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Autoregulation of rhodopsin synthesis in *Chlamydomonas reinhardtii*

(carotenogenesis/gene expression/action spectra/retinal/phototaxis)

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ABSTRACT **A sensitive assay for the induction of carotenoid and rhodopsin synthesis, based on the phototactic response, has been developed in a mutant of the unicellular alga *Chlamydomonas reinhardtii*. In the dark, the mutant fails to synthesize carotene and retinal, but it contains the apoprotein opsin. When retinal synthesis is induced by light treatment, the retinal combines with opsin to form rhodopsin, and the cells swim away from a source of light. Since the amount of light required to trigger a phototactic response is inversely proportional to the concentration of rhodopsin, the decrease in amount of light necessary to generate that response can serve as a measure of the amount of retinal synthesized in cells after induction. Using this assay, we found that (*i*) light induction of retinal depends linearly on light exposure and rhodopsin concentration during the exposure; (*ii*) the action spectrum of light induction is identical with that for phototaxis for which the receptor pigment is rhodopsin; and (*iii*) incubation with all-*trans*-7,8-dihydroretinal before light exposure shifts the action-spectrum peak for light induction 0.41 eV (−71 nm). We conclude that the photopigment for induction of retinal synthesis is a rhodopsin. The time lag required for induction of retinal synthesis and preliminary experiments with transcription or translation inhibitors suggest that alterations in gene expression could be involved in the induction process. Its control could be similar to other processes in which membrane receptors for hormones, neurotransmitters, or growth factors regulate gene expression.**

Rhodopsin plays multiple roles in animals. It is the photoreceptor for vision and a regulator of circadian rhythm and melatonin production. In vertebrates, it acts as a regulator both in the pineal gland (1) and, to a lesser extent, in the retinal photoreceptor cells (2). In *Chlamydomonas*, a flagellated eukaryotic alga, we found that a bovine-like rhodopsin serves as the photoreceptor pigment for phototaxis (3). Rhodopsin in eukaryotes is thus of ancient origin. Analysis of 5S RNAs (4) has shown that the *Chlamydomonas* evolutionary line branched from the plant line soon after the plant and animal lines diverged. The question naturally arises whether the regulatory function of rhodopsin is also ancient.

The identity of the light receptors that regulate carotene synthesis in bacteria, fungi, algae, and angiosperms has been uncertain. Flavoproteins, porphyrins, and carotenoid proteins have all been suggested on the basis of action spectra in various organisms (5).

The measurement of phototaxis threshold (3) has given us a sensitive assay for the presence of rhodopsin. We noticed that carotenoid mutants exposed to light recovered their phototaxis. Here we report that rhodopsin excitation induces synthesis of carotene and retinal. We do not know the nature of the induced products or how they modulate the synthetic pathway. Nevertheless, since retinal, the chromophore for rhodopsin, is synthesized as a result of rhodopsin excitation, we have an autoregulated pathway with respect to the chromophore. Preliminary accounts of this work have been given elsewhere (6, 7).

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MATERIALS AND METHODS

Culture Conditions and Cell Preparation. A colorless mutant strain of *Chlamydomonas reinhardtii* was obtained from W. Y. Wang (University of Iowa) and plated on plate medium (3). Colonies were tested for negative phototaxis and phototactic threshold. Strain FN68 (Car-1) had a high phototactic threshold but regained the sensitivity of the wild type on incubation with retinal (as below). Growth and manipulation of this mutant were done in darkness or under a Kodak safelight (no. 2 red filter). Cells were grown at 18°C for 7–10 days on plate medium. On the day before experiments, cells from two plates were suspended in 10–20 ml of nitrogen-free minimal medium (NMM), which does not support growth, to induce opsin synthesis (ref. 3; P. Hegemann and K.W.F., unpublished results). The suspension was centrifuged for 2 min at 354 × *g*, and the pellet was resuspended in about 14 ml of NMM to give 5–7 × 10^6 cells per ml. This suspension was shaken at 150–180 rpm, 18°C, overnight in a 50-ml tube.

In some experiments, 4–5 hr after transfer to NMM the cells were incubated in all-*trans*-retinal, 7,8-dihydroretinal, or retinol. Then 0.025% of the antioxidant *d*-α-tocopherol acetate (10% wt/vol solution in HPLC-grade methanol) and 1 μl per ml of cell suspension of sufficient chromophore dissolved in HPLC-grade methanol to give the final concentration was added before the cells were returned to the shaker overnight.

