# Algal Sensory Photoreceptors

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## Key Words

aureochrome, blue-light photoreceptors, BLUF-proteins, channelrhodopsin, enzymerhodopsin, neochrome, phototropin, photoactivated cyclase

## Abstract

Only five major types of sensory photoreceptors (BLUF-proteins, cryptochromes, phototropins, phytochromes, and rhodopsins) are used in nature to regulate developmental processes, photosynthesis, photoorientation, and control of the circadian clock. Sensory photoreceptors of algae and protists are exceptionally rich in structure and function; light-gated ion channels and photoactivated adenylate cyclases are unique examples. During the past ten years major progress has been made with respect to understanding the function, photochemistry, and structure of key sensory players of the algal kingdom.

## Contents





## INTRODUCTION

The photobiology of algae is extremely rich, unusual, and a gold mine for new proteins that may be far from our traditional thinking, which is based on knowledge from prokaryotes, higher plants, or animals. Animals are motile organisms that use their photoreceptors preferentially for orientation when they move fast, search for food, or need to avoid obstacles, enemies, etc. Plants, in contrast, are usually immobile and use their photoreceptors to adjust photosynthesis and developmental steps to optimize the photosynthetic apparatus according to the available light. Algae are photosynthetic and either mobile per se or at least during some developmental stages as gametes or zoospores. Algae need photoreceptors for their general development, as is the case in plants, for the switch between different stages of the life cycle, for the release of gametes, for controlling the photosynthetic apparatus, and for orientation during their motile life stages. Microalgae and related flagellates are especially convenient to work with owing to their fast life cycle, the ease of quantifying their behavioral responses, and in some selected cases the well-established genetics. In contrast to prokaryotes, unicellular algae are large enough to harbor optical devices for the detection of light direction, which opens attractive evolutionary aspects for the study of eye development. Thus, photoreceptors of at least some microalgae are much better suited for study than those of macroalgae.

This review does not give an overview of the general photobiology of algae; I leave this task to more qualified botanists or plant physiologists. I instead focus on the reaction mechanisms of only a few selected algal photoreceptors. Our understanding of these photoreceptors has grown tremendously during the past ten years, and their study might have a broad impact on the understanding of algal physiology and/or promote applications in other nonrelated scientific areas.

## Phototaxis and Photoactivated Electrical Processes in *Volvocaceae*

Phototaxis is the ability of motile microbes to track a light source—normally the sun—and move toward or away from it depending on the amount of light that is actually required for photosynthesis. True phototaxis requires an optical system that allows detection of the direction of the light source. The direction of the sun must be determined when light is very weak. But, it is equally important to record the light direction in conditions of intense diffuse background light. Classical tools for the study of phototaxis are microalgae with two or four flagella and a bright orange eyespot. Most studies were carried out on Volvocales such as *Chlamydomonas*, *Volvox*, and *Dunaliella*, but *Dinophyceae* such as *Gonyaulax* and *Peridinium* were also investigated quite carefully (28). Mast (59) originally recognized the eyespot in 1916 as the organelle cells use to orient their

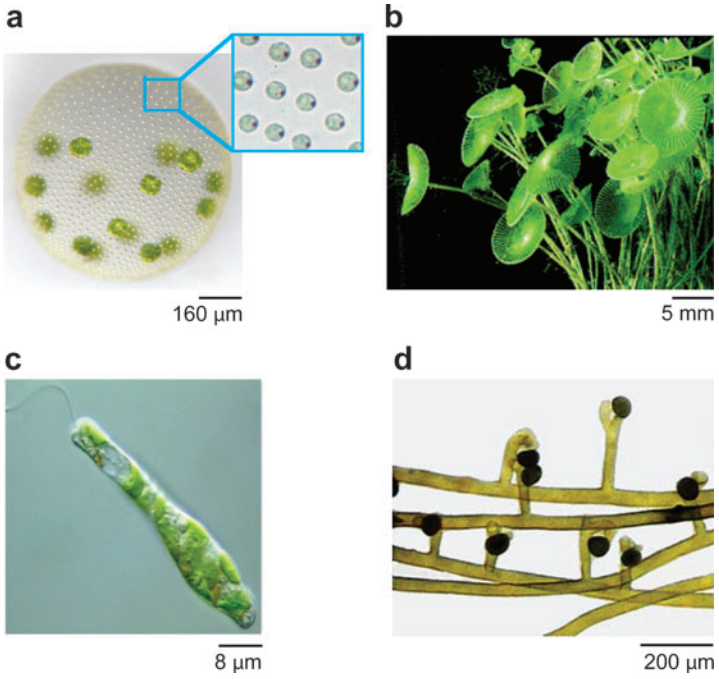


**Figure 1**

Photographs of (*a*) the colonial alga *Volvox carteri* (courtesy of Armin Hallmann); (*b*) the marine alga *Acetabularia acetabulum* (**http://hoopermuseum.earthsci.carleton.ca/Bermuda/maralga/BERM7.HTML**); (*c*) the unicellular flagellate *Euglena gracilis* (**http://www.fortunecity.de/lindenpark/hundertwasser/517/Euglena.html**); and (*d*) the filamentous alga *Vaucheria terrestris* (*Xanthophyceae*, *Chrysophyta*) (**http://www.biol.tsukuba.ac.jp/∼inouye/ino/st/x/vauch.jpg**).

swimming with respect to light incidence. Macroalgae release motile spores or gametes that are quite similar to unicellular microalgae in behavior. Phototaxis of gametes from *Ulva mutabilis* or *Acetabularia mediteranea* (**Figure 1*b***) was studied as early as a century ago (see Reference 28 and included literature).

Algae developed eyes to monitor the light direction; apparent pigmented eyespots serve as the optical system. Algal eyes may operate as simple shading devices or screens (Chrysophyceae, Xanthophyceae, Phaeophyceae, Eustigmatophyceae, Dinophyceae), quarter wave layers or quarter wave stack antennas (Chlorophyceae), lens antennas (Dinophyceae), or dielectric slab wave guides (Chryptophyceae). Foster & Smyth (19) elucidated and summarized the relevant optical principles in their fundamental review titled "Light antennas in phototactic algae."

