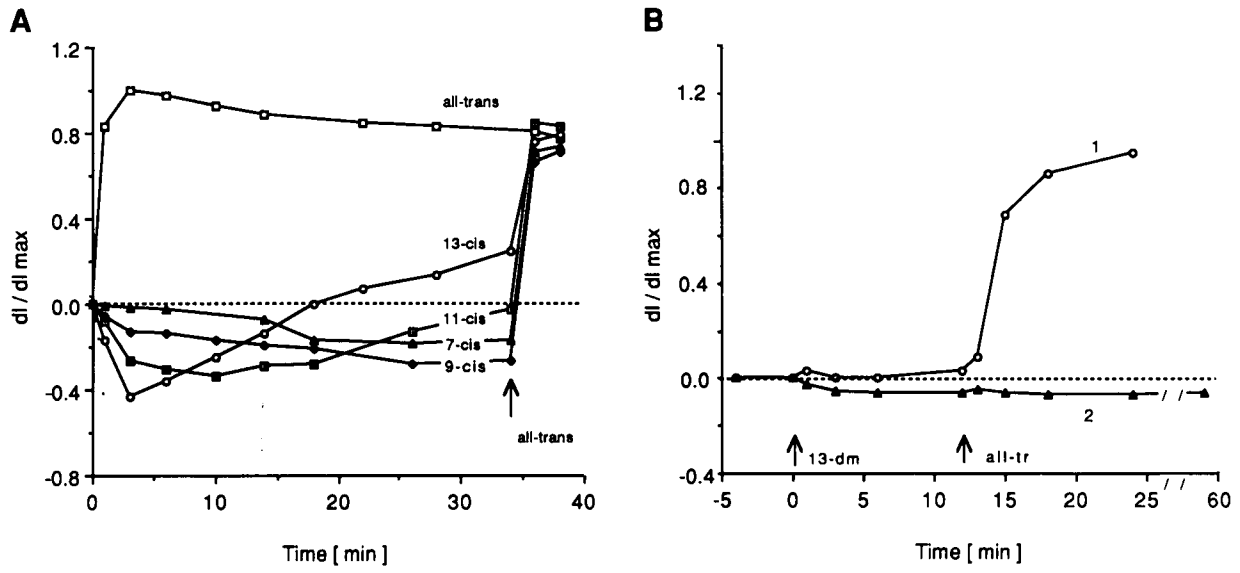


FIGURE 3 (*A*) Reconstitution kinetics of light scattering transients after addition of the various retinal isomers at 1 nM final concentration to CC2359 cells. (481 nm flash, 4.8×10^{17} photons m^{-2}). After 34 min, 10 nm all-*trans*-retinal was added to each sample. (*B*) Light scattering changes in presence of 100 nm (1) or 1 μM (2) 13-dm-retinal in response to flashes of 5.4×10^{16} photons m^{-2} (481 nm). After 12 min, 100 nM all-*trans*-retinal was added. The LS-amplitudes 200 ms after the flash are plotted versus the reconstitution time.

TABLE 2 Results from reconstitution with retinal and analog compounds

Compound	Stop*	*t* 1/2 (conc.)	Phototaxis	Action spectra/Lit. max. (Lit)
1	+	1′ (1nM)	+	501nm/Foster et al., 1984
2	+	35′ (1nM)	(+)	
3	+	60′ (1nM)	(+)	505nm/Foster et al., 1984
4	+	190′ (1nM)	(+)	488nm/Foster et al., 1984
5	+	190′ (1nM)	(+)	
6	+	10′ (1nM)	(+)	511nm/Foster et al., 1984
		1′ (10nM)		
7	+	4′ (100nM)	+	
8	—	10′ (100nM)#	—	
9	+	8′ (100nM)	n.d.	
10	—	<1h (10μM)#	—	476nm/Foster et al., 1988[a]
11	—	<4h (10μM)#	—	
12	+	10′ (2μM)	+	459nm/Foster et al., 1988[a]
13	+	12′ (2μM)	+	
14	—	— (40μM)	n.d.	
15	—	— (100μM)	n.d.	
16	—	— (100μM)	—	
17	—	— (100μM)	—	354nm/Foster et al., 1988[a]
18	—	— (100μM)	—	339nm/Foster et al., 1988[a]
19	+	6′ (100nM)	+	
20	+	4′ (100nM)	+	

*Stop response was tested with all compounds between 360 and 500 nm; # indicates tested by competition experiments.