Phototaxis Assay. Contamination-free suspensions whose cells were at least 70% motile and oval were used. A 1.5-ml sample was transferred to a plastic Petri dish (Falcon, 34 mm inside diameter), one dish for each photon energy (i.e., wavelength). The light sources were tungsten/halogen passed through three-cavity interference filters blocked at extreme wavelengths (Microcoating, Westford, MA); these had 10-nm bandwidths and peaked at 420, 460, 500, 540, 546, and 600 nm, respectively, For higher irradiances a series of three-cavity interference filters with constant-energy bandwidth of 0.159 eV (Barr Associates, Westford, MA) were used; their peak wavelengths and wavelength bandwidths were 400 (20), 428.6 (24), 461.5 (27), 500 (32), 545.5 (38), and 600 (42) nm, respectively. At 600 nm blue-green light was blocked by placing the 42-nm-wide and 10-nm-wide filters in series. Light irradiance was measured with a photodiode radiometer model 88XLA (Photodyne, Newbury Park, CA) calibrated to within 5% accuracy at each wavelength and corrected for Petri dish absorption. Irradiance was adjusted by insertion of neutral density filters and by varying the distance between the dish and the light source. Temperature was controlled at 20 ± 1°C.

Abbreviation: Ephoton, exaphoton (10^{18} photons).
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Cells usually accumulated in small dots all over the dish, with a narrow clear zone all around the periphery; both phenomena are probably caused by hydrodynamic effects (8). The width of the clear zone was independent of the light intensity, except on the side of the dish facing light as a result of negative phototaxis. After 10 min of side illumination we determined the phototactic rate from the width of the clear zone near the light minus the width of the peripheral clearing on the far side of the dish. The resultant phototactic rate was always intensity dependent. Rates of phototaxis down to 1 μm/s could be measured.

To obtain a phototactic threshold at each photon energy (wavelength), at least three measurements of phototactic rate were made, one at an irradiance near threshold, the second and third at 2- and 4-fold higher. Since all these measurements are in fact very near threshold (the maximal intensity that would give no response), the largest phototactic rate was typically 5 μm/s, much less than the free swimming rate of more than 100 μm/s (9). The three points were plotted semilogarithmically as phototactic rate vs. irradiance (as in Fig. 1). Threshold was determined within 0.087 log unit by linearly extrapolating to zero phototactic rate. Sensitivity was defined as 1/threshold irradiance.

Action spectra (Figs. 4–6) were plotted semilogarithmically as sensitivity vs. photon energy (eV), and a nonlinear wavelength scale (nm) was included at the top (1 eV = 1239.85/wavelength in nm). A fit of the standard absorption curve of rhodopsin was made to the data points; both the sensitivity and peak energy of the standard curve were allowed to vary, except as indicated in Fig. 6*B*. Because of variations in sensitivity from culture to culture, a complete action spectrum was determined in 1 day from cells taken from the same culture.

Induction. The three steps were exposure to inducing light, dark incubation, and threshold measurement. Cell suspension (1.5 ml) was placed in a 34-mm Petri dish, one for each photon energy (wavelength) and intensity. To get uniform stimulation of cells the inducing light was incident from above the 1.65-mm-deep layer of cells, preventing phototactic aggregation and third- and higher-order scattering. After induction, the cells were incubated between 30 min and 120 min in the dark before the phototaxis threshold was measured. As shown below, the duration of the dark period within these limits had no effect on the induction process or the final level of retinal synthesis.

All chemicals were from Sigma, except 7,8-dihydroretinal (synthesized by G.Z.).

RESULTS

Characteristics of the Light-Induction System. Fig. 1 (solid lines) shows that the phototactic threshold of cells incubated overnight in NMM was lowered by a factor of 1000 within 15 min of addition of 10 μM retinal in the dark. Since, as shown below, the rhodopsin concentration is inversely proportional to the phototactic threshold, the rhodopsin concentration must have increased 1000-fold. This rapid appearance of rhodopsin in the dark was not inhibited by the inhibitors of protein synthesis, cycloheximide and chloramphenicol, added individually or together (Fig. 1), at concentrations demonstrated to block light induction of retinal synthesis. These results show that opsin must have been present prior to retinal incubation. When, instead, the mutant was incubated under conditions in which opsin is not induced (in liquid medium: plate medium without agar; cf. ref. 3), incubation in retinal or light illumination did not restore any phototaxis sensitivity.

