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In Vitro Identification of Rhodopsin in the Green Alga *Chlamydomonas*[†]

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ABSTRACT: The unicellular alga *Chlamydomonas* can detect both intensity and direction of the ambient light and adjust its swimming speed and direction accordingly. On the basis of physiological experiments, the functional photoreceptor for this *visual* process has recently shown to be a rhodopsin. We here report the in vitro identification of endogenous retinal and a rhodopsin in *Chlamydomonas* cell extracts and purified membrane preparations. The rhodopsin absorption spectrum has fine structure with the maximum at 495 nm and matches the action spectra for the behavioral light responses. The rhodopsin can be bleached and subsequently reconstituted with exogenous retinal. Labeling with [^{3}H]retinal occurs in the final preparation only with a single protein with a molecular weight of 32 000. We conclude that this protein is the visual photoreceptor in *Chlamydomonas*.

The unicellular alga *Chlamydomonas* can use light as an energy source (photosynthesis), as a regulator for developmental processes (photomorphogenesis), and as a sensory stimulus for orientation to or away from a light source (phototaxis and stop responses; Pfeffer, 1904; Schmidt & Eckert, 1976; Foster & Smyth, 1980; Nultsch & Häder, 1988; Hegemann & Bruck, 1988). While the photoreceptors for photosynthesis and photomorphogenesis, e.g., reaction centers and phytochrome, have been studied extensively for many years, the photoreceptor for behavioral light responses has only recently been dealt with. Phototaxis and light-induced stop responses of *Chlamydomonas reinhardtii* show rhodopsin action spectra and are retinal dependent (Foster et al., 1984; Uhl & Hegemann, 1990). Retinal can restore phototaxis in

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blind FN68 cells, and the action spectrum is shifted when retinal analogues are used (Foster et al., 1984, 1988b, 1989). Furthermore, the phototaxis in *Chlamydomonas* wild-type cells can be inhibited by hydroxylamine and subsequently reconstituted with retinal (Hegemann et al., 1988). These physiological experiments gave conclusive evidence that a rhodopsin serves as the photoreceptor for movement responses in *Chlamydomonas*. This is the first example for a rhodopsin in a lower plant system. Here we prove this conclusion by presenting experiments that identify retinal and a rhodopsin in *Chlamydomonas* by spectroscopic and biochemical methods.

Materials and Methods

All chemicals were purchased from Sigma and are of analytical grade.

FN68 cells have been provided by K. W. Foster's laboratory. The strain has been isolated by W.-Y. Wang and has originally been named F1. Strain FN68 and strain 806 cells were grown on plates and converted to gametes in liquid culture as described (Foster et al., 1984; Hegemann et al., 1988). Gametes were used for all experiments.

For retinal induction, FN68 cells were exposed to green light of high photon irradiance (520 nm, 60-nm half-bandwidth (bw), selected filter combination SFK (Schott, Mainz, Germany), 1.5×10^{20} photons m^{-2} s^{-1}) and kept in the dark for another 30 min before phototaxis was measured or pigments were extracted. For phototaxis measurements, 1.5 mL of a cell suspension in a small Petri dish (34-mm i.d.) was placed in front of a stabilized light source at various photon irradiances (500 nm, 10-nm bw, single-cavity interference filter (Schott). The distance traveled by the trailing edge of the population was measured after 10 min. The phototactic rate was plotted versus the photon irradiance of the stimulating light (Foster et al., 1984; Smyth et al., 1989). Pigments were extracted from 2×10^8 strain FN68 cells or from S2 membrane preparations by the methods of Groenendijk et al. (1979) or Suzuki et al. (1986). Both methods gave comparable results. Aliquots were applied to a LiChrosorb Si-60 (5-μm) HPLC column and eluted according to Suzuki and Makino-Tasaka (1983). The absorbance of the eluate was recorded continuously between 300 and 500 nm with a 2-nm resolution by a multichannel detector (Waters) during elution, which allowed the detection of spectra during chromatography.

