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Diversion of the sign of phototaxis in a *Chlamydomonas reinhardtii* mutant incorporated with retinal and its analogs

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The blind mutant FN68 of the unicellular flagellate green alga *Chlamydomonas reinhardtii* is negatively phototactic in the presence of the native chromophore all-*trans* retinal. In contrast, analog chromophores such as a ring-acyclic retinal and those in which *trans/cis* isomerization about the C11=C12 double bond was blocked induced predominantly positive phototaxis in the same strain under the same experimental conditions. These observations can be interpreted by assuming that the negative and the positive phototaxis is mediated distinctively by two rhodopsin species which differ in their affinities with the exogenous chromophores. However, a more reasonable explanation, which requires fewer assumptions, is that the sign of phototaxis depends on a delay in intracellular photosignal transduction. This novel view was deduced directly from the widely accepted hypothesis [1980, Microbiol. Rev. 44, 572–630] on phototaxis mechanisms.

Rhodopsin; *Chlamydomonas reinhardtii*; Photoreceptor; Retinal analog; Phototaxis; Eyespot

1. INTRODUCTION

Phototactic flagellate algae orient their movement axis with respect to the direction of environmental light stimulus. In general, the cells show positive phototaxis (swimming toward the light source) to the stimulus at moderate intensities, but at high light intensity they show negative phototaxis (swimming away from the light source). These responses are important for the cells to keep themselves under optimal conditions for both phototropic growth and survival. Attention has been focused on the question as to how these small organisms are capable of precisely detecting the direction of light [1,2].

Using a blind mutant strain of the unicellular green alga *Chlamydomonas reinhardtii*, Foster et al. [3] demonstrated that this eukaryotic alga uses a rhodopsin. This mutant FN68 is itself not phototactic because of its incapability of carotenoid biosynthesis, but showed exclusively negative phototaxis after incubation with retinal (vitamin A aldehyde) or its analogs. Recent studies on another retinal-deficient strain of *C. reinhardtii* showed that all-*trans* retinal is the functional chromophore, which isomerizes its C13=C14 double bond upon photoreception [4–6]. These properties of the chromophore suggest that the *Chlamydomonas* rhodopsin is evolutionarily more closely related to a family of rhodopsins in archaebacteria [7,8] than to visual pigments in animals. Unlike archaebacterial rhodopsins, however, the photoreceptor in *C. reinhardtii* has been suggested to localize in a membrane region close to the organelle called the eyespot [9].

By use of a computer-linked movement analysis system for individual cell tracking [6] in combination with the facilities of the Okazaki Large Spectrograph (OLS) [10], we have investigated photobehavioral responses of the two mutant strains of *C. reinhardtii* incorporated with a variety of retinal isomers and analogs. Here, we show that the FN68 cells exhibit predominantly positive phototaxis when analogs of the chromophore are incorporated.

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Abbreviations: C. reinhardtii, *Chlamydomonas reinhardtii*; OLS, Okazaki Large Spectrograph; NMM, nitrogen-free minimal medium; HPLC, high performance liquid chromatography; 9,11-di*cis*(7)-retinal, 9,11-di*cis* retinal with fixed 11-ene by 7-membered ring; 11-*trans*(5)-retinal, 11-*trans* retinal with fixed 11-ene by 5-membered ring; pentaenal retinal, all-*trans* 3,7,11-trimethyl-10-isopropyldodeca-2,4,6,8,10-pentaenal.

2. MATERIALS AND METHODS

C. reinhardtii strain FN68 was a gift from Kenneth Foster and Juree Saranak (Syracuse University, New York). The cells were grown in the dark in a standard medium [3–6]. Differentiation of the cells into gametes was induced by washing and resuspending the cells with nitrogen-free minimal medium (NMM) [3], followed by mild agitation under fluorescent light (2 W/m^2) for 18 h. Calcium and magnesium