Because algae lack a brain, the photoreceptor system must be directly connected to an output system that modulates the swimming direction according to the deviation from the desired tracking direction. Most unicellular Chlorophyceae accomplish this by placing their eye in a position that advances the beating plane of the flagella by ≈30° during rotational swimming. If they swim perpendicular to the light source, they receive a modulated signal owing to the contrast of the eyespot, and correct their swimming until the modulation amplitude is small (19). The cells modulate the flagellar beating plane, the beat frequency, and the three dimensional beating pattern in an anticyclic manner depending on changes of the light intensity (74, 75) and the

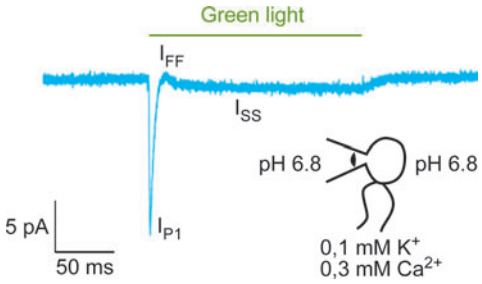


**Figure 2**

Photocurrents recorded from the eye of a *Chlamydomonas* cell at pH = 6.8. The green bar indicates the duration of the light pulse. $I_{P1}$, fast photoreceptor current; $I_{SS}$, stationary current; $I_{FF}$, fast flagellar current.

direction the cell swims with respect to the light source. The flagellar beating pattern is regulated by $Ca^{2+}$ but the exact mechanism remains unclear.

Light excitation of a *Chlorophycean* alga results in a cascade of electrical events that may be recorded from individual cells or from a cell population. Photocurrents are similar in all chlorophyceae analyzed so far, namely *Haematococcus pluvialis* (58), *Chlamydomonas reinhardtii* (33), *Volvox carteri* (5), *Polytomella* and *Spermatozopsis similis* (25), and even the freshwater cryptophyte, *Cryptomonas sp.* (85). The initial event after light excitiation is always a cation influx across the eyespot overlaying part of the plasma membrane (photoreceptor current) (**Figures 2** and **3**). A flash results in a single transient peak $I_P$, whereas the signal seen upon step-up stimulation $I_P$ is followed by a small stationary current $I_{SS}$ [reviewed by Sineshchekov & Govorunova (82)]. The most amazing and most discussed property of the photoreceptor current is that it starts without any detectable delay ($\tau < 50$ μs). The photocurrent peaks after 1–2 ms and decays within approximately $\tau \leq 20$ ms, depending on the flash energy (12, 40, 85, 87).

In a *Chlamydomonas* cell, the amplitude of the photoreceptor current $I_P$ may reach 40 pA, which is 50% of the total current, if it is recorded directly from the eye. This value corresponds to $10^6$ elementary charges entering the eyespot area. Taking a membrane capacity of 1 μF/cm$^2$ into account, the cell is transiently depolarized by approximately 80 mV (34). If the number of rhodopsin molecules per eye is in the range of $10^4$ to $10^5$, as determined from freeze fracture images of the eye (63), by retinal extraction (1), and recent calculations of the number of rhodopsin molecules per cell (26), each photoreceptor channel would carry only 10 to 100 charges after a saturating flash. Thus, it is more than sufficient for the cell if one photon activates a single ion channel. There is simply no need for any chemical signal amplification, as in animal vision, as we pointed out many years ago (34).

Several aspects of the recorded photocurrents are still a matter of debate, resulting mainly from different recording techniques used in the different laboratories. One such aspect is the rise of the photoreceptor current, which is a critical issue for understanding the mechanism of ion channel activation. In single cell experiments at saturating light flashes (>$10^{21}$ photon m$^{-2}$s$^{-1}$ green light), the photoreceptor current $I_P$ rises monoexponentially with a time constant τ of 0.2 ms and reaches the peak after 1.5 ms (11). There is no indication of a second component (12, 40) (**Figure 3**). This extremely fast rise led to the direct coupling model (DCM), which proposes that the rhodopsin and ion channel are directly coupled, forming a single protein complex (34). Photocurrents of chlorophycean algae were also retrieved from free-swimming cells in populations (83, 84). The advantage of this technique is that currents can be recorded from wild-type cells, whereas single cell measurements are restricted to cell wall–deficient mutants (82). In population experiments the photoreceptor current rises biphasically, which the Sineshchekov group (86, 88) interpreted as follows: The initial slow rise is a charge movement within the photoreceptor molecules, whereas the second component is the current that flows through a secondary channel (remote coupling model, RCM). However, the population assay is a

**Direct coupling model (DCM):** implies that the photoreceptor current of a chlophyceaen eye is carried by cations conducted by channelrhodopsins

**Remote coupling model (RCM):** implies that the charge movement within channelrhodopsin is only the trigger for activation of a secondary ion channel

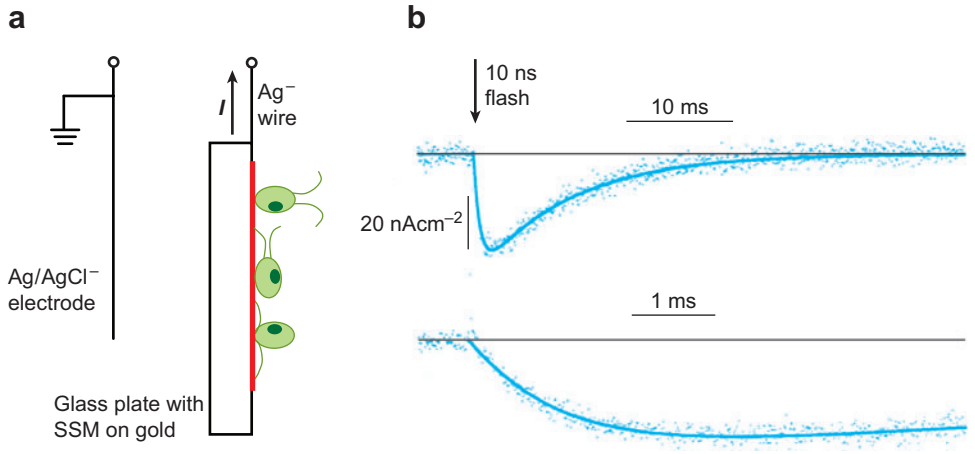


**Figure 3**

(*a*) Schematic representation of *Chlamydomonas* cells adsorbed to a solid supported membrane (SSM), prepared according to Reference 80 (1.5% phosphatidylcholine, 0.1% phosphatidylserine). (*b*) Flash-induced photocurrents recorded at higher time resolution ($\lambda = 530$ nm). Only one fifth of the recorded data points are shown. Exponential function is shown as a solid line, taken from Reference 44.

differential assay; it records differences between cells oriented differently with respect to the light source or between cells of different sensitivity. The resulting current is less than 0.01% of the total current (1 nA from $>10^7$ cells) (83). This extremely small fraction makes an interpretation of the signals extremely difficult.