All membranes were prepared from strain 806 cells. The cells were grown for 5 days on HSM+S agar plates (HSM plus 250 mM sorbitol and trace elements; Sueoka et al., 1967; Hutner et al., 1950), for 3 days in 50 mL of HSM+S liquid medium, for 3 days in 1 L, and for another 4 days in 20 L of HSM (without sorbitol). During growth, the cells were irradiated with "cool fluorescent white" light (2 W m^{-2}) at 18–20 °C under slight agitation or stirring in liquid. At a cell density of 6×10^6 cells mL^{-1}, the cells were transferred to 10 L of nitrogen-deficient medium (NMM; Foster et al., 1984) and stirred with 120 rpm under an aeration of 2 L min^{-1} and irradiation (2 W m^{-2}) at 18 °C. As found by the criterion of the oval cell shape, the cells had converted into gametes after 2 days. Then, the 10-L culture was harvested and used as starting material for cell fractionation. All subsequent work was carried out in the dark or in dim red light. The cells were resuspended in 100 mL of MOPS buffer, pH 6.8 (3 mM $CaCl_2$, 10 mM $MgCl_2$, 10 mM NaCl, 1 mM EDTA, 0.05% NaN_3, 0.5% PEG 20000, 0.5% Dextran P40, 10 mM MOPS, pH 6.8, 0.1 mM PMSF, 0.0005% aprotinin, 0.0002% leupeptin, and 0.0002% pepstatin), and incubated for 30 min under nitrogen at 1750 psi in a Parr cell-disruption bomb. The lysate was released into 5 mL of 5 M sorbitol in MOPS buffer (250 mM final sorbitol concentration = MOPS+S) and centrifuged at 80000g for 90 min. The total membrane fraction was resuspended in MOPS buffer, sucrose was added to a final concentration of 1.2 M, and the solution was stirred for 30 min. Portions of 15 mL were overlaid with 13 mL of 0.6 M sucrose and 10 mL of 0.3 M sucrose, all in MOPS+S buffer. The gradients were developed for 13 h at 62000g and 4 °C. The fractions S1–S4 were collected separately and washed twice with a NaCl/MOPS buffer (100 mM NaCl, 1 mM EDTA, 0.5% NaN_3, 10 mM MOPS, pH 6.8, plus inhibitors). The final pellets were resuspended in NaCl/MOPS buffer. Fraction S2 was extracted with 1% octyl β-glucoside (Chemex, CA) in NaCl/MOPS buffer at a detergent to protein ratio of 30 at 18 °C for 30 min. The extracted membranes were washed twice and resuspended in NaCl/MOPS buffer.

Spectroscopy was carried out with 2 μL of the membrane suspension with a total absorbance of 1–1.5 cm^{-1} at 500 nm. Spectra were recorded on a UV–vis microspectrophotometer (UMSP-80; Zeiss, Oberkochen, Germany) equipped with a 6.3× Ultrafluar objective (Zeiss). A second Ultrafluar objective with 10× magnification was used as a condenser. The spectrophotometer was interfaced with a computer (HP 300 series) and controlled by a λ-scan software package (Zeiss). Slit width and step width were 2 nm. The light path length through the sample was 0.2 mm. The presented spectra are all averages of three recordings.

For bleaching, membranes were irradiated with 5×10^{22} photons m^{-2} s^{-1} of green light (maximum 520 nm, filter combination of GG 475 (Schott), and a low-pass 550-nm filter (Melles Griot, Darmstadt, Germany)) for 30 min. Some experiments were carried out in the presence of 100 mM hydroxylamine, pH 7.0. The irradiated area had a diameter of 50 μm. Free retinal may diffuse out of the measuring area, thus resulting in a lower concentration of free retinal compared to that of rhodopsin before bleaching. For this reason, the extinction coefficient was determined from reconstitution and not from bleaching experiments. S2 membranes that have been extracted with detergent and were bleached in green light for 5 min under conditions described above were reconstituted by the addition of 5 μL of a 2 μM *all-trans*-retinal solution in 1% ethanol to 5 μL of the membrane suspension.