Reconstitution with compounds of modified retinal structure

The all-*trans* configuration of the chromophore suggests a closer relation of *Chlamydomonas* rhodopsin to bacterial rhodopsins than to vertebrate or invertebrate visual rhodopsins. To elucidate structural requirements of the chromophore with respect to its ability to trigger behavioral light responses, a series of retinal analog compounds was tested.

In BR, the 13-methyl group plays an important role for 13–14 isomerisation and for the exact fit of the retinal in the binding site. This was demonstrated by experiments with retinal derivatives carrying substituents of varying size at position 13 (Gärtner et al., 1988). 13-demethyl(dm)-retinal (8) is a particularly interesting example, because it reconstitutes a bacterorhodopsin with almost unchanged absorption spectrum but low transport activity (Gärtner et al., 1983). According to a brief abstract, the chloride pump halorhodopsin (HR) behaves analogously (Iwasa and Oesterhelt, 1987). We have applied 13-dm-retinal (8) to *Chlamydomonas* CC2359 cells and found no reconstitution of any flash-induced response, independent of the time of incubation (Fig. 3 *b*). Subsequent addition of all-*trans* retinal gave no immediate functional reconstitution. Instead, long incubation times ranging from many minutes to hours were required, the time depending on the excess of retinal over 13-dm-retinal. We conclude that although 13-dm-retinal fails to restore light sensitivity, it must be

able to occupy the rhodopsin binding site and is only slowly replaced by all-*trans* retinal.

The removal of the methyl group from position 13 of a protonated retinal Schiff base has a marked influence on the 13–14 bond length and its rotation energy barrier, but leaves the other double bonds almost unchanged (Tavan et al., 1985). Thus, the observation that 13-dm-retinal is functionally ineffective suggests that rotation around the 13–14 bond is important for activation of *Chlamydomonas* rhodopsin. To prove the proposed electronic interaction and to distinguish between an electronic and a steric influence of the 13-substituent, experiments were carried out with 13-ethyl-retinal (7), in which the 13–14 rotation energy barrier is kept unchanged or even slightly lowered compared to normal retinal (Tavan et al., 1985). 13-ethyl-retinal restored light responses within a few minutes. The maximal sensitivity was 5 × lower and the action spectrum was slightly blue shifted by ~30 nm.

9-dm retinal (9), on the other hand, similar as in BR (Gärtner et al., 1983), mediated normal function after slow reconstitution. The peak sensitivity was found near 465 nm, i.e., 30 nm blue shifted compared to the native photoreceptor. Compared to the 13-methyl group, the 9-methyl substituent seems to be of less functional importance in *Chlamydomonas* rhodopsin. Apparently it plays a role for the proper fit of the retinal in the binding site, thereby influencing the retinal affinity.

The conclusion that photoisomerisation is a critical step in the sensory transduction chain of *Chlamydomonas* is supported by the lack of function in the presence of 9,12-phenylretinal (10) and its 13-dm derivative (11). In (10) the phenylring blocks the 9–10 and 11–12 double bonds and, for sterical reasons, also prevents isomerization to an undistorted 13-*cis*. In 11, similarly as in 13-dm-retinal, a planar 13-*cis* conformation is impossible, and thermal 13-*cis*/all-*trans* rotation is hindered. Consequently, as expected for a mechanism involving 13–14 isomerisation, no light responses were found after incubation with these two phenylretinal derivatives. However, both compounds enter the rhodopsin binding site as shown by competition experiments with all-*trans* retinal.