To find out if measurements of light induction were affected by the time between the exposure of the cells and the phototaxis assay, three samples were exposed for 30 min,

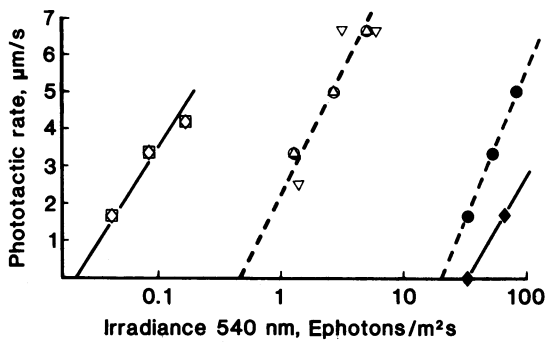

FIG. 1. Phototactic rate as a function of irradiance, measured with photons of energy 2.30 eV (540 nm); 1 exaphoton (Ephoton) = 10^{18} photons = 1.661 microeinstein. Solid lines, experiment illustrating restoration of phototaxis sensitivity after the addition of retinal. Rates of phototaxis were measured at irradiances near threshold of cells incubated overnight in NMM (♦), or incubated overnight in NMM and then exposed to retinal (10 μM, 15 min; ◇) or to retinal (as above) in the presence of inhibitors of protein synthesis (cycloheximide at 0.2 μg/ml plus chloramphenicol at 40 μg/ml, 15 min; □). Broken lines illustrate insensitivity of light induction to duration of subsequent incubation in the dark. Cells were exposed to 2.51×10^4 Ephotons/m^2 of photon energy 2.58 eV (480 nm) and incubated in the dark for 1 (○), 2 (▽), or 4 (△) hr. Cells in the control (●) were not exposed to light prior to measurement of phototactic rate.

incubated in the dark for 1, 2 and 4 hr, respectively, and then tested for phototaxis. Fig. 1 (broken lines) shows that the intensity–response curves for these three samples overlap with the same threshold, implying that each sample contained the same amount of rhodopsin, 40 times that of the unexposed control (see below).

If rhodopsin is responsible for induction, the amount of induction should depend on the amount of rhodopsin present at the time the cells are exposed to inducing light. To test this, the cells were incubated with various amounts of retinal or retinol and induced with a constant amount of light (Fig. 2). Retinal and retinol (which is oxidized to retinal, presumably by a dehydrogenase within the cells) gave similar results. The logarithmic plots of phototactic sensitivity vs. retinal or retinol concentration were linear, with slope 1. This shows that the amount of rhodopsin formed is directly proportional to the concentration of the compound added. This also shows that the amount of retinal added did not saturate the available opsin and in fact that we are working at relatively low levels of rhodopsin. Light induction at constant light exposure shifted the plot upward by a constant amount. This implies that the amount of rhodopsin induced was directly proportional to the amount of rhodopsin present.

Cells were incubated overnight with a subsaturation dose (0.5 μM) of retinal or retinol and induced for a variable length of time (Fig. 3). Control cells received no retinal or retinol. Both plots in Fig. 3 were linear, with slope 1, showing that the amount of rhodopsin induced was directly proportional to the amount of inducing light even when the initial concentration of rhodopsin varied by as much as 150-fold. Retinal and retinol were equally effective in the same batch of cells.