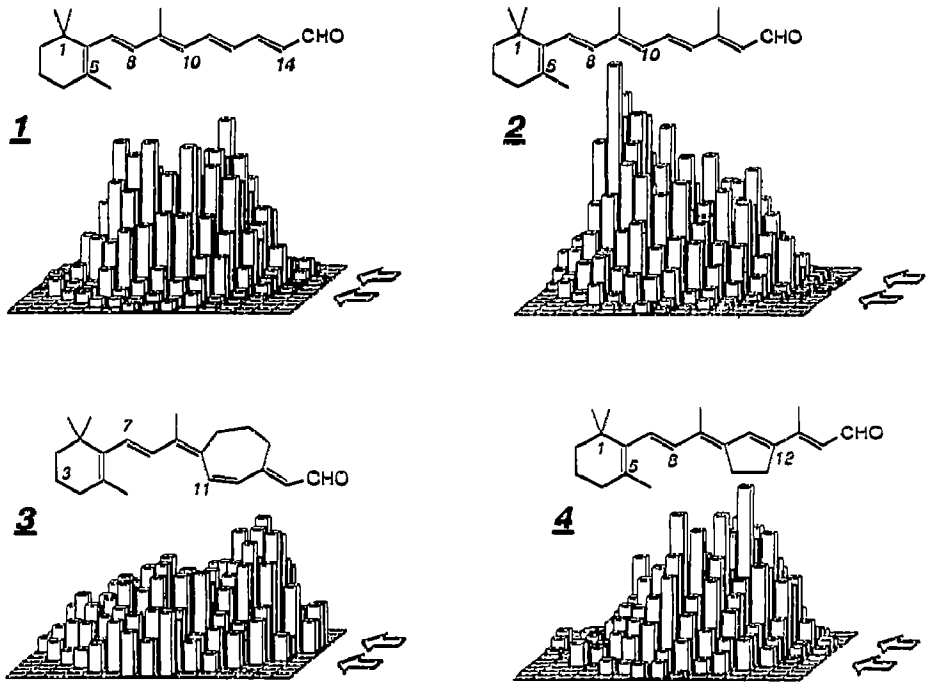


Fig. 1. Typical histograms of swimming vectors showing two-dimensional displacements of the vegetative FN68 cells during actinic irradiation at 489 nm (10 s after the onset of the irradiation). Cells were incubated before measurements for 4 h with 0.01 μM of analog (**1**), for 2.5 h with 0.02 μM of all-*trans* retinal (**2**), for 3.5 h with 0.4 μM of analog (**3**), or for 5 h with 0.15 μM of analog (**4**). Arrows indicate the direction of actinic light. The intensities of the actinic light for the cells incubated with (**1**) or (**4**) and for those with (**2**) or (**3**) were 1.3×10^{12} and 1×10^{14} photons/mm^2· s, respectively.

concentrations in the culture medium (and in NMM) were 0.68×10^{-4} (1×10^{-4}) M and 0.81×10^{-4} M, respectively.

All-*trans* retinal (Sigma Chemical Co.) and retinal analogs [11–14] were purified before use by HPLC (Cosmosil Si-60, Nacalai Tesque Inc., Kyoto; eluted with 10% ethyl ether in hexane), and used as ethanolic solutions.

Phototactic orientation of *C. reinhardtii* was determined at 23°C from ca. 200 individual swimming tracks obtained from a cell population in a quartz chamber ($10 \times 10 \times 0.5$ mm) within 15 min, by use of a computerized movement analysis technique [6]. Five equivalent movement analyzers were connected to video-microscope systems (observing light, >750 nm) aligned at different positions (corresponding to the desired wavelengths) on the OLS. The swimming tracks were registered and analyzed real-time after 10-s period of actinic irradiation (half band width <3 nm). Each experiment consisted of ~10 cycles of the actinic irradiation plus ca. 50 s of dark period. The orientation of a swimming cell was characterized as a 'swimming vector', which was defined as the two-dimensional displacement of the cell detected from two consecutive frames recorded on a video digitizer. The frame rates were 2.2 and 1.7 s^{-1} for the gametes and vegetative cells, respectively. Before experiments, the cells were incubated at 23°C under dim red light (>600 nm), in the presence or absence of retinal and analogs.