Short chained retinal analogs

The hexenylring can be omitted from the retinal without detrimental effects for its function. Dimethyl-octatrienal (12), merely consisting of the retinal backbone from position 8 to the aldehyde group, i.e., containing three double bonds in conjugation with the aldehyde, was the shortest compound to restore normal function (Fig. 4 *a*). However, micromolar concentrations (k_m = 1 μM) were needed instead of nM concentrations as with full-length analogs (Table 2). The reconstitution time course was

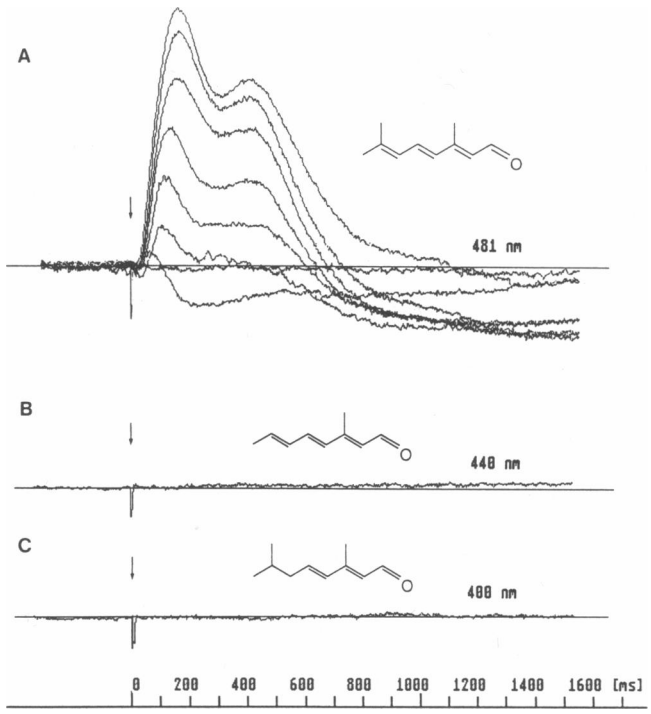


FIGURE 4 Transient light scattering changes from CC2359 cells (*A*) to flashes of 481 nm (6.8×10^{17} photons m-2) at 0, 1, 3, 6, 12, 24, 30, and 40 min after addition of 3.4 μM dimethyl-trienal (12), (*B* and *C*) responses in presence of 40 μM methyl-trienal (14) and 100 μM dimethyldienal (15) to flashes of 440 and 400 nm, respectively (4.8×10^{16} and 4.1×10^{16} photons m^{-2}).

clearly slowed down, but was very similar for the all-*trans* (12) and the *cis* isomer (13). In both cases, the cells showed maximal sensitivity around 470 nm, in close agreement with earlier phototaxis measurements with 12 (Foster et al., 1989, Table 2).

Removing the methyl group from position 9 (according to retinal numbering) of derivative 12 yields trienal 14, which still contains three double bonds. This compound, however, completely failed to restore light responses (see Fig. 4 *b*). Analog compounds with less than three double bonds in conjugation with the aldehyde function were also ineffective, no matter if the 13-related methyl group (15–18) was present or not. This was tested with flashes of 360, 400, 464, or 481 nm light, an incubation time of 3 h, and drug concentrations of up to 100 μM. These short chained compounds, including hexenal (17) or hexanal (18), did neither enter the rhodopsin binding site, nor influence its behavior in any way. In all cases subsequent addition of 10 nM all-*trans* retinal mediated full resensitization within one or two minutes. These results emphasize the importance of the above described structural requirements, including the

9-methyl group, for the formation of the *Chlamydomonas* rhodopsin chromophore.

PHOTOTAXIS

Because it is conceivable that flash-induced stop responses and phototaxis, i.e., the net movement of the cells to or away from continuous light, are regulated by different rhodopsin-type photoreceptors, the ability of the retinal compounds to restore phototaxis was tested in a separate set of experiments.