Another so far underestimated approach for recording photoreceptor currents with excellent time resolution from *Chlamydomonas* cells is the use of black lipid membranes (BLM) or solid supported membranes (SSM) (**Figure 3*a***). An SSM consists of a lipid monolayer on a gold-sputtered glass substrate, coated with a long-chained mercaptan [$CH_3(CH_2)_nSH$, n = 15, 17] (80). *Chlamydomonas* cells were adsorbed to the SSM and photocurrents recorded in a capacitive mode from a few hundred cells with high time resolution. The rise of the photocurrent is clearly monoexponential (44) (**Figure 3*b***), consistent with the DC-model.

The kinetics of the photocurrent does not necessarily reflect the kinetics of the photoreceptor, nor does it parallel the conductance change of the plasma membrane, especially not at high light intensities. The ion influx transiently depolarizes the cell and causes a rapid reduction of the influx. Thus, the current peaks earlier and decays faster at high flash energies. The key properties of the current are as follows: (*a*) high light saturation, (*b*) extremely fast rise, and (*c*) voltage-dependent decay; these properties were explained by the presence of a directly light-activated rhodopsin–ion channel complex (40). The DCM was the basis for the identification of light-gated channels (see below).

The identity of the ions that carry the algal photoreceptor current is also a matter of debate since the discovery of photocurrents in *Haematococcus* (58). In *Chlamydomonas* at neutral pH the photoreceptor current is mostly carried by $Ca^{2+}$, but $H^+$ and $K^+$ also contribute when the driving force is high enough, i.e., at low pH or high extracellular $K^+$ (12, 40, 70). Meanwhile, it is clear that $H^+$ is by far the best-conducted ion but owing to low $H^+$ abundance, $Ca^{2+}$ contributes the most to the current under nearly all physiological conditions.

Upon step-up stimulation, photoreceptor currents show a transient phase that decays to

**SSM:** solid supported membranes

**Channelrhodopsin (ChR):** rhodopsin with intrinsic cation conductance that is regulated/gated by retinal isomerization (after light absorption)

a stationary level, $I_{SS}$, of a few pA, which is only a percent of the transient current. This adaptation makes sense for an alga that may be permanently exposed to high light intensities during the day. Despite our knowledge from in vitro studies of photoreceptor inactivation in stationary light (see below), the more extreme adaptation of the photoreceptor current in vivo remains quite unclear.

After a light flash a secondary $K^+$ efflux repolarizes the cell toward the $K^+$ equilibrium potential; this secondary $K^+$ efflux is in equilibrium with the local $Ca^{2+}/H^+$ influx in continuous light. Steady state currents deserve more attention because modulated stationary currents constitute the basis for phototaxis at high light levels. Owing to the extremely small amplitude steady-state currents have not yet been studied in detail.

**Flagellar currents.** When the integral of the photoreceptor current exceeds a critical level in unicellular algae, flagellar currents are triggered. Because unicellular flagellates are nearly isopotential, the flagellar channels sense the primary depolarization originating from the eye. Thus, flagellar current activation does not require signal amplification or a transmitter. In *C. reinhardtii* and *H. pluvialis* researchers observed a fast action potential–like flagellar current, $I_{FF}$. $I_{FF}$ is the trigger for switching from forward to backward swimming (phobic response) (78), as shown by simultaneous detection of photocurrents and flagellar beating (41). At low intensity flashes or dim continuous light, when no $I_{FF}$ currents appear, flagella undergo only weak beating changes without changing the beating mode. The signaling that occurs between the eyespot and the flagella under these low-light conditions remains unclear. Because depolarization is in the range of only 1 mV at 1% rhodopsin bleaching, modulation of the flagellar beat frequency and the beating plane might be supported by intracellular signaling from the eye to the flagellar base (88).

Freshwater algae have developed special mechanisms for surviving in conditions of low extracellular ionic strength. At low extracellular $Cl^-$, a $Cl^-$-efflux $I_{Cl}$ can be observed between $I_P$ and $I_F$. Because photocurrents are conventionally recorded at a few mM $Cl^-$, which inhibits the $Cl^-$ efflux, this current is hidden in most experiments (11, 32).

## CHANNELRHODOPSIN

### Physiology

The clear demonstration that the photoreceptor for phototaxis is rhodopsin came originally from action spectroscopy on white chlorophyll- and carotenoid-deficient strains. After complementation of these blind cells with retinal or analogous compounds, phototaxis recovered within one minute and the action spectrum shifted depending on the retinoid used (18). After researchers excluded the two animal rhodopsin–related proteins chlamyrhodopsin-1 and chlamyrhodopsin-2, which occur in the algal eyespot as dominant proteins, as phototaxis photoreceptors (20), three research groups independently identified two microbial rhodopsin–related sequences in the *Chlamydomonas* cDNA database. The encoded proteins were named channelrhodopsin 1 and 2 (ChR1 and ChR2), *Chlamydomonas* sensory rhodopsin A and B (CSRA and CSRB), or archaeal-related sensory rhodopsin 1 and 2 (ASR1 and ASR2); the genes in the *Chlamydomonas* database are known simply as *Chlamyopsin 3* and *4* (*COP3* and *COP4*). This confusion is a consequence of the closed deposition of gene names into the genome database. Sineshchekov and colleagues (26, 86) immediately employed an antisense approach. Cells with reduced ChR1 or ChR2 content showed small photoreceptor currents and were less light sensitive than wild-type. The authors recorded action spectra with two maxima; in cells with reduced ChR1 content the photocurrent action spectra reshaped in favor of the 470 nm side peak. Although the action spectrum bands are quite narrow compared with rhodopsin absorption spectra, the initial conclusion that ChR1

absorption peaks at approximately 500 nm whereas the ChR2 absorption peaks at 470 nm is correct and has been confirmed by in vitro studies. The second important finding that resulted from these early experiments on ChRs was that phobic responses are impaired in cells with reduced ChR content, independent of the species or the ChR isoform used (26, 86). The extent to which phototaxis is supported by ChR1 and ChR2 remains unclear, and more behavioral studies that employ strains completely lacking one of the ChRs are urgently needed. But as long as nuclear gene targeting is not routinely possible in *Chlamydomonas*, such mutants might remain an unrealistic dream. In any case, the finding that two similar photoreceptor proteins are involved in behavioral responses explains the broad-banded high-intensity *Chlorophycaean* action spectra of earlier times (28).