3. RESULTS

Typical histograms showing orientation of the swimming vectors of vegetative FN68 cells are illustrated in Fig. 1. The swimming vectors (defined as the displacements of the cells during 0.6 s) of the cells incubated with 13-demethyl retinal (**1**) are evenly distributed in all directions around the origin (Fig. 1, upper left), confirming that this analog is ineffective in reconstituting the active photoreceptor for phototaxis [4]. The histogram for the cells incubated with all-*trans* retinal (**2**) show biased orientation toward the direction away from the light source, indicating that the cells are negatively phototactic (Fig. 1, upper right). On the other hand, the two histograms in the bottom of Fig. 1 show biased orientation toward the opposite direction; this means that the analog chromophores 9,11-di*cis*(7)-retinal (**3**) and 11-*trans*(5)-retinal (**4**) caused positive phototaxis in the strain FN68.

To quantitate these photoresponses, indexes for the biased orientation both toward and away from the light source were introduced, and plotted against the intensity of the actinic light at four different wavelengths (Fig. 2). Negative phototaxis is evident in the vegetative cells incubated with all-*trans* retinal (Fig. 2A), because the negative phototactic index is higher than the positive phototactic index at various light intensities ranging from 10^{10} to 10^{14} photons/mm^2 · s. After incubation with all-*trans* retinal, gametes of the strain FN68 also exhibited negative phototaxis at moderate light intensities (10^{10}–3×10^{12} photons/mm^2 · s, Fig. 2B), as was reported by previous investigators [3,15,16].

By contrast, the gametes incubated with analog (**4**) showed positive phototaxis to the 450–520 nm light at any intensity between 5×10^{10} and 10^{14} photons/mm^2·s (Fig. 2C). The vegetative cells incubated with acyclic

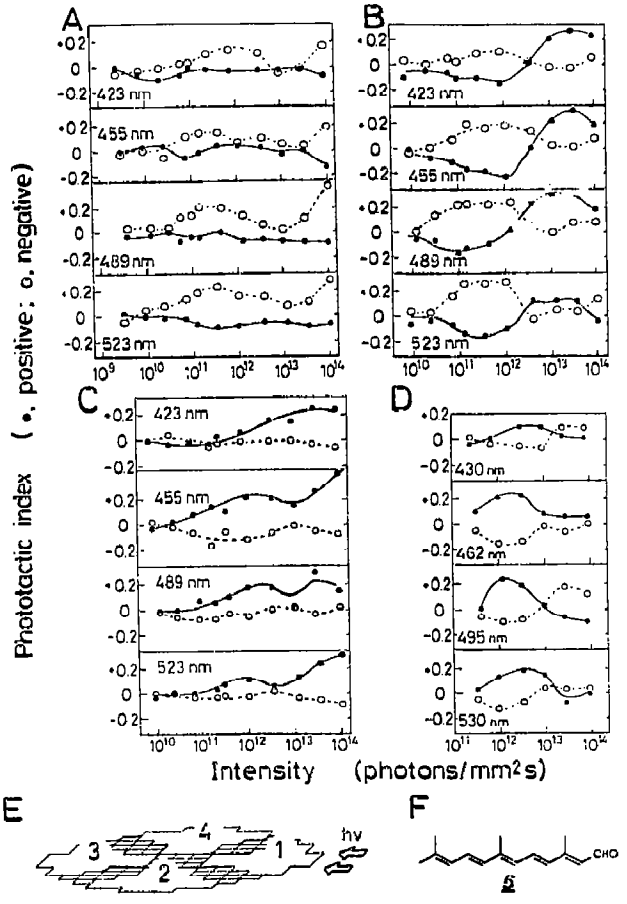


Fig. 2. (A–D) Dependence of phototactic indexes on the intensity of actinic light. Each point represents the average orientation of ca. 200 swimming cells after 10 s of monochromatic irradiation. Vegetative (A and D) or gametic (B and C) FN68 cells were almost simultaneous subjected to independent measurements at the four different wavelengths indicated in the figures. The cells were incubated for 5–8 h before the measurements with 0.02 μM of all-*trans* retinal (A and B), 0.15 μM of analog (4) (C), or 0.1 μM of analog (5) (D). Phototactic indexes were calculated from the populations of the swimming vectors in the four clover-leaf domains assumed in the plane of the vectors (E). Positive (•) and negative (o) phototactic indexes were defined as $\{p_1/(p_1+p_2+p_3+p_4)-0.25\}/0.75$ and $\{p_3/(p_1+p_2+p_3+p_4)-0.25\}/0.75$, respectively, where p_n (n = 1,2,3,4) is the population of the swimming vectors that belong to the domains designated by n. Arrows in E indicate the direction of actinic light. (F) The structure of pentaenal retinal.