Phototaxis was assayed in a semiautomatic taxigraph by optical measurement of the cell concentration in the front and the rear half of a cuvette, which was irradiated from one side (Uhl and Hegemann, 1990 *b*). This allows to determine the net movement of cells towards or away from light of variable photon irradiance. Because phototaxis has to be observed for an extended time period, phototaxis measurements are not well suited for the determination of reconstitution kinetics in the minute range. Therefore phototaxis was tested after the cells had equilibrated with the retinal compound, usually after 3 to 5 h. As seen from Fig. 5, CC2359 cells switch from negative to positive phototaxis at irradiances only ~100-fold higher than threshold. The switch depends on the wavelength and the duration of the stimulating light, on the pH and the oxygen concentration in the medium (Nultsch, 1977, and P. Hegemann unpublished observation). In fully reconstituted cells, switching commences as early as 80 s after turning the light on, when 5 $\mu W/cm^2$ of 500-nm light is used (Fig. 5). As a quantitative measure of phototaxis, the initial phototactic rate, i.e., the average phototactic rate within the 60 s after light on, was plotted against the stimulus photon irradiance (Fig. 6).

The phototactic sensitivities of strain CC2359 cells incubated with different isomers of retinal (1–5), with retinol (19), β-carotene (20), with 3-en-al (12) or 9-dm-retinal (9) were all similar. They fell within one log unit when light of 481 nm was used for stimulation (shown in Fig. 6). 13-dm-retinal (8), phenylretinals (10 and 11) and the short chain analogs (14 to 17) could not restore phototaxis when tested with actinic light between 360 and 550 nm.

The above phototaxis experiments lend further support to the conclusions drawn from flash-induced LS-responses: (*a*) all-*trans* retinal is the most efficient retinoid and (*b*) the 13-methyl group of the retinal and at least three double bonds in conjugation with the aldehyde function are essential for an analog compound to form a phototactically active rhodopsin. The core part of the retinal molecule which is pivotal for its physiological function in *Chlamydomonas* rhodopsin is emphasized by bold lines in Fig. 7.

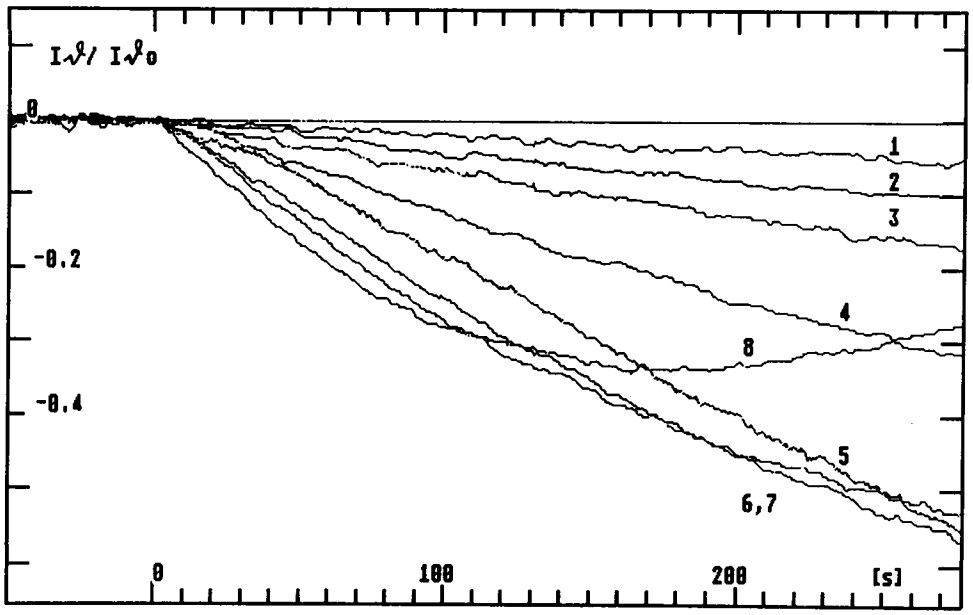


FIGURE 5 Phototaxis of CC2359 cells away from light of 500 nm at photon irradiances between 6.3×10^{14} and 1.3×10^{17} photons $m^{-2} s^{-1}$ at 100 nM all-*trans* retinal. The irradiance was varied in steps of 2.