Flash experiments were performed on the same instrumentation. The membrane preparations were stimulated with a 5-ns laser flash of 510 nm (Eximer laser EMG 53 MSC, dye laser FL 3001 (Lambda Physics, Göttingen, Germany); the dye was coumarin 307).

[11,12-^{3}H]-*all-trans*-Retinol (Amersham) was oxidized to retinal (Arnth, 1975). Retinal (1 μCi) was added to the membranes (50 μg of protein) under the conditions described for the reconstitution. Reduction was carried out as described by Schreckenbach et al. (1977). The proteins were separated on a 16.5% polyacrylamide gel in a tricine–SDS buffer system (Schägger & Jagow, 1987). The left lane of Figure 5 indicates the sizes of marker proteins in multiples of thousand.

Results

The phototactic sensitivity of a *Chlamydomonas* cell population is defined as the reciprocal of the lowest photon irradiance that the cells can respond to (threshold criterion). It can be practically determined by extrapolation of the stimulus–response curve to zero response. *Chlamydomonas* strain FN68 cells are almost completely blind if grown in darkness, showing phototactic activity only at very high photon irradiance. The phototactic sensitivity of these cells can be reconstituted by addition of external retinal but also, alternatively, by exposure of the cells to green light (Figure 1). The

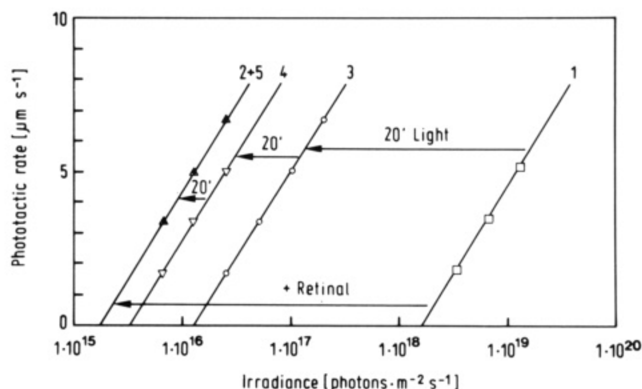

FIGURE 1: Phototactic sensitivity of FN68 cells reconstituted by retinal or green light. Dark-grown FN68 cells are phototactic only at high photon irradiances (1); the phototactic sensitivity recovers by addition of 2 μM *all-trans*-retinal (2) or gradually after preirradiation with green light for 20 min (3), 40 min (4), or 60 min (5).

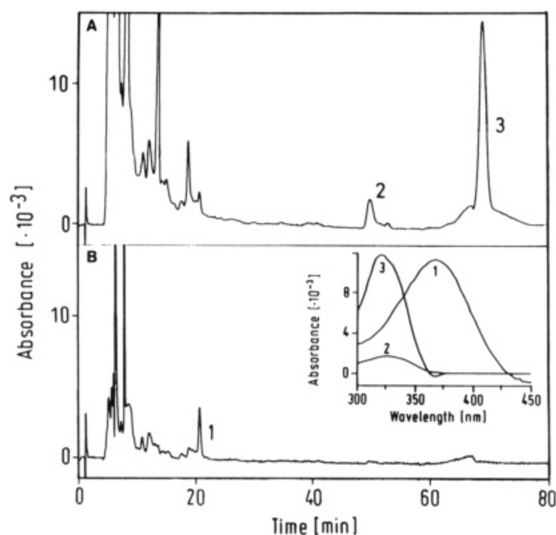

FIGURE 2: Identification of retinoids. HPLC analysis of a methanolic extract of FN68 cells that were exposed to green light for 60 min (one out of four experiments) is shown. Retinol compounds were measured at 320 nm (A), whereas retinal is detected at 370 nm (B). The spectra of compounds 1–3 show absorption spectra (insets) of *all-trans*-retinal and 13-*cis*- and *all-trans*-retinol. Spectrum 1 was normalized to spectrum 3, whereas 2 and 3 are shown in their absorption ratio. In separate experiments, the identity of the retinoids was further verified by coelution with added standard retinal and retinol isomers (9-cis, 11-cis, 13-cis, and all-trans).