pentaenal retinal ((5), structure shown in Fig. 2F) also exhibited clear positive phototaxis at moderate (5×10^{11}– 10^{13} photons/mm^2 · s) light intensities (Fig. 2D). The tendency toward positive phototaxis in these analog-incorporated cells was highly reproducible. Vegetative FN68 cells never exhibited negative phototaxis after incubation with analog (3) or (4), regardless of the phase of cell culture: the negative phototactic index was always lower than 0.08, suggesting that the sign of phototaxis is chromophore specific. These responses are not transient because we observed accumulation of cells at the corresponding edges of the swimming chamber after

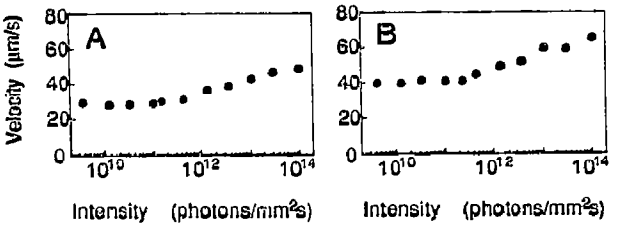


Fig. 3. Dependence of swimming velocity on the intensity of the actinic light at 489 nm. The absolute values of swimming vectors from ca. 200 vegetative (A) and gametic (B) cells were obtained from the data for Figs. 2A and 2B, respectively, averaged and plotted.

several minutes of actinic irradiation. Because we compared the analog-incorporated cells (e.g. Fig. 2C) with the control cells incubated with all-*trans* retinal (e.g. Fig. 2B) within 4 h on the same day using same batches of cell culture, it seemed difficult to expect differences in any other cellular or experimental condition such as the age of cell culture, diurnal rhythmic phase, membrane potential, intracellular or extracellular Ca^{2+} concentration. We therefore account for the unexpected positive phototaxis in this strain as described below.

4. DISCUSSION

4.1. *Possibilities*

Since the sign of the phototaxis is chromophore specific, it would be natural to assume two photoreceptor (rhodopsin) species, each of which corresponds distinctively to the negative or positive phototaxis. Because exclusively positive phototaxis was induced by analog chromophores in which isomerization was blocked about a double bond C11=C12, it is possible that the other receptor mediates *the negative* phototaxis via photoisomerization either about the C11=C12 bond or about a double bond other than C13=C14 of the chromophore (the isomerization about the C13=C14 double bond was demonstrated in another retinal-deficient strain, CC-2359, which predominantly shows positive phototaxis after incubation with retinal or analogs [4–6]). The supposition that the two rhodopsin photoreceptors isomerize their chromophore at different sites (e.g. the 13-ene and the 11-ene) seemed attractive, because it would partly account for previous observations on the same strain by Foster et al. [15] who observed negative phototaxis in the FN68 cells incorporated with a variety of analogs including those in which isomerizations about the 7-, 9-, 11-, or 13-ene were blocked. We think this supposition to be unlikely, however, because under our experimental conditions no phototaxis was observable in the same strain after incubation with analogs in which the 13-ene was locked in either the *trans* or *cis* configuration (data not shown). The positive phototaxis observed in the cells incorporated with analog (5) also disfavors the supposition because isomerization at any double bond is possible in (5). However, regardless of

isomerization specificity, the possibility remains that the strain FN68 contains two distinctive *Chlamydomonas* rhodopsins having different affinities with the exogenous chromophores.