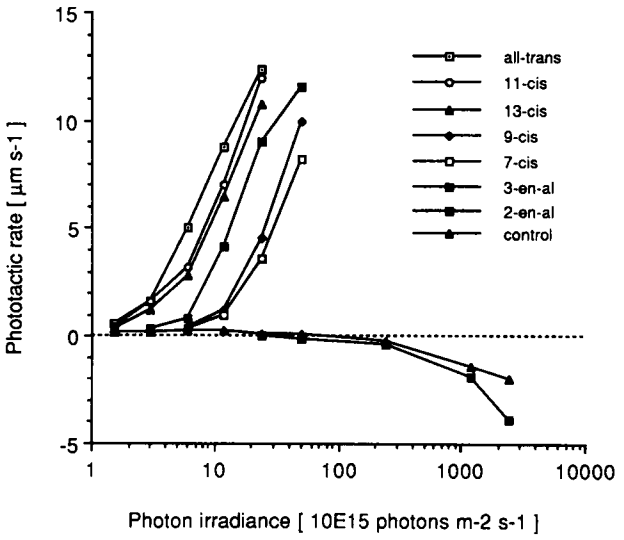


FIGURE 6 Phototaxis of strain CC2359 cells before and after reconstitution with various retinal compounds, which are: 1 nM of all-*trans* (1), 13-*cis* (2), 11-*cis* (3), 9-*cis* (4), 10 nm 7-*cis* retinal (5), 3 µM dimethyl-trienal (12 3-en-al), or 100 µM dimethyl-dien-al (15). The average phototactic rate during 1 min after light on is plotted versus the stimulus photon irradiance. The wavelength of the stimulation light was 492 nm in all cases except for compound (15), where 440 nm was used.

DISCUSSION

The predominance of all-*trans* retinal in *Chlamydomonas* rhodopsin has been previously concluded from a retinal analysis of light-sensitized white strain cells, from reconstitution of rhodopsin in membrane preparations, and from the fine structured rhodopsin absorption spectrum (Beckmann and Hegemann, 1991). Here we provide evidence that the same holds true for wild type cells. The functional identification of all-*trans* retinal as the native chromophore became feasible due to an improved reconstitution assay which allows to work with exceedingly low retinal concentrations and to resolve rapid reconstitution kinetics. Furthermore, because the assay probes an intact in vivo system, studies using modified retinal compounds permit to probe structural requirements for a functional *Chlamydomonas* rhodopsin chromophore and to compare these with those of other retinal proteins.

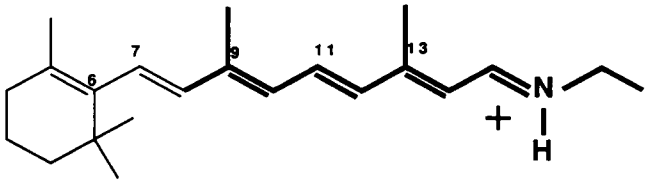


FIGURE 7 Structure of the *Chlamydomonas* rhodopsin chromophore as derived from absorption spectroscopy and from the behavioral studies presented above. The bold part of the retinal shows the minimal chromophore that formed a functional rhodopsin in our hands when CC2359 cells were used.

With its preference for the all-*trans* configuration and its sensitivity towards changes in the region around the C_{13} position of the chromophore, *Chlamydomonas* rhodopsin appears to be closer related to bacterial rhodopsins than to visual rhodopsins from higher animals. The greatest similarity seems to exist between *Chlamydomonas* rhodopsin and phoborhodopsin (SR II), the photoreceptor for repellent photomovement responses to blue-green light in *Halobacteria.* Both contain an all-*trans* and 6-s-*trans* retinal chromophore (Foster et al., 1987; Takahashi et al., 1990; Beckmann and Hegemann, 1991), both exhibit a fine structured absorption spectrum not found in any other rhodopsin, and both absorb at very similar wavelengths (maxima at 487 vs. 495 nm; Takahashi et al., 1990, Beckmann and Hegemann, 1991).