ChRs are light-gated ion channels that belong to the class of microbial-type rhodopsins because they occur in archaea, eubacteria, and fungi; bacteriorhodopsin (BR) is the prototype of this class. Amino acids that form the $H^+$-conducting network in BR are conserved, but are not connected to the intracellular or extracellular bulk phases. Thus, the conserved $H^+$-network was considered to be part of the switch between the closed and open states of the channel, but was not considered as the conducting pore (51, 90). ChRs exhibit some striking features that distinguish them from other microbial rhodopsins (ion pumps and sensors). ChRs contain six glutamates in helix two as well as many cysteines and serines distributed all over the hypothetical transmembrane regions. This increase in polar residues is partially compensated for by an excess of aromatic amino acids. The increase in polarity leads to the expectation of higher water content in the transmembrane region, which is necessary for a functional channel. In the 3D models that were calculated on the basis of the bacteriorhodopsin X-ray structure, the polar groups of helix two point to the outside of the ring formed by the seven transmembrane (7TM) helices, toward the lipid face (37). A ChR trimer may be the functional unit and the conducting pore may be formed at the contact site between three monomers. However, a trimer as a functional unit would suggest cooperativity in ChR activation, which has not been found (36, 72). Thus, on the basis of the experimental results, the channel is most likely a monomer and the 3D models are probably incorrect with respect to helix two.

## Transport Activity

Two channelrhodopsins from *Chlamydomonas* and one from *Volvox carteri* (GenBank Accession No. DQ094781) were functionally studied in host cell systems such as *Xenopus* oocytes, HEK cells, and PC12 cells (36, 48, 66, 67). Flash stimulation generates a photocurrent that rises with a time constant of roughly 200 μs, peaks after 1.5–2 ms, and decays with $\tau \approx 15$ ms under standard conditions (**Figure 4*a***). The photocurrent kinetics resemble photocurrents from intact algae, although the latter decay faster owing to the unclamped potential. Stimulation of ChR with light pulses in host cells under voltage clamp generates photocurrents that relax toward a stationary level, as seen in **Figure 4*b***. Compared with ChR1, ChR2 inactivates more strongly in constant light and especially at high extracellular pH (pHo) and low membrane voltage. All three known ChRs conduct primarily protons and, to a lower extent, cations, with variable efficiency. In the first publication the cation conductance of ChR1 was overlooked (66). The calculated unitary conductance (for example, defined for 1 mM substrate and –100 mV) is more than 100-fold larger for $H^+$ compared with $Na^+$ or $K^+$. In freshwater algae, $Ca^{2+}$ is in most cases more available than any other cation, and because ChR binds $Ca^{2+}$ very tightly ($k_M$ = 100 μM), $Ca^{2+}$ needs specific consideration. Larger photocurrents recorded from ChR2-expressing cells compared with those from ChR1-expressing counterparts (68) are due to higher expression levels and are not caused

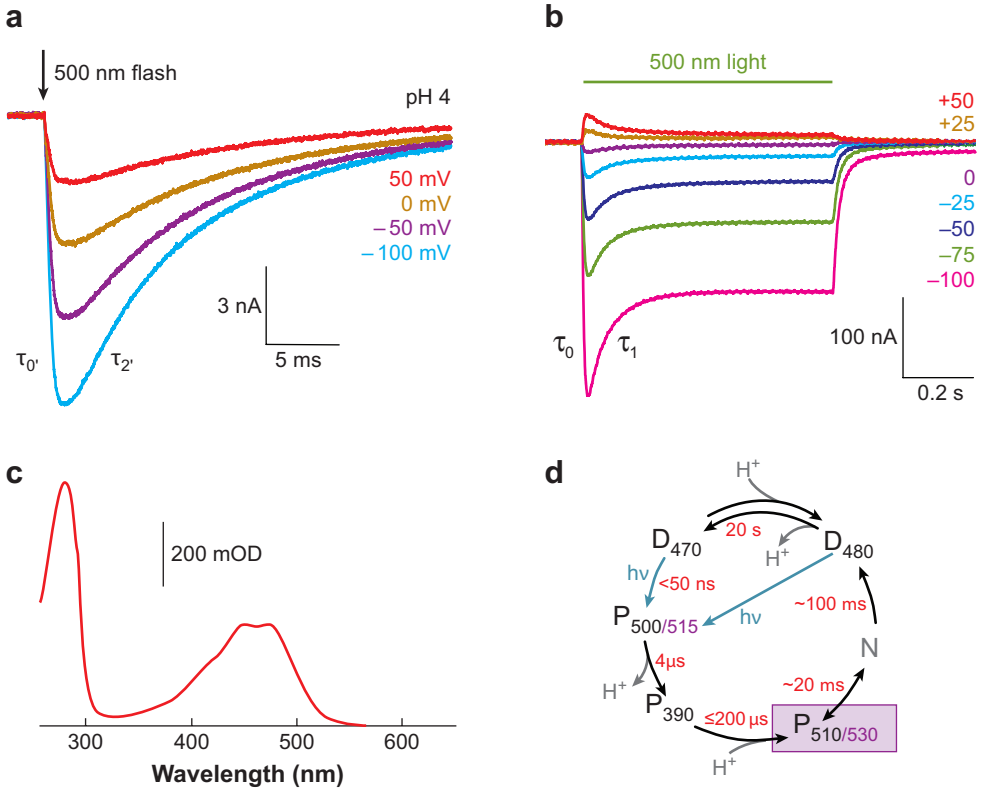


**Figure 4**

(*a*) Flash-induced currents recorded from *Volvox carteri* channelrhodopsin (VChR1/2)-expressing *Xenopus* oocytes (10 ns). (*b*) Photocurrents in response to 600 ms light pulses at variable membrane voltage. (*c*) Absorption spectrum of ChR2 (purified from COS cells) at pH 6. (*d*) Simplified reaction scheme of VChR from spectroscopic data. $P_{510}$ is the conducting state that absorbs maximally at 530 nm under acidic conditions (adapted from Reference 16).

by a higher unitary conductance (S.Tsunoda, unpublished data).