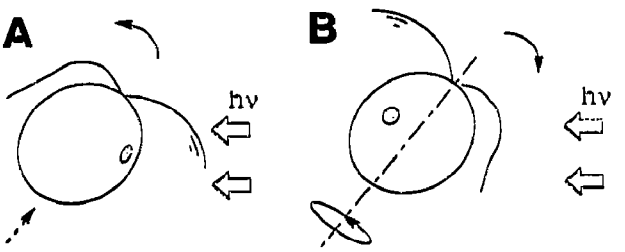
An alternative explanation is that the analog-incorporated cells showed positive phototaxis because these analogs are less effective in reconstituting the active photoreceptor protein. This view is drawn from the fact that wild-type strains (except in old cell cultures) generally show positive phototaxis only when the intensity of actinic light is lower than 10^{12}–10^{13} photons/mm^2 · s [17,18]. Also, this view is based on the assumption that a reciprocity holds between the intensity of actinic light and the concentration of the active photoreceptor protein. Such a reciprocity has already been observed in another retinal-deficient strain, CC-2359 [6]. To test this possibility, we decreased the light intensity until no phototaxis became observable in the cells incorporated with all-*trans* retinal. However, no positive phototaxis appeared even at very low light intensities (Fig. 2A), thus suggesting that such a reciprocity does not hold in the strain FN68, or that this second alternative explanation is incorrect.

4.2. *Hypothesis*

We propose here a hypothesis which consistently accounts for our observations without too many presumptions. This hypothesis also accounts for the abnormality in the presumed reciprocity; this abnormality is one of the two alternative consequences of the above discussion and has been in fact observable in some cases in this strain (data not shown).

The hypothesis is directly deduced from the previously proposed phototaxis mechanisms that are widely accepted [1,2,18]. In such mechanisms, periodical shading of the photoreceptor by the eyespot (and also exposure to the light reflected by the eyespot), resulting from rotational motion of the *Chlamydomonas* cell body, is important for the phototaxis [1]. The cell changes its swimming axis so that constant signal is generated from the photoreceptor irrespective of the phase of the rotation.

In such mechanisms, the sign of the phototaxis depends not only on the motion of the flagellar apparatus that causes reorientation of the swimming axis, but also on the rotational phase angle at the moment when light-induced intracellular signal is actually transduced to the motion of the flagellar apparatus. In other words, the sign can be determined by a phase delay that corresponds to the time consumed by both photoreception and intracellular photosignal transduction. Our hypothesis is as follows: when light intensity or the concentration of the effective photoreceptor is so low that the delay becomes comparable to half the period of the rotational motion of the cell body, this phase delay becomes a determinant of the sign of the phototaxis. This situation is possible because generally intracellular photosignal transduction depends on the number of absorbed photons, and because the probability of photon absorption is proportional to the effective photoreceptor concentration multiplied by both light intensity and the period of exposure. Furthermore, considering that analog (5) prolongs the lifetime of signaling states 5- to 15-fold in archaebacterial sensory photoreceptors [19,20], we think it very likely that the signal processing time also differs between the native and analog-incorporated *Chlamydomonas* rhodopsins. In either case, the delay causes the flagellar response at a wrong phase of the cellular rotation, thereby, in a certain case, causing the phototaxis toward the opposite direction (Scheme I).



Scheme I. A model scheme for phototactic orientation of swimming *Chlamydomonas* cells. (A) When signal from the photoreceptors is transduced quickly to the flagellar apparatus, inactivation of the flagellum distal to the eyespot reorients the cell so that the cell swims away from the light source [2,18,22]. (B) If the signal is recognized by the flagellar apparatus after a certain phase delay, the corresponding flagellar response would result in orientation towards the opposite direction.

This phase delay depends both on the time required for photosignal transduction and on the angular velocity of the cellular rotation, and thus it should be sensitive to the swimming velocity of the cells. In this connection, it is noteworthy that the swimming velocity is affected by actinic irradiation even in the FN68 cells (Fig. 3), although they exhibit much less chlorophyl content than wild-type strains [16]. The above-mentioned abnormality in reciprocity, a reciprocity between light intensity and the effective photoreceptor concentration, is readily explained, if we accept that the positive phototaxis appears under conditions where the swimming velocity is relatively high (thus the angular velocity is high) and the effective concentration of the photoreceptor (or the actinic light intensity) is relatively low.