The analogy between bacterial rhodopsins and *Chlamydomonas* rhodopsin is further emphasized by the finding that 9-dm-retinal (9) is functional in these two systems, whereas it fails to restore sensitivity in bovine rhodopsin, where it forms a chromophore but cannot initiate the visual transduction process (Ganter et al., 1989). This observation is understood from a much greater influence of the 9-methyl group on a C_{11}–C_{12} isomerisation (sterically and electronically), as it takes place in metazoan rhodopsins, than on a 13–14 isomerisation. The 13-methyl group, on the other hand, has only a minor influence on the C_{11}–C_{12} rotation but an impact on 13–14 isomerisation. Consequently, 13-dm retinal (8) reconstitutes functional bovine rhodopsin (Ebrey et al., 1980). In bacterial rhodopsins, 13-dm-retinal also enters the binding pocket and slowly forms spectroscopically normal rhodopsins. In the case of the ion pump BR, ion transport activity of the *trans* form is restored with the stochiometry of the native system, i.e., 1 proton per cycle (Fendler et al., 1987), but the photocycle is slowed down. The slow cycling together with a higher percentage of the *cis* form leads to a low transport activity and a low photophosphorylation rate in constant light (Gärtner et al., 1983). In the *halobacterial* light sensor SRI, the reconstituted 13-dm-SR is not functional, i.e., it shows neither photocycling nor phototactic activity, although the chromophore is 100% *trans* (Yan et al., 1990 and J. L. Spudich, personal communication). The similarity between SR and *Chlamydomonas* rhodopsin, where 13-dm-retinal enters the binding pocket without restor-

ing function, suggests a similar activation mechanism including *trans/cis* isomerisation for both light sensors.

Because a correct interpretation of the above result would obviously be an important piece in the puzzle of how *Chlamydomonas* rhodopsin is light activated, we would like to discuss several factors possibly responsible for the above observations.

(*a*) Electronic effects: from studies of the BR photocycle it is known that the primary process, an all-*trans* to 13-*cis* isomerisation, is only very little affected by alteration of the 13-substituent (Trissl and Gärtner, 1987), i.e., the energy barriers in the excited state are not altered. The thermal backreaction with its *cis/trans* back isomerisation, on the other hand, is slowed down by a factor 3 to 5 (Fendler et al., 1987, Gärtner et al., 1988). This is explained by an increased ground state energy barrier for a C=C rotation around the 13–14 double bond in a protonated 13-dm-retinal Schiff base. According to quantum chemical calculations (MNDOC), this barrier should be raised from 11.5 to 16.4 kcal/mol, leading to a retarded reisomerisation around the 13, 14 bond and hence a slowing of the photocycle by a factor of 1,000 (Tavan et al., 1985). A similar retardation of the *Chlamydomonas* photocycle by a factor of ~1,000 could explain the observed lack of light responses in continuous light (phototaxis), but not the lack of response to flashes of light applied after long dark adaptation. To explain this, other (sterical) factors need to be considered.

(*b*) Steric effects: BR of reconstitution with retinal proceeds via several precursors, which can be trapped at low temperature (Schreckenbach et al., 1978). The final transition of this reconstitution process, the formation of a fully red shifted rhodopsin, is the rate limiting step. With dm-derivatives this step is slowed down considerably. If similar precursors existed in *Chlamydomonas* rhodopsin, sterical factors could preclude the final transition or make it exceedingly slow (>24 h for compound 8), thus explaining the lack of reconstitution with all-*trans* retinal after preincubation with 13-dm-retinal. Alternatively, 13-dm retinal could restore a red shifted rhodopsin, but retinal-protein coupling in the photocycle could be blocked by the missing 13-substituent. Having no spectroscopic assay at hand yet, we cannot decide between the two possibilities but from the similarity to the SR system we would rather believe that the second alternative is correct. This would assign the 13-methyl group an important relay function for retinal-protein coupling in sensory systems not found in ion pumps.