Action Spectrum of the Pigment Formed by Light Induction. In *Chlamydomonas* carotenes and retinal are normally made in the dark as well as in the light. We wished to determine in the mutant cells used in the present experiments whether the rhodopsin formed from retinal induced by light treatment had the same action spectrum as that in wild-type cells [peaked at 2.46 eV (503 nm); cf. ref. 9]. In Fig. 4, phototaxis sensitivity is plotted as a function of photon energy for two batches of cells after different exposures to light. These action spectra peaked at 2.46 eV (503 nm) and therefore are indistinguishable from the action spectrum of the wild type. The close fit

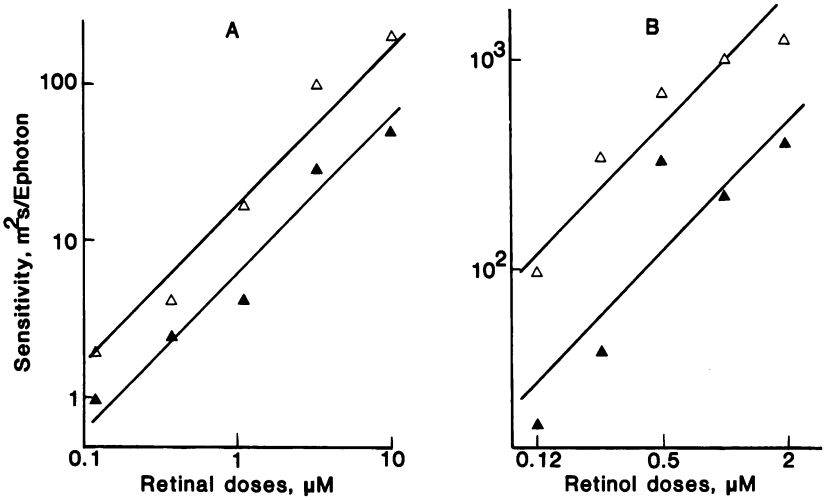

FIG. 2. Change in threshold sensitivity for phototaxis as a function of concentration of retinal (*A*) or retinol (*B*) with (△) or without (▲) light induction. Experiments *A* and *B* were done on different days with different batches of cells. Cells were incubated overnight with retinal or retinol. Exposure was 4.52×10^4 Ephotons/m^2 of energy 2.27 eV (545.5 nm) for retinal and 4.52×10^4 Ephotons/m^2 of energy 2.30 eV (540 nm) for retinol. Incubation time after induction was 30–45 min. Phototaxis was measured with photons of energy 2.27 eV (546 nm) for retinal and of energy 2.30 eV (540 nm) for retinol. Each point represents one threshold measurement.

to the rhodopsin standard curve suggests only one predominate form of retinal. Having shown previously (3) that rhodopsin is the sole pigment for phototaxis, we conclude that light induces formation of native rhodopsin.

Rhodopsin analogs can be found that have their action spectra significantly shifted from the spectrum of the natural compound; for example, the rhodopsin generated when opsin is incubated with *trans*-7,8-dihydroretinal has a peak at 2.86 eV (434 nm). Since cells synthesize retinal rather than analogs, the action spectrum of cells containing such a blue-shifted rhodopsin analog should shift back toward the natural action spectrum after induction by light. To test this we incubated the cells with *trans*-7,8-dihydroretinal (Fig. 5). A 1.5-ml sample was kept in the dark, while the rest was induced. The asterisk is the phototactic sensitivity at 2.30 eV (540 nm) for the uninduced cells. This activity is caused by the analog-substituted rhodopsin plus the endogenous rhodopsin. For the cells exposed to light, the activity is caused by the pigments present in the unexposed cells plus the induced rhodopsin. The solid curve is the weighted sum of the action spectra of 7,8-dihydroretinal-opsin (peak 2.86 eV) and retinal-opsin (peak 2.46 eV) that best fits the data points. The weights are 3:1 analog to retinal, showing that about ¾ of the rhodopsin molecules contain analog and ¼ contain retinal. The increased sensitivity at 2.30 eV shows the amount of induced rhodopsin is 2.3 times the endogenous amount. Since the induced rhodopsin is the difference between the exposed and unexposed cells and has a known spectrum with a peak at 2.46 eV, we have enough information to construct what the spectrum of the unexposed cells would have been if we had measured it (the broken line).

This provides us with an assay for the amount of the induced rhodopsin—namely, the difference between the exposed and unexposed sensitivity at 2.30 eV. We chose this photon energy to test for induced pigment in subsequent action spectra, because at this energy the difference between the two curves is near maximum.