## Photochromism and Photoactivation

Although ChR2 was functionally expressed in a variety of mammalian cell culture systems and used for the generation of action potentials with light (see below), ChR only very recently could be expressed and purified in sufficient amounts to allow a preliminary spectroscopic analysis. VChR was expressed in COS1 cells (green monkey kidney cells) and functionally purified, which made it possible to record absorption spectra of the dark states. The VChR spectrum is vibrationally fine-structured with a maximum at 470 nm ($D_{470}$) (**Figure 4*c***). $D_{470}$ is in equilibrium with a $D_{480}$ species in a pH-dependent manner. The ratio $P_{480}/P_{470}$ increases at low pH and after preillumination (light-adaptation). Excitation of $D_{470}$ with laser flashes causes immediate absorbance changes ($\tau \leq 100$ ns) and the appearance of a red-shifted photoproduct. This early photoproduct, $P_{500}$, deprotonates with $\tau = 4$ µs owing to the formation of $P_{390}$, which reacts further to form $P_{510}$ with $\tau = 200$ µs. $P_{510}$ is considered to be the main conducting state, G1. If this is true, the channel should be gated within 200 µs after light absorption (16). $P_{480}$ undergoes a photocycle with $P_{530}$ as the dominant intermediate but without

visible deprotonated species. The photocycle model, as seen in **Figure 4*d***, unifies photocurrents measured under different light, pH, and voltage conditions with spectroscopic data.

Despite the enormous progress made toward understanding ChR1 and ChR2 since the appearance of the two sequences in the expressed sequence tags (EST) database, we cannot make a clear discrimination between the DCM and the RCM from the experiments carried out so far. J.L. Spudich and colleagues (26, 50, 86) argued that ChR might not work as a channel in vivo but instead may activate a transducer or a remote channel, as sensory rhodopsins do in archaea and eubacteria. This hypothesis cannot be ruled out and would not even be possible to test via the use of ChR knockout mutants. Only the extreme similarity of flash-induced ChR photocurrents in oocytes or HEK cells to the photocurrents measured from single *Chlamydomonas* or *Volvox* cells makes it quite unlikely that ChR does not carry the photocurrents in the living alga. That other signaling systems may contribute to the phototactic response at low light is certainly not in doubt.

## Application to Neuroscience

Originally, three groups tested the potential of ChR to control nerve cell firing (2, 4, 48, 57). They all used ChR2 because no cation conductance has yet been reported for ChR1 and the photocurrents in ChR2-expressing oocytes were larger than their ChR1-expressing counterparts (68). All three groups demonstrated that in cultured hippocampal neurons, as well as in hippocampal slices, light pulses of only a few ms in duration are sufficient to trigger action potentials. Moreover, neurons can follow light protocols with action potential firing up to 30 Hz. These observations nicely correlate with the decay of the photocurrent ($\tau = 20$ ms). "It is a very simple system, because all you have to do is express this one protein, and now you can control the activity of neurons with light," said the neurophysiologist Edward Callaway (64). Retinal is made in most mammalian cells on demand and there is no need to add retinal to the culture. Meanwhile, researchers have expressed ChR2 in *C. elegans* (65), chicken embryos (57), and mouse neurons (95), and action potentials could be specifically generated in neurons from all species by the application of light, either directly or via thin optical fibers. When Bi and coworkers (2) delivered ChR2 to the retina of a blind mouse using lentivirus as a vector, they found that light could elicit electrical activity similar to that of a seeing animal (prosthetic vision).

By combining ChR2 with halorhodopsin (HR), an eubacterial light-driven $Cl^-$ pump that absorbs light red-shifted 100 nm compared with ChR2 and hyperpolarizes the cells in the light, the cellular membrane voltage could be controlled even more precisely and driven in both directions by changing the intensity and the ratio of 470/570 nm light (29) (**Figure 5**). Applications of ChR were already discussed in a larger context in References 38 and 98. Two limitations exist for the wider application of ChR in neuroscience and technology: First, ChR conducts only up to a few hundred ions per absorbed photon, summing up to a current of a few femtosiemens per ChR in continuous high intensities. This limitation can be overcome by high expression levels, by tailoring ChR toward a larger conductance, or possibly by purifying ChR from larger algal cells that require larger cation influxes for depolarization. Second, ChRs that absorb green or even red light would be extremely desirable. This could be achieved through modification of the retinal binding pocket (39) or the expression of ChR from algae with red-shifted action spectra, such as *Prorocentrum* (maximum at 570 nm) (28).

## Ion Pumps and Enzyme Rhodopsins

The first algal rhodopsin purified in sufficient amounts for direct flash photolysis analysis

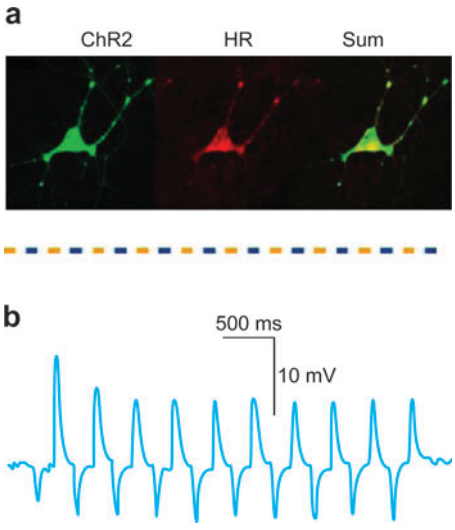


**Figure 5**

(*a*) Expression of algal channelrhodopsin 2 (ChR2), fused to the red fluorophore mCherry, and bacterial halorhodopsin (HR), fused to GFP, in a hippocampal neuronal cell. (*b*) The cells were stimulated alternatively with yellow and blue light; the graph shows hyperpolarization (downward signal) and depolarization depending on HR and ChR activation. (adapted from Reference 29, under provision of the Creative Commons Attribution License).

was a rhodopsin from the marine cryptophyta *Guillardia theta* (GtR1) (85). *Guillardia theta* is a protist and is not classified as a plant. The plastid originates from a rhodophyte. The rhodopsin was functionally expressed in *E.coli*. In flash photolysis experiments GtR1 behaves similarly to the well-characterized archean rhodopsins in that it shows K-, M-, and O-like photocycle intermediates and a photocycle turnover time with $\tau = 80$ ms. GtR1 lacks proton pumping activity (or passive channel activity), although the amino acids specific for a light-driven proton pump are conserved. A microbial-type rhodopsin gene was also found in the dinoflagellate *Pyrocystis*. This rhodopsin is controlled by the internal clock but no information about other functions is available (71). A rhodopsin with proven proton pumping activity was found in the marine Ulvophyceaen alga *Acetabularia acetabulum* (**Figure 1*b***). In *Xenopus* oocytes *Acetabularia* rhodopsin (AR) pumped protons out of the cell in the light under all tested conditions, with an action spectrum maximum at 518 nm (94). AR is the first ion-pumping rhodopsin found in a green plant organism; it is very similar to bacteriorhodopsin and its eubacterial relatives. Discouragingly, no information could be collected regarding the in vivo function of either rhodopsin from *Guillardia* or *Acetabularia*. Mechanistically these rhodopsins may prove uninteresting because they represent no new principle, but it would be intriguing to discover why photosynthetic algae need a light-driven proton pump.