If the 13-methyl group is replaced by ethyl rather than by a hydrogen atom, the rotation energy barrier for the 13–14 bond of a protonated Schiff base is lowered (10.3 instead of 11.5 kcal/mol, Tavan et al., 1985). Consequently, a normal or slightly favoured 13-cis to trans transition is expected. However, due to the interaction of the bulkier ethyl-group with the C_{15} hydrogen, the *trans* state is energetically less favorable compared to 13-*cis* ($E_{t\text{-}c}$ +1.2 instead of 1.0 kcal/mol), which should lead to an increased 13-*cis* fraction in 13-ethyl-rhodopsin. The latter would explain the lowered light sensitivity of *Chlamydomonas* cells in presence of this analog.

As in BR and HR, the phenyl-retinals 10 and 11 cannot reconstitute an active *Chlamydomonas* rhodopsin. In organic solvents 9,12-phenyl-retinal (10) can photoisomerise from the all-*trans* to 13-*cis* and 7-*cis* forms (Kölling et al., 1984). 13-*cis* formation, however, introduces a steric hindrance between the hydrogen atoms at C-11 and C-15, which enforces a distorted conformation and prevents the molecule from becoming planar. As a consequence, the 13-*cis* isomer cannot reconstitute BR nor is a 13-*cis* formed upon irradiation from the reconstituted all-*trans* state (Kölling et al., 1984, Polland et al., 1984). Because the absorption of *Chlamydomonas* rhodopsin in purified membranes is fine structured, a planar retinal conformation has been suggested. Therefore it is conceivable that a planar retinal binding site does not accept a twisted 13-*cis* retinal and renders the 9,12-phenyl-rhodopsin inactive.

The similarity between *Chlamydomonas*-rhodopsin and bacterial retinal proteins is further emphasized by the finding that the activity is drastically diminished if the number of C=C double bonds in conjugation with the aldehyde function drops below a critical number. Its value is four in bacterial rhodopsins and three in *Chlamydomonas* rhodopsin. Dimethyl-octatrienal (12) or 7,8-dihydro-retinal (Spudich et al., 1986) form BRs with proton transport activities of only 1% or less (Kölling, 1987). In *Chlamydomonas,* the activity drops to <1% if the polyene system of the chromophore is shortened from three to two C=C bonds. Whereas in retinals with regular number of conjugated double bonds the 9-methyl group appears to be of less functional significance, with short analogs both methyl groups in the polyene chain exhibit an essential contribution to the chromophore function. The 9-methyl group seems to keep the shortened polyene system in the proper position of the binding site. In case of the full length retinal structure, this is achieved by the ring. The importance of the ring structure is further demonstrated by the slow reconstitution of all-*trans*-3,4-dehydroretinal (A_2 retinal [6]).

COMPARISON WITH PREVIOUS BEHAVIOURAL STUDIES

The above results show that all-*trans* retinal is the chromophore in *Chlamydomonas* rhodopsin and suggest

that *cis*-isomers do not enter the binding site. Moreover, they point to a *trans*/13-*cis* isomerisation as an essential constituent of the activation process, similarly as found for the sensory rhodopsins of *Halobacteria* (Yan et al., 1990).

With respect to all three conclusions we are in close agreement with preliminary results from another laboratory (Zacks et al., 1991), which studied the stop response of the same mutant.

However, there are major discrepancies between our results and previous studies by Foster and colleagues, who measured phototaxis and used a different mutant (strain FN68, summarized in Nakanishi et al., 1989). They proposed that the chromophore is 11-*cis,* that various *cis*- and the all-*trans* form of retinal enter the binding site within 10 min, yielding different rhodopsin species, and that no isomerisation is necessary for excitation. It cannot be ruled out that the discrepancies are due to the use of different mutants, but we find this explanation unlikely. In the following we discuss some of the conflicting points and attempt to resolve at least part of the discrepancies.