sensitivity increase is seen as a shift of the stimulus–response curve to low stimulus photon irradiance (Foster et al., 1988a). The phototactic sensitivity increased up to 1000-fold and reached the level of wild-type cells (strain 806; Hegemann et al., 1988), provided high photon irradiances had been used for a limited period of time (up to 1 h, Figure 1). Upon longer irradiation, the motility of the cells began to decrease.

Using these resensitized cells rather than wild-type cells is advantageous for retinal analysis because they are virtually free of carotenoids that are present in large amounts in wild-type cells (Krinsky & Levine 1964). From irradiated FN68 cells, organic compounds have been extracted and retinoids have been identified by HPLC (Figure 2). No extractable retinal was found in cells that have not been exposed to light. Retinal was, however, detectable in irradiated cells (Figure 2B), and the amount of retinal increased with time and paralleled the increase in phototactic sensitivity (data not shown). These experiments support two earlier drawn conclusions: Retinal is the chromophore of the visual photoreceptor in *Chlamydomonas* (Foster et al., 1984), and the phototactic sensitivity is linearly proportional to the amount of retinal present in the cell (Foster et al., 1988a). When the cells had reached the wild-type sensitivity level, the retinal concentration did not further increase. Both 3-dehydroretinal and 3-hydroxyretinal, which function in some fishes, amphibians, or insects as rhodopsin chromophores (Rodiek, 1973; Vogt & Kirschfeld, 1984), have not been detected in *Chlamydomonas* cells. Retinol was detectable only after irradiation periods of more than 1 h when the phototactic sensitivity had already completely recovered (Figure 2A). Retinol seems to be a storage form of retinoids in the cell. Besides the all-trans isomers, small amounts of 13-cis, but no 11-cis, retinoids were detectable. This result was unexpected because based on phototaxis experiments 11-*cis*-retinal has been suggested as the natural retinal isomer of *Chlamydomonas* rhodopsin (Foster et al., 1984). The highest amount of extracted retinal corresponds to about 30 000 molecules/cell. Since the percentage of retinal that is bound to rhodopsin in intact cells is not known, this number does not necessarily represent the actual number of rhodopsin molecules per cell. The number is, however, within the limits of the estimates from electron microscopical determination of the intramembrane particle density in the eyespot-overlaying membranes (30 000) (Melkonian & Robenek, 1984; Smyth et al., 1989).

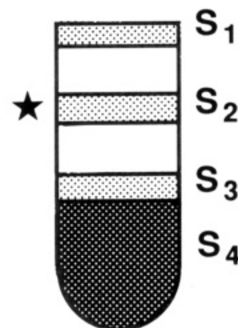

FIGURE 3: Fractionation of strain 806 cell material by sucrose density centrifugation. S2 was the retinal- and rhodopsin-containing fraction (*). S1–S3 were yellow whereas S4 was dark green.

Under the assumption that the number of rhodopsin molecules per cell is close to 30 000, the spectroscopic identification of rhodopsin seemed to be possible if starting from a 20-L cell culture. Since *Chlamydomonas* is a plant cell with a large chloroplast, it contains amounts of photosynthetic pigments and carotenoids that by far exceed those of retinal. This requires extensive membrane fractionation before retinal proteins can be identified. Sucrose density centrifugation of the cell membranes (Figure 3) and subsequent extraction with the mild detergent octyl glucoside yielded a membrane preparation (S2) having an absorption spectrum of an overall shape similar to that of bovine rhodopsin, an absorption maximum centered at 495 nm, and a half-width of about 90 nm (Figure 4A, spectrum 1).