Action Spectrum of the Pigment Responsible for Induction. Using the assay for the induced rhodopsin described above, we obtained the action spectrum for the pigment responsible for the induction by measuring the amount of rhodopsin as a function of various inducing photon energies. The experimental design was dictated by two experimental limitations. First, there is a small and variable amount of endogenous rhodopsin that causes variation in sensitivity of cells from

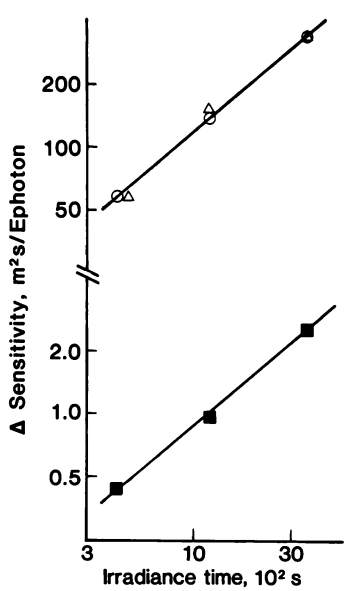

FIG. 3. Change in sensitivity for phototaxis as a function of light induction for cells incubated overnight with 0.5 μM retinal (△) or retinol (○). Cells in the control (■) were not incubated with either compound. Irradiance was 41.9 Ephotons/m^2·s of energy 2.27 eV (546 nm) for the times indicated on the abscissa. The cells were incubated for 60 min before phototactic threshold was measured at 2.27 eV (546 nm). The differences in sensitivity between induced and uninduced cells were plotted vs. the irradiance time.

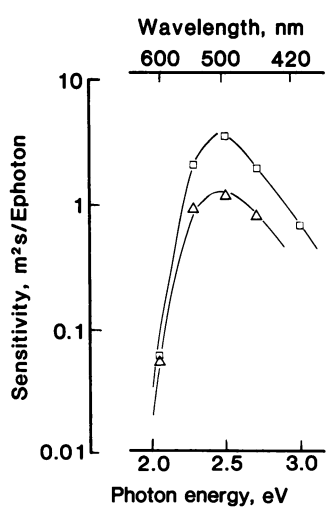

FIG. 4. Phototaxis action spectrum of strain FN68 after light induction. Suspensions of two different batches of cells were exposed to 7.53×10^4 Ephotons/m^2 (□) and 4.50×10^4 Ephotons/m^2 (△) of energy 2.30 eV (540 nm) to induced synthesis of the chromophore. Phototaxis threshold was measured after incubation for 60–90 min.

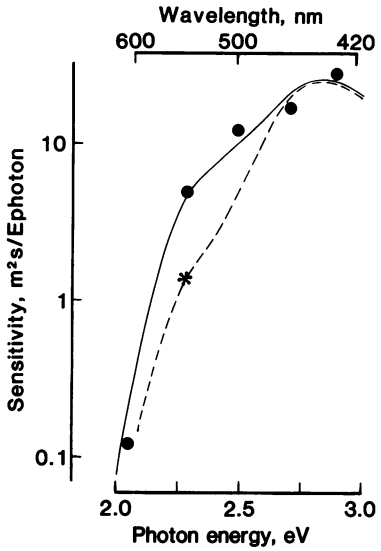

FIG. 5. Phototaxis action spectrum of cells that were induced by light after incubation with *trans*-7,8-dihydroretinal (4 μM overnight). Cells were exposed to 4.60×10^3 Ephotons/m^2 of energy 2.90 eV (428 nm) and incubated for 100–120 min. The solid line is the fit of the data to a weighted sum (in proportion 3:1) of the action spectra of rhodopsins containing dihydroretinal or retinal. The broken line uses the same amount of dihydroretinal-opsin but reduces the weight of retinal-opsin (now in proportion 10:1) to go through the data point (*) of the uninduced cells measured with photons of energy 2.30 eV (540 nm). The difference between the two curves is the sensitivity due to the induced rhodopsin.