(*a*) The original proposal that 11-*cis* is the native isomer (Foster et al., 1984) was based on a three times higher sensitivity of FN-68 cells reconstituted with 11-*cis* retinal compared with that in presence of all-*trans.* In these studies, cells were incubated with 25μM of the respective retinal-isomer. In our hands 1 nM, i.e., 25,000× less, of the *trans* isomer suffices for rapid reconstitution, and 10–100 nM were used routinely. Equally low concentrations of *cis*-isomers, on the other hand, require greatly increased incubation times (Fig. 3*A*), and the addition of all-*trans* retinal on top of a *cis*-isomer, increases the rate of reconstitution to the value found with all-*trans* retinal alone. Thus the specificity for all-*trans* appears so high that at 25 μM even small contaminations with all-*trans* should allow full reconstitution. It appears impossible to rule out that 25 μM contain nM impurities of all-*trans.*

(*b*) The peaks of the published action spectra for *Chlamydomonas,* rhodopsin reconstituted with all *trans,* (501 nm), 11-*cis*- (503 nm), and 9-*cis* retinal (488 nm) are remarkably close to the absorption maximum of the rhodopsin in purified membranes (495 nm, Beckmann and Hegemann, 1991). With the experimental accuracies of our assays we would not be able to rule out that it is the same chromophore in all cases which is responsible for the successful reconstitution.

(*c*) The third class of discrepancies is not easily understood. In fact, we have not even a hint as to how they may be resolved: in our hands only retinals with three to seven conjugated double bonds can restore sensitivity, both when flash-induced LS-responses or phototaxis are tested. Substances with zero to two double bonds not only show no signs of effectivity, they do not even enter the protein. In previous studies, however, (Foster and Saranak, 1989, Foster et al., 1984, 1988*a*), substances of the latter group were reported to reconstitute excitability, sometimes with a higher effectivity than the native chromophore. Surprisingly, this even holds for hexanal, which has neither a double bond nor a methyl group. Moreover, the opsin shift, which ranges from −0.21 to 0.52 eV (1,660–4,190 cm^{-1}) with compounds having 3–7 double bonds, reportedly assumes values between 0.95 and 1.37 eV (7,630–11,000 cm^{-1}) for these short chained analogs. The fact that this exceeds known values from other rhodopsins by a large margin does not constitute an argument against the validity of the data, however.

From the physiological activity of rhodopsins containing a chromophore unable to photoisomerise, Foster and colleagues have concluded that another intramolecular activation mechanism must exist (Foster and Saranak, 1989, Foster et al., 1988*a*, Nakanishi et al., 1989). They proposed a charge redistribution in the excited state of the chromophore, similar to the mechanism once discussed for vertebrate rhodopsin or BR (Salem and Brukmann, 1975, Lewis, 1978, Warshel, 1978), or an isomerisation of the C=N bond of the Schiff base as possible trigger for signal transduction. Our results, e.g., all-*trans* retinal in dark-adapted rhodopsin, the high sensitivity of the physiological function to modifications of the 13–14 double bond, the influence of ligands at the 13 position, and the requirement for at least three conjugated double bonds, are consistent with a photoisomerisation, most likely from all-*trans* to 13-*cis.* A *trans* to 11-*cis* conversion as known for Cephalopod retinochrome (Hara and Hara, 1982) is less likely. However, we cannot rule out the existence or at least participation of the alternative mechanisms as discussed by Foster and colleagues (Nakanishi et al., 1989).

In conclusion, some discrepancies between the present study and previous ones could be explained by the high retinal concentrations used in earlier studies and the extremely high reconstitution effectivity of all-*trans* reported here. The puzzling differences with respect to the minimum number of double bonds and the necessity for a photoisomerisation remain unresolved. It should be kept in mind, however, that not only the strains were different in the conflicting studies, but also the experimental techniques. But a careful consideration of artifacts and pitfalls possibly connected with the different techniques could not provide any insight. The fact that our results are backed by three different approaches, i.e., measuring light responses to continuous light in a taxigraph and measuring responses to pulses of light using motion analysis and light scattering techniques, makes us confident that artifacts, which should be

different for the three approaches do not greatly distort our conclusions.

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