To determine whether the major absorption in the visible range was attributable to a retinal protein, the membranes were bleached in the microspectrophotometer by prolonged irradiation with green light of the measuring beam (50 μm in diameter). In parallel, free retinal appeared as seen from the rising absorption at 370 nm (Figure 4A, panels A and B). The rhodopsin difference spectra match the absolute spectrum, including the main peak at 495 and the shoulder at 465 nm. The isobestic point at 412 nm indicates the bleaching of a single species. This kind of fine structure is highly unusual for a rhodopsin spectrum detected at room temperature, but interestingly, a similar fine structure has been found in the

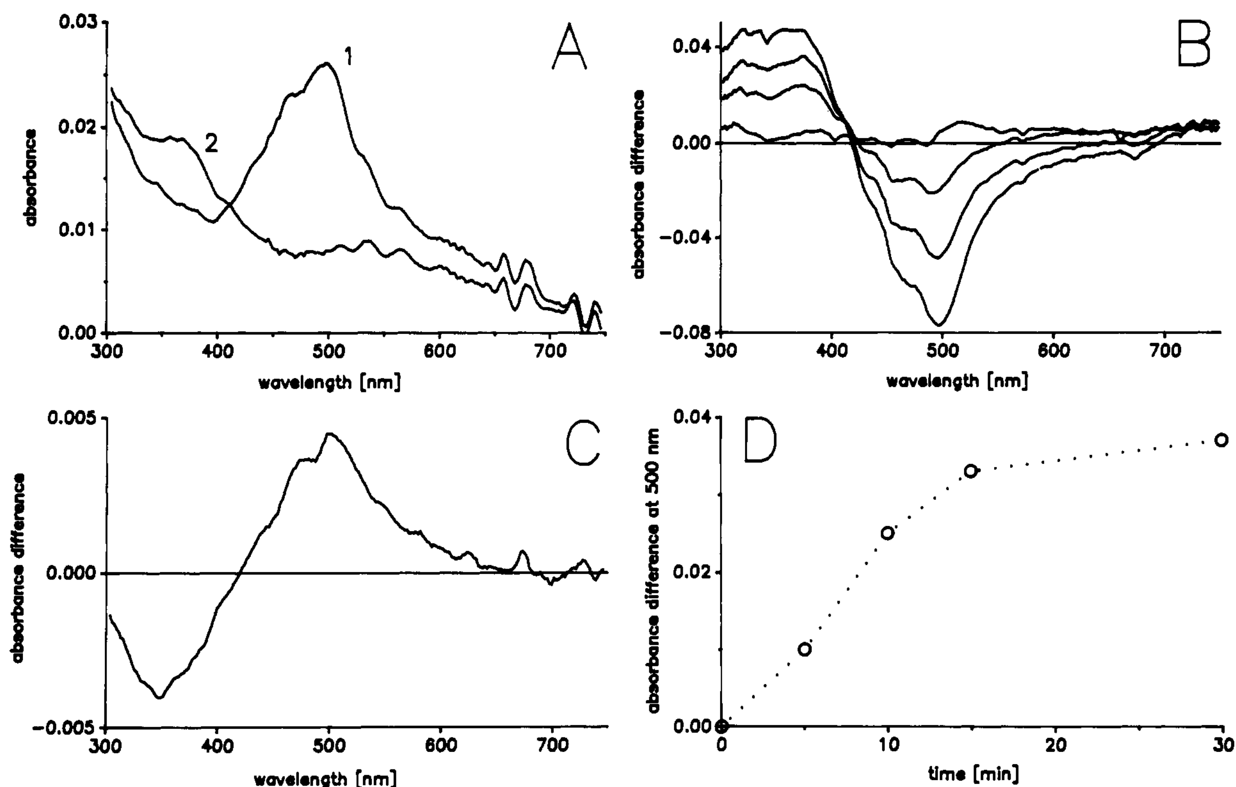

FIGURE 4: Spectroscopic identification of rhodopsin in S2 membranes from strain 806 cells. Panel A shows absorption spectra of detergent-treated S2 membranes before (1) and after 30 min of bleaching (2). Panel B shows the absorbance difference of bleached minus unbleached membranes after 1, 10, 20, and 30 min of bleaching. Panel C shows the reconstitution of bleached membranes with 1 μM *all-trans*-retinal (absorbance difference after minus before reconstitution). Panel D shows the time course of the rhodopsin reconstitution.