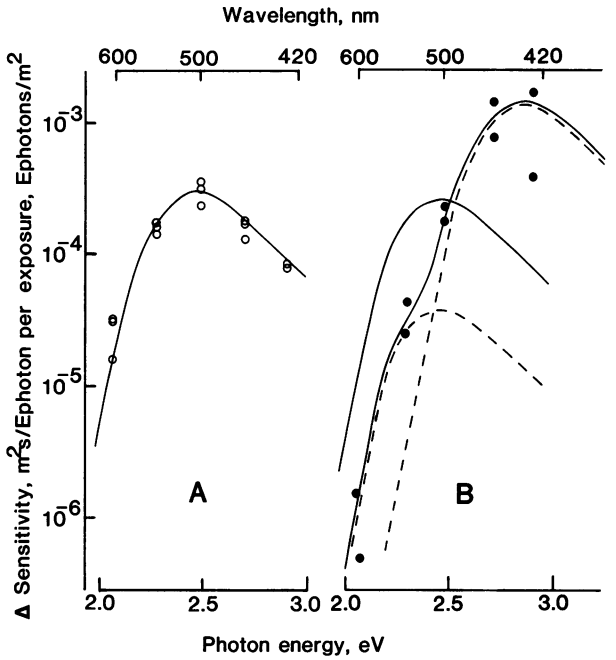

FIG. 6. Action spectra of the pigments responsible for light induction of rhodopsin with endogenous pigments only (*A*) and with the addition of 4 μM *trans*-7,8-dihydroretinal (*B*). Cell suspensions were exposed to two or three different irradiances of each photon energy for 20 min. Phototactic threshold at energy 2.30 eV (540 nm) was tested after 60 min of incubation to give a measure of rhodopsin induced. The inducing endogenous pigment action spectrum (*A*) shows a peak sensitivity at energy 2.45 eV (505 nm). In *B* the right solid curve shows the best fit (least squares) to a weighted sum (in proportion 36:1) of action spectra of an exogenous pigment peaking at energy 2.86 eV (434 nm) and the endogenous pigment (2.45 eV). The broken curves show individually the two action spectra. The left solid curve was drawn as the best fit to the data, assuming only a nonshifted endogenous pigment.

one batch to another, requiring the entire spectrum be measured on one batch of cells during one day. Second, only a narrow range of inducing exposures gave useful results. If the inducing exposure was too low, the difference between induced and uninduced phototaxis sensitivity was too imprecise, and if the inducing exposure was too high, the induction saturated. We chose the inducing exposure that increased the sensitivity of the cells 10 to 20 times that for no exposure. Each data point in Fig. 6 is the difference in phototaxis sensitivity between the exposed and unexposed cells measured at 2.30 eV (Δ sensitivity) divided by the inducing light exposure. This method is valid because the amount of rhodopsin formed by induction is linearly proportional to the amount of inducing exposure (irradiance × time) and the phototactic sensitivity is linearly proportional to the amount of rhodopsin. A plot of these data points as a function of photon energy gives the action spectrum for the pigment responsible for the induction. The observed scatter results from uncontrollable experimental variation. The line in Fig. 6*A* is the standard curve for rhodopsin adjusted by eye to best fit the data. The resulting peak is at 2.45 eV (505 nm). The pigment responsible for inducing rhodopsin has an action spectrum close to that of rhodopsin, but the possibility of another pigment with the same action spectrum is not excluded.

Proof that retinals function as the active chromophore in the receptor pigment was obtained by incorporating the retinal analog *trans*-7,8-dihydroretinal. A shift in the action spectrum of the pigment responsible for inducing rhodopsin can only be interpreted as due to the analog playing an activating role. In this experiment we incubated the cells with the analog at the concentration that kept the level of analog-substituted rhodopsin low enough to avoid saturation of the opsin during induction, but high enough to predominate over the endogenous rhodopsin present. Otherwise, the experiment and analysis of the data (Fig. 6*B*) were the same as above (Fig. 6*A*). The solid curve on the right of Fig. 6*B* is the weighted sum of the action spectra of retinal-opsin and 7,8-dihydroretinal-opsin that gave the best fit to the induction action spectrum; the broken curves show the individual contributions. Assuming no shift, the best fit to the data points of a standard rhodopsin curve (peaked at 2.45 eV) is shown by the solid curve on the left. Application of the *F* test to the variance ratio obtained by comparing the variance of the data points to the right and left solid curves suggests a probability of less than 0.001 that the data are not shifted by the incorporation of the analog relative to the endogenous pigment (10). This experiment provides convincing evidence that a rhodopsin is the inducing agent.