Another new subfamily of algal rhodopsins is defined by rhodopsin sequences that contain extremely long C-terminal extensions. These sequences encode large proteins that comprise a microbial sensory rhodopsin, a histidine kinase, a response regulator, and an effector protein, such as a nucleotide cyclase [adenylate or guanylate cyclase (AC or GC)] (**Figure 6**). These rhodopsins present a classical two-component system, which is widely used in prokaryotes for a variety of signaling processes and in eukaryotes, including plants, for very selected purposes (45). The unusual feature of the algal sequences is that all the elements, from the photoreceptor to the final enzymatic output domain, are assembled in one protein. Examples include chlamyrhodopsin-5, -6, and -7 (CR5, CR6, and CR7) (51). CR5 and CR6 contain cyclase domains, whereas the function of the terminal enzyme domain of CR7 remains unclear. Researchers have found related enzyme rhodopsins in the genome of *Ostreococcus taurii*. *O. taurii* belongs to the *Prasinophyceae*, an early diverging class within the green plant lineage, and is a globally abundant, single-celled alga thriving in the upper (illuminated) water column of the oceans. The most striking feature of *O. tauri* and related species is their minimal cellular organization: They consist of a naked, nearly 1 micron cell without flagella, but with a single

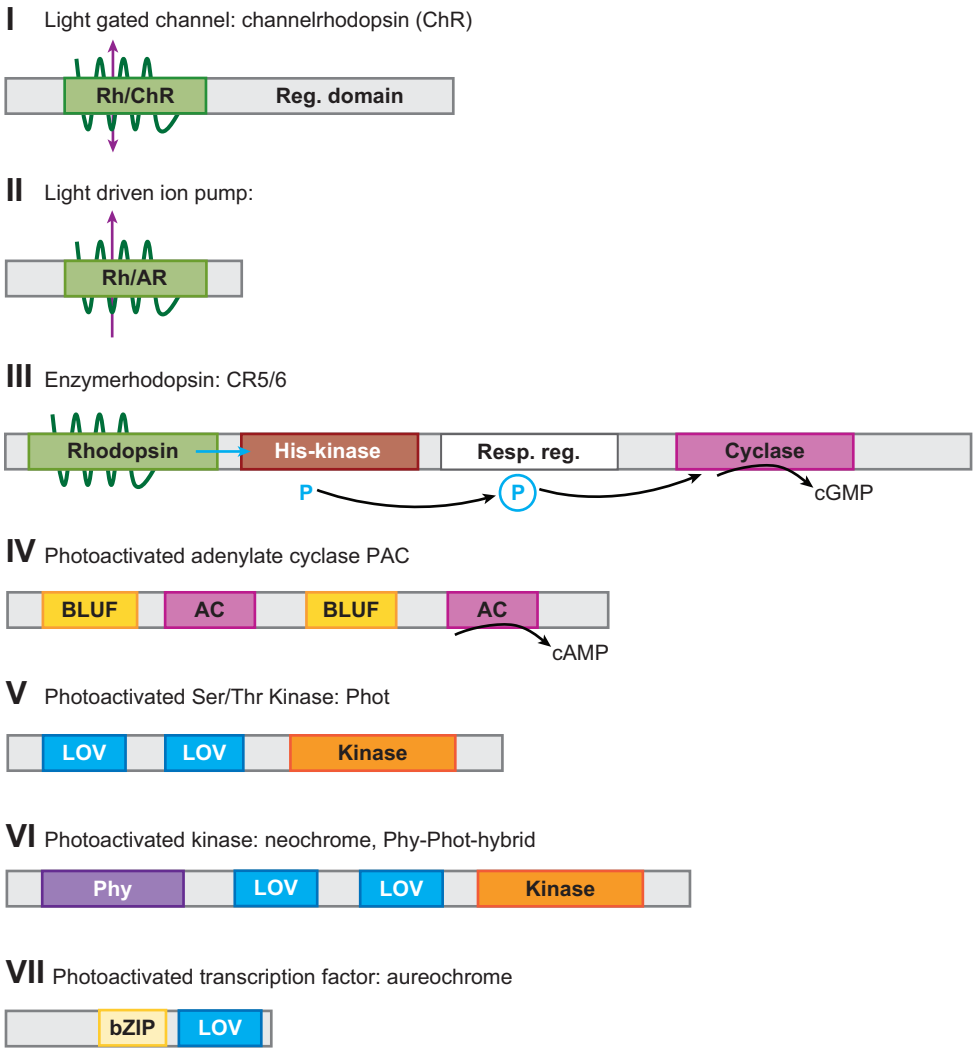


**Figure 6**

Seven principal sensory photoreceptor types as they occur in algae and/or flagellates (protists). Rh, rhodopsin; ChR, channelrhodopsin; AR, Acetabularia rhodopsins; Resp.reg, response regulator; BLUF, blue-light receptor using flavin adenine dinucleotide (FAD); AC, adenylate cyclase; LOV, light, oxygen, voltage sensor; Phy, phytochrome; CR, chlamyrhodopsin; AUREO, Aureochrome; bZIP, basic-region/leucine-zipper.

chloroplast and mitochondrion. Researchers have defined three different ecotypes or potential species on the basis of their adaptation to light intensity. One ecotype (*O. lucimarinus*) is adapted to high light intensities and corresponds to surface-isolated strains. The second (RCC141) is a low-light species that prefers deep areas of the water column. The third (*O. tauri*) belongs to a group of strains isolated from a coastal lagoon and is considered to be light polyvalent. All three enzyme rhodopsin sequences are highly interesting but so far their function is unknown (S. Kateriya, personal communication).