absorption spectrum of phoborhodopsin, the repellent photoreceptor of *Halobacteria* (Takahashi et al., 1990). The unusual band shape can be explained by the vibrational fine structure of a rigid retinal chromophore. The difference between the observed absorption maxima is 1300 cm^{-1}, which is close to the spacing expected for the vibronic transitions of polyene systems similar to that of retinal (1450 cm^{-1}; Stern & Timmons, 1970). A chromophore rigidity can originate from a forced coplanar conformation that is possible for a retinal with all-trans and 6-s-trans conformation. This has been discussed in detail for retrobacteriorhodopsin and phoborhodopsin (Schreckenbach et al., 1977; Takahashi et al., 1990). That retinal in *Chlamydomonas* rhodopsin is in a planar 6-s-trans conformation has been already suggested from in vivo reconstitution experiments with 6–7 locked retinal analogues (Foster et al., 1987).

Hydroxylamine, which has been widely used for animal rhodopsins to eliminate back-reactions after bleaching ("Dartnall effect"; Dartnall, 1968), shifted the maximum of the absorption increase to 360 nm due to the formation of retinal oxime but did not accelerate bleaching as has been found for bacteriorhodopsin (Oesterhelt et al., 1974). In detergent-treated membranes, the absorption in the visible range of the spectrum was not stable, even in darkness. It decomposed at 4 °C within a few days. Conditions have been found that allowed the reconstitution of the chromophore in the presence of exogenous *all-trans*-retinal. The spectral changes during this reconstitution process were very similar to the bleaching difference spectrum, with a maximum near 495 nm and the typical fine structure (Figure 4C). In membranes that had been left in darkness for 4 days, a rhodopsin absorption with a maximum near 495 nm had also been reconstituted, but under these conditions the fine structure was almost completely lost. This loss of fine structure was favored when MOPS was used as a buffer or the detergent was not properly washed out. The reconstitution with *all-trans*-retinal had a half-time of 7 min (Figure 4D) and was about 10 times slower than the reconstitution of light responses in blind cells (P.H., unpublished observation). The reconstitution process was much slower with 9-*cis*-, 11-*cis*-, and 13-*cis*-retinal isomers, and no clear spectra have been recorded. This result is consistent with the faster in vivo reconstitution of flash-induced light responses in blind cells with *all-trans*-retinal compared to that with the cis isomers (P.H., unpublished observation).

From the spectroscopic experiments described above, it was convincing that the major visible absorption of the final membrane preparation (S2) is due to a retinal protein.

From the reconstitution experiments, the extinction coefficient ϵ_{495nm} was determined to be around 50 000 M^{-1} cm^{-1}, which is higher than that of bovine rhodopsin (40 000 M^{-1} cm^{-1}) and comparable to that of octopus rhodopsin or halorhodopsin in *Halobacteria* (50 000 M^{-1} cm^{-1}).

The visible absorption of detergent-extracted S2 membranes is only 8 nm blue shifted compared to the threshold action spectra for phototaxis of wild-type or irradiated FN68 cells that were reported to peak at 503 nm (Foster et al., 1984) and accurately matches the action spectra for flash-induced responses (maxima near 495 nm; Uhl & Hegemann, 1990). Slight changes of the absorption maximum may be caused by physical and chemical alterations of the rhodopsin environment during preparation, as it has been observed for example in the case of gecko rhodopsin (Crescitelli, 1989) and halorhodopsin from *Halobacteria* (Grossjean & Tavan, 1987).