In wild-type cells, the sign of phototaxis changes drastically and in a complex manner [17,18]. An outwardly directed Ca^{2+} pump is presumably involved in the mechanisms of the regulation of the sign of phototaxis [21,22]. The time required for the photosignal transduction started by a light flash (duration, 0.3 ms) is reported to be 11 and 30 ms (for total fluences of 2×10^{13} and 2×10^{12} photons/mm^2, respectively [21]), which are considerably shorter than half the period of the cellular rotation (ca. 250 ms). Because currently we

have no method for measuring the signal transducing time with weak continuous actinic light, it is unclear whether our hypothetical mechanism is also operative in wild-type cells. Nevertheless, we think our hypothesis to be the most probable explanation for the observed diversion of the sign of phototaxis in the strain FN68.

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REFERENCES

[1] Foster, K.W. and Smyth, R.D. (1980) Microbiol. Rev. 44, 572–630.
[2] Harris, E.H. (1988) The Chlamydomonas Sourcebook, Academic Press, New York.
[3] Foster, K.W., Saranak, J., Patel, N., Zarilli, G., Okabe, M., Kline, T. and Nakanishi, K. (1984) Nature 311, 756–759.
[4] Hegemann, P., Gärtner, W. and Uhl, R. (1991) Biophys. J. 60, 1477–1489.
[5] Lawson, M.A., Zacks, D.N., Derguini, F., Nakanishi, K. and Spudich, J.L. (1991) Biophys. J. 60, 1490–1498.
[6] Takahashi, T., Yoshihara, K., Watanabe, M., Kubota, M., Johnson, R., Derguini, F. and Nakanishi, K. (1991) Biochem. Biophys. Res. Commun. 178, 1273–1279.
[7] Takahashi, T., Yan, B., Mazur, P., Derguini, F., Nakanishi, K. and Spudich, J.L. (1990) Biochemistry 29, 8467–8474.
[8] Beckmann, M. and Hegemann, P. (1980) Biochemistry 30, 3692–3697.
[9] Melkonian, M. and Robenek, H. (1980) J. Ultrastruct. Res. 72, 90–102.
[10] Watanabe, M., Furuya, M., Miyoshi, Y., Inoue, Y., Iwahashi, I. and Matsumoto, K. (1982) Photochem. Photobiol. 36, 491–498.
[11] Akita, H., Tanis, S., Adams, M., Balogh-Nair, V. and Nakanishi, K. (1980) J. Am. Chem. Soc. 102, 6370–6372.
[12] Jayathirtha Rao, V., Zingoni, J.P., Crouch, R., Denny, M. and Liu, R.S.H. (1985) Photochem. Photobiol. 41, 171–174.
[13] Gärtner, W., Towner, P., Hopf, H. and Oesterhelt, D. (1983) Biochemistry 22, 2637–2644.
[14] Albeck, A., Friedman, N., Sheves, M. and Ottolenghi, M. (1986) J. Am. Chem. Soc. 108, 4614–4618.
[15] Foster, K.W., Saranak, J., Derguini, F., Zarrilli, G.R., Johnson, R., Okabe, M. and Nakanishi, K. (1989) Biochemistry 28, 819–824.
[16] Hegemann, P., Hegemann, U. and Foster, K.W. (1988) Photochem. Photobiol. 48, 123–128.
[17] Feinleib, M.E.H. and Curry, G.M. (1971) Physiol. Plant. 25, 346–352.
[18] Morel-Laurens, N. (1987) Photochem. Photobiol. 45, 119–128.
[19] Yan, B. and Spudich, J.L. (1991) Photochem. Photobiol. 54, 1023–1026.
[20] Takahashi, T., Yan, B. and Spudich, J.L. (1992) Photochem. Photobiol. (in press).
[21] Harz, H. and Hegemann, P. (1991) Nature 351, 489–451.???
[22] Kamiya, R. and Witman, G.B. (1984) J. Cell Biol. 98, 97–107.