**FAD:** flavin adenine dinucleotide

**PAC:** photoacivated adenylate cyclase

**Light-regulated enzyme:** Fusion of photoreceptor and enzyme; the photoreceptor domains activate or drastically stimulate the activity of the enzyme in the light

## PHOTOACTIVATED ENZYMES FROM *EUGLENOPHYTA*

### Function and Physiology

Besides chlorophycean algae, the flagellate *Euglena* (especially *E. gracilis*) (**Figure 1*c***) is the classical organism for the study of photomovement responses in lower eukaryotes (6, 13, 60). *Euglenozoa* are protists and are not considered to be plants. In *Euglena* the sensory photoreceptor for behavioral responses is concentrated in the swelling at the base of the long flagella, called the paraflagellar swelling (PFS) (**Figure 7*a***). The PFS is a three-dimensional crystal; Piccinni & Mammi (73) recorded refractive reflexes of the flagellar membrane many years ago. After 20 years of unsuccessful attempts by many research groups to functionally purify the photoreceptor, the Watanabe group (47) finally succeeded. Their breakthrough involved denaturation and subsequent acidification of the *Euglena* samples. Flavin adenine dinucleotide (FAD) becomes brightly fluorescent at low pH, allowing easy quantification of the photoreceptor complex. The photoreceptor is a heterotetrameric complex of two homologous photoactivated adenylate cyclases (PAC), PACα and PACβ. The purified complex shows basic adenylate cyclase activity that is stimulated 80-fold by light (47, 96). PAC proteins define a new family of directly light-stimulated enzymes.

To gain insight into the evolution of this unique light-regulated enzyme, researchers

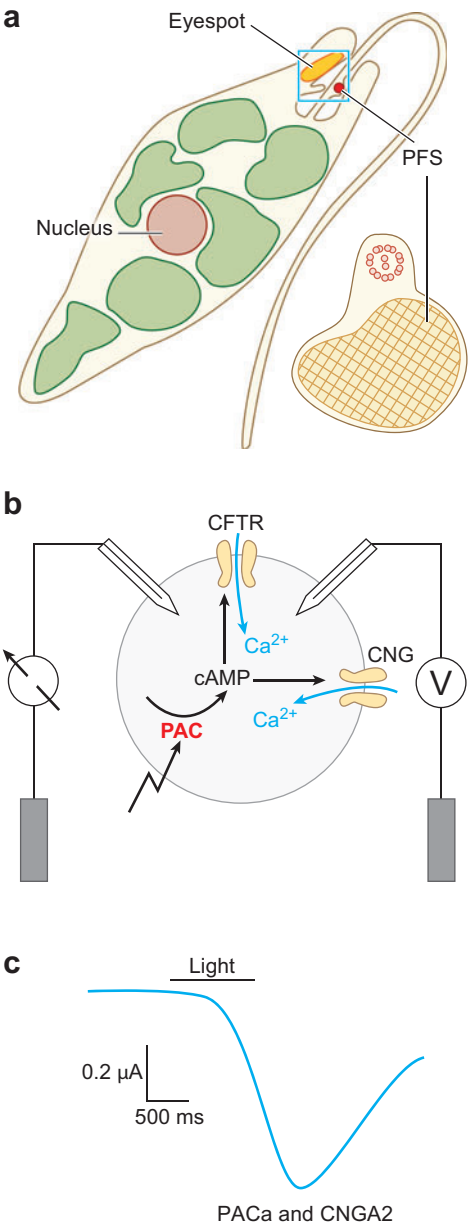


**Figure 7**

(*a*) Scheme of *Euglena*. The crystalline paraflagellar swelling (PFS) is attached to the base of the long flagellum as seen from the magnified view. (*b*) Time-resolved studies on photoactivated adenylate cyclase (PAC) in *Xenopus* oocytes. Broken arrow depicts light pulse. In this assay, PAC is expressed in conjunction with the cystic fibrosis transmembrane conductance regulator (CFTR) in such a way that cyclic adenosine monophosphate (cAMP) activates a cAMP-dependent protein kinase (PKA), which subsequently modifies and activates CFTR. This assay is highly sensitive but relatively slow. Alternatively, PAC can be expressed along with a cAMP-sensitive cation channel from olfactory neurons that directly opens upon binding of cAMP. The latter approach is faster but less sensitive; an example result is given in *c*. Adapted from Reference 79, with permission from the Nature Publishing Group. Abbreviation: CNG, cyclic nucleotide–gated channel.

searched for related sequences in various euglenoids by RT-PCR. Two PAC-like transcripts were found in four phototrophic euglenoids: *E. sideropus*, *E. viridis*, *E. gymnastica*, and the osmotrophic species *Khawkinea quartana*. All the encoded proteins are expected to function as light-activated cyclases. Because a phylogenetic analysis revealed that the cyclase domains all belong to a bacterial cluster, the authors proposed that PAC might have been transferred to Euglenoids during secondary endosymbiosis (55).

## In Vitro Analysis of Photoactivated Adenylate Cyclase and Related Proteins

Photoactivated cyclases are modular proteins; each comprises two photoreceptor domains (F1 and F2) as well as two catalytic domains (C1 and C2) (**Figure 6**). The F domains bind FAD and belong to a group of blue light receptors using FAD (BLUF) that is widely distributed in prokaryotes but rare in eukaryotes (24). The first identified BLUF member is AppA (for activation of photopigment and puc expression), which functions as a blue light–dependent derepressor of photosynthesis genes in *Rhodobacter sphaeroides* (23). The photochemistry of BLUF is radically different from that of traditional sensory photoreceptors such as rhodopsins and phytochromes. In rhodopsin and phytochrome the isomerizing chromophores react similarly in solution and in the protein, whereas flavoproteins generally transfer electrons and the reaction is quite variable depending on the protein environment or the reaction partner. Upon light excitation BLUF undergoes a conformational change (seen as a *ca*. 10 nm red-shift in absorption) but the chromophore remains in its oxidized state (49, 61). The photocycle is slow and requires several seconds to recover to the dark state. In contrast, the primary photochemistry of BLUF domains is ultrafast and the red-shifted signaling state is reached after a few picoseconds (22). Excitation of FAD from the electronic $S_0$ to the $S_1$ state is followed by an electron transfer from a conserved Tyr in the chromophore binding pocket to FAD to form the radical anion $FAD^{*\bullet-}$. Next, a $H^+$ is transferred, most likely onto N5 to form the neutral radical $FADH^{*\bullet}$. This neutral radical causes isomerization of a conserved Gln and subsequent modification of the hydrogen bonding network between FAD and associated amino acid residues (62). The positions of the reactive Tyr and Gln are conserved in the different species and are precisely known from four available X-ray structures (97). The radical species were visualized only in the BLUF domain of the Slr1694 protein from *Synechocystis sp*. PCC6803 (21), but there is no doubt that the results are valid for all BLUF-domains, including those of the PAC.