The total amount of the retinal protein as determined in the reconstitution assay in S2 membranes allows an estimation of at least 7200 molecules/cell. This is the lowest possible number not regarding the yield during preparation. Assuming the number of retinal protein molecules in the cell to be very

Table I: Purification Efficiency

	total protein (mg)	rhodopsin (nmol)	purification factor
total membrane fraction	150	3.5[a]	1
final preparation (OG-extracted S2)	0.11	0.85[b]	330[a]

[a] Under the assumption of 30 000 rhodopsin molecules/cell. [b] The amount of rhodopsin that can be reconstituted with *all-trans*-retinal.

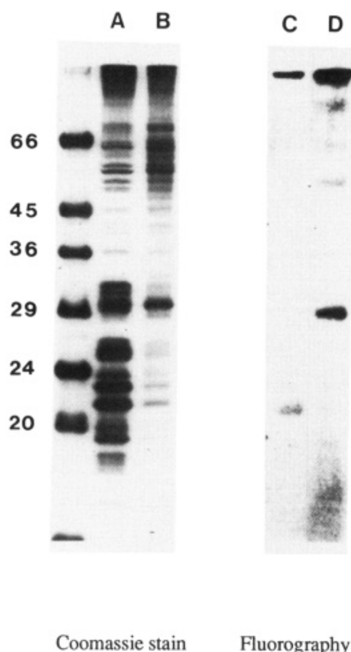

FIGURE 5: Molecular identification of rhodopsin. Electrophoretic fractionation of [^{3}H]retinal-labeled proteins of total membrane fraction (A, C) and S2 membranes (B, D) from strain 806 cells is shown: Coomassie stain (A, B) and fluorography (C, D).

close to the number of retinal molecules per cell (30 000) as it is in rod outer segments or halobacteria, i.e., a very low concentration of free retinal, the yield of *Chlamydomonas* rhodopsin in the final preparation (S2) is about 24% (starting from 30 000; Table I). This is an indirect guess, but a more accurate value is not available because rhodopsin cannot be detected and quantified in whole cells or cell extracts.

For the molecular identification of retinal proteins, radioactive retinal was used for reconstitution followed by reduction of the formed retinylidene protein into a nonhydrolyzable retinyl protein that could be identified after gel electrophoresis by fluorography. Only one retinyl protein with a MW of 32 000 was found in the S2 membrane fraction where rhodopsin has been demonstrated spectroscopically (Figure 5). As shown by 2-D electrophoresis, no other protein comigrates with the retinal protein. Its abundance in these membranes was about 25% of total protein (data not shown). In previous experiments small amounts of retinal proteins with higher molecular weights were found in membranes that were prepared by the use of Percoll gradients (Hegemann et al., 1987), but these retinal proteins have never been correlated with any retinal-dependent absorption in the visible range.

Starace and Foster (1989) briefly reported the spectroscopic identification of rhodopsin in eyespot membrane fragments from *Chlamydomonas*. In their experiments a rhodopsin absorption had been reconstituted after addition of 5 μM 11-*cis*- or 9-*cis*-retinal to bleached membranes but, in contrast to our experiments, not after addition of *all-trans*-retinal. The material they used, however, was probably quite different from the membranes described here.

Discussion

The experiments described above demonstrate the presence of *all-trans*-retinal in *Chlamydomonas* cells and a correlation between the number of retinal molecules per cell and the phototactic sensitivity of the cell population. The spectroscopic experiments identify a retinal protein absorption very similar to the action spectra for movement responses, and the labeling experiments identify a single retinal protein species in the same membrane fraction. From these lines of evidence, we strongly suggest that this retinal protein is the rhodopsin that functions as the photoreceptor for behavioral responses in *Chlamydomonas*. Since light-regulated movement responses are very similar in many unicellular algal species and the threshold action spectra are rhodopsin shaped with maxima between 470 and 510 nm (Foster & Smyth 1980; Foster et al., 1984), it can be anticipated that the *Chlamydomonas* rhodopsin is a representative of a class of rhodopsin photoreceptors that is widely distributed.