The incorporated retinal analog gave a specific shift in the induction action spectrum, just as it did in the phototactic action spectrum (Fig. 5 and ref. 3). The enhancement of induction by added retinal (Figs. 2 and 3) was suggestive evidence that rhodopsin is the photoreceptor for induction, but it did not rule out an indirect effect. The analog shift of the action spectrum provides convincing evidence that a rhodopsin-like pigment is *directly* involved in induction.

DISCUSSION

The normal dark pathway for carotene synthesis is inactive in strain FN68, making it easier to study the light regulation of the carotene and retinal synthesis pathway (11, 12). We have shown here that the photoreceptor responsible for this light-dependent pathway is rhodopsin, by showing that the light induction action spectrum fits the rhodopsin standard curve, and that incorporation of 7,8-dihydroretinal shifts this spectrum appropriately. Previously we used this technique to show that rhodopsin is responsible for visual excitation in

Chlamydomonas (3). Since rhodopsin is the photoreceptor responsible for stimulation of retinal synthesis by light, and since retinal appears to be the limiting substrate for rhodopsin synthesis, it is evident in the mutant FN68, and perhaps also in wild-type cells, that rhodopsin regulates its own biosynthesis. In this mutant and under the conditions of these experiments the protein opsin, which forms the rhodopsin with retinal, is made in the dark.

We found that phototactic sensitivity was linearly related to the concentration of exogenously supplied chromophore (at least for low concentrations). Since the amount of retinal or retinol incorporated probably depends linearly on its concentration in solution, the observed sensitivity must be directly proportional to the concentration of pigment. This is reasonable, since the total capture cross-section for excitation should be proportional to the number of active rhodopsin molecules.

The amount of rhodopsin synthesis that is induced depends linearly on the initial amount of rhodopsin present in the dark (at least for low concentrations) as shown by the parallel rise in sensitivity on the logarithmic plot of light-induced cells compared to cells kept in the dark. Also the amount of induction depends linearly on the length of exposure up to 1 hr. Both results imply that the number of rhodopsin excitations dictates linearly the extent of induction, suggesting that each excitation counted equally. This kind of linearity is not observed in the behavioral response, where cells adapt to changes in light intensity over a time span of about a minute. Therefore, either more than one rhodopsin is involved or there is more than one signal-processing pathway.

To obtain clues as to where the signal generated by the excitation acts in the chain of events leading to retinal synthesis, we did a preliminary study with chemical inhibitors that suggested to us that the most likely point at which rhodopsin excitation mediates control of retinal synthesis is that of transcription. That study, along with work on light-induced changes in mRNA, will be discussed in a future paper. If indeed it is transcription that is being regulated by rhodopsin, then it must be turned on and off rapidly, because the amount of rhodopsin synthesized was found to be the same over a period of 1–4 hr after the exposure to light. This also suggests that the rhodopsin is not degraded during 4 hr.

Other possible regulatory roles of rhodopsin include the induction of ribulose carboxylase (13) in *Volvox*, photomorphogenesis in the dinoflagellate *Scrippsiella trochoidea* (14), the dark and light photomechanical movements found in frogs and squid (15), and in circadian rhythm and melatonin production in vertebrates (1). Other evidence suggesting a regulator role for rhodopsin is the increased synthesis of mRNA in rat photoreceptor cells exposed to light (2) and the existence of photoreceptor cell diseases apparently involving intracellular signaling and a light dependence. These mammalian diseases cause photoreceptor cell degeneration (reviewed in refs. 16–18) and are characterized by alteration of cyclic nucleotide levels and induction by light or phosphodiesterase inhibitors.

Rhodopsin belongs to a homologous family of membrane receptors that are involved in GDP–GTP exchange with guanine nucleotide-binding proteins (G proteins) (19). These receptors and their signaling pathways are ubiquitous throughout the eukaryotes and are variously involved in sensory reception, growth regulation, hormone and neurotransmitter activity, and significantly, where studied, in regulation of gene expression. We believe it likely that rhodopsin in *Chlamydomonas* might also control retinal synthesis via regulation of gene expression and that this system will be useful for studying this pathway in cellular and molecular detail.

Our study shows that in *Chlamydomonas* a rhodopsin pigment does more than merely function as the photoreceptor for vision. It also functions in cell regulation by controlling the synthesis of retinal. Rhodopsin's dual sensory/regulatory role is thus evolutionarily ancient.

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