In basically all rhodopsins studied so far, light-induced absorption changes have been detected on a time scale ranging from femtoseconds to seconds. One exeption is a halobacterial species with maximal absorbance at 590 nm that bleaches in 1 mM hydroxylamine but does not show any photochemical activity in the >1-μs time range (Scherrer et al., 1987). In flash experiments with *Chlamydomonas* rhodopsin, we also have not been able to detect such absorption changes on a time scale of 100 μs to seconds after stimulation of the membranes with an intense laser flash of green light (510 nm). Thus, the conformational changes that are required for signaling in the living cell either are not connected with absorption changes in the visual range or occur on a faster time scale. One explanation for the absence of absorption changes is a rhodopsin activation without isomerization of the retinal chromophore. That neither isomerization nor the protonation state of chromophore (C=N— bond) plays an essential role for the activation of *Chlamydomonas* rhodopsin has recently been stated from phototaxis experiments with FN68 cells that have been supplemented with retinal analogues that cannot isomerize or deprotonate after retinal Schiff base formation (Foster et al., 1988b, 1989). This, however, contrasts with the general assumption that isomerization is required for rhodopsin activation (Hubbard & Wald 1952). On the other hand, application of the suction pipet technique on *Haematococcus* and *Chlamydomonas* cells demonstrated that rhodopsin regulates calcium channel activities in these two related algal species within less than 1 ms (Seneshchekov et al., 1990; H. Harz and P. Hegemann, manuscripts in preparation), indicating that the signal transduction in these algal species is a very rapid process. This requires a photoreceptor activation much faster than that in mammalian cells and may be faster than the time resolution of our flash photolysis experiments.

Acknowledgments

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Registry No. Retinal, 116-31-4.

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Guanine Nucleotide Dependent Formation of a Complex between Choleragen (Cholera Toxin) A Subunit and Bovine Brain ADP-Ribosylation Factor

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ABSTRACT: Cholera toxin activates adenylyl cyclase by catalyzing the ADP-ribosylation of $G_{s\alpha}$, the stimulatory guanine nucleotide binding protein of the cyclase system. This toxin-catalyzed reaction, as well as the ADP-ribosylation of guanidino compounds and auto-ADP-ribosylation of the toxin A_1 protein (CTA_1), is stimulated, in the presence of GTP (or GTP analogue), by 19–21-kDa proteins, termed ADP-ribosylation factors or ARFs. These proteins directly activate CTA_1 in a reaction enhanced by sodium dodecyl sulfate (SDS) or dimyristoylphosphatidylcholine (DMPC)/cholate. To determine whether ARF stimulation of ADP-ribosylation is associated with formation of a toxin–ARF complex, these proteins were incubated with guanine nucleotides and/or detergents and then subjected to gel permeation chromatography. An active ARF–toxin complex was observed in the presence of SDS and GTPγS [guanosine 5′-*O*-(3-thiotriphosphate)] but not GDPβS [guanosine 5′-*O*-(2-thiodiphosphate)]. Only a fraction of the ARF was capable of complex formation. The substrate specificities of complexed and noncomplexed CTA differed; complexed CTA exhibited markedly enhanced auto-ADP-ribosylation. In the presence of GTPγS and DMPC/cholate, an ARF–CTA complex was not detected. A GTPγS-dependent ARF aggregate was observed, however, exhibiting a different substrate specificity from monomeric ARF. These studies support the hypothesis that in the presence of guanine nucleotide and either SDS or DMPC/cholate, ARF and toxin exist as multiple species which exhibit different substrate specificities.

Choleragen activates adenylyl cyclase by catalyzing the ADP-ribosylation of the stimulatory guanine nucleotide binding protein of the cyclase system ($G_{s\alpha}$)[1] (Stryer & Bourne, 1986; Gilman, 1987; Moss & Vaughan, 1988). Toxin-cata-

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