Unfortunately, neither electrical measurements nor fluorescence imaging of $Ca^{2+}$ has been successful on any *Euglenoid* flagellate. The invaginated cell wall and the underlying plasma membrane make the cell surface inaccessible for a suction pipette and impermeable for $Ca^{2+}$-dyes such as Fura or Fluo. To study PAC function further, researchers injected PAC complementary RNAs into *Xenopus* oocytes. Oocytes are wonderful systems for studying algal proteins because the proteins are generally well expressed even if expression in prokaryotes and yeast is impossible. The kinetics were monitored electrically by the coexpression of various cAMP-sensitive ion channels (**Figure 7*b*** and ***c***). Both PAC variants are active but PACα is 100-fold more active than PACβ (79). The reason why PACβ is less active remains unclear and more detailed studies are needed to determine how the BLUF-domains activate the enzyme.

Light-activated enzymes such as PAC are as valuable for applied science as ChRs, because cAMP is a key messenger in all eukaryotes. The PAC expression level needed for light stimulation of cAMP in the host cell is small. Nagahama and coworkers (47) carried out the first experiments in this direction. Serotonergic modulation of sensory neurons

**BLUF:** blue light photoreceptors using FAD

**cAMP:** cyclic adenosine monophosphate

**Phototropin protein (Phot):** photoreceptor comprising two FMN-binding LOV-domains and a C-terminal kinase; regulates hypocotyl phototropism, chloroplast relocation and guard cell opening in higher plants as well as sexual differentiation in algae in blue light

**LOV:** light, oxygen, voltage

**FMN:** flavin mononucleotide

in *Aplysia* increases intracellular cAMP levels and promotes synaptic transmission by increasing the spike width. Expression of PAC in motor neurons and subsequent illumination with blue light decreases the spike amplitude and increases the spike width, which demonstrates that PAC successfully modulates cAMP levels in living animals.

What is the next step? To gain a profound understanding of the primary processes of BLUF at the same level of detail as is available for the primary processes of photosynthesis or retinal proteins (bacteriorhodopsin), we first need molecular dynamic (MD) calculation of the flavin in its protein environment on the basis of available X-ray structures. Second, to gain a deeper insight into the dynamics of the amino acid side chains that interact with the isoalloxacine, femtosecond stimulated Raman experiments (56) and femtosecond infrared spectroscopy (27) are required. No other photoreceptor shows such a complex behavior on an ultrafast time scale and so little change on a longer time scale. To study the signal transfer to the effector domains, the PACs from protists are ideal, because most other BLUFs act on partner proteins, most of which are unknown and/or are only in transient contact with the photoreceptor.

## PHOTOTROPIN

Phototropins (Phot) are light-activated serine/threonine kinases. These proteins were originally discovered in *Arabidopsis* and other higher plants, where they control phototropism, chloroplast relocation, and stomatal opening, among other functions (8). Phot sequences were found in many algae but these proteins were studied functionally only in *Chlamydomonas* to some extent. In this alga, differentiation from the vegetative state into the sexually active state (gametic state) is mediated by nitrogen starvation in combination with blue light (76). Gametes of different mating types fuse to form zygotes and when the conditions are appropriate, zygotes germinate to release the progenies. However, the progeny release is again light-dependent and blue light is more efficient than other colors. Beck and colleagues (42, 43) localized Phot in the cell body and the flagella (4%). By comparing the development of wild-type and Phot-antisense strains at low photon irradiance, they showed that Phot is the photoreceptor responsible for both developmental processes (42, 43). Unlike higher plant phototropins, the activation of *Chlamydomonas* Phot causes major changes in expression of specific gene targets, in particular genes encoding chlorophyll and carotenoid biosynthesis enzymes (42, 43, 46). The formation of mating-competent gametes is accompanied by a loss of chemotaxis toward ammonia (7). Phototropin in *Chlamydomonas* gametes also seems to mediate the light control of this type of chemotaxis (14, 15). Whereas vegetative cells move toward the ammonium source in the light and in darkness, chemotaxis is switched off in the late phase of gamete formation, and this is mediated by phototropin (14).

Phot proteins comprise two light sensor domains, LOV1 and LOV2, and a C-terminal kinase. LOV (light, oxygen, voltage) domains are a subclass of the much larger family of PAS (Per-ARNT-Sim, where PER stands for *Drosophila* period clock protein, ARNT stands for aryl hydrocarbon receptor nuclear translocator and SIM is *Drosophila* single-minded protein) proteins, which participate in a diverse array of biological signaling pathways. In vitro studies on LOV1 from *Chlamydomonas* Phot, LOV2 from *Avena sativa*, and Neochrome from the fern *Adiantum capillus-veneris* (discussed below) contributed the most to our knowledge about the reaction mechanism of LOV photoreceptors. Upon illumination, LOV domains undergo a photocycle that comprises a triplet state, LOV-715, which converts within one or a few microseconds into the signaling state to form a thioadduct between a formerly protonated Cys and the C(4a) position of the flavin mononucleotide (FMN)-isoalloxacine ring (**Figure 8*a***). The resulting LOV-390

reconverts to the dark state within many seconds to minutes (54, 91). The X-ray crystal structures of dark state LOV-447 and the photoproduct LOV-390 were determined for both LOV1 and LOV2 (9, 10, 17). The *Chlamydomonas* LOV1 dark state is shown in **Figure 8*b***. The transition from the dark state to the signaling state is accompanied by surprisingly minimal changes in the surrounding protein. However, the contact between LOV2 and the downstream Jα-helix is interrupted upon formation of the thioadduct in the light (31). If the Jα-LOV contact is prevented by mutation, the kinase is permanently active, which demonstrates the relevance of this particular interaction (30).

The mechanism of thioaddict formation is not yet completely clear. Results from ultrafast spectroscopy originally suggested that a $H^+$ transfer from the thiol to the N(5) position leads to a protonated triplet state that is subsequently attacked by the thiolate (52). However, theoretical studies on a model system via the use of ab initio formalism showed that a radical mechanism via transfer of a hydrogen atom from the Cys to the flavin is energetically favored (69). The preference of an electron over a $H^+$ transfer was supported by studies on a mutant in which the reactive Cys was replaced by Met (3, 53). Likewise, time-resolved electron paramagnetic resonance (EPR) measurements on several LOV domains supported the conclusion that a radical pair mechanism dominates the pathway under most conditions (3, 77). However, depending on the conditions, the radical pair may be formed by a hydrogen atom transfer or in a two-step process that transfers an electron first and a $H^+$ in a second step (77), as shown in **Figure 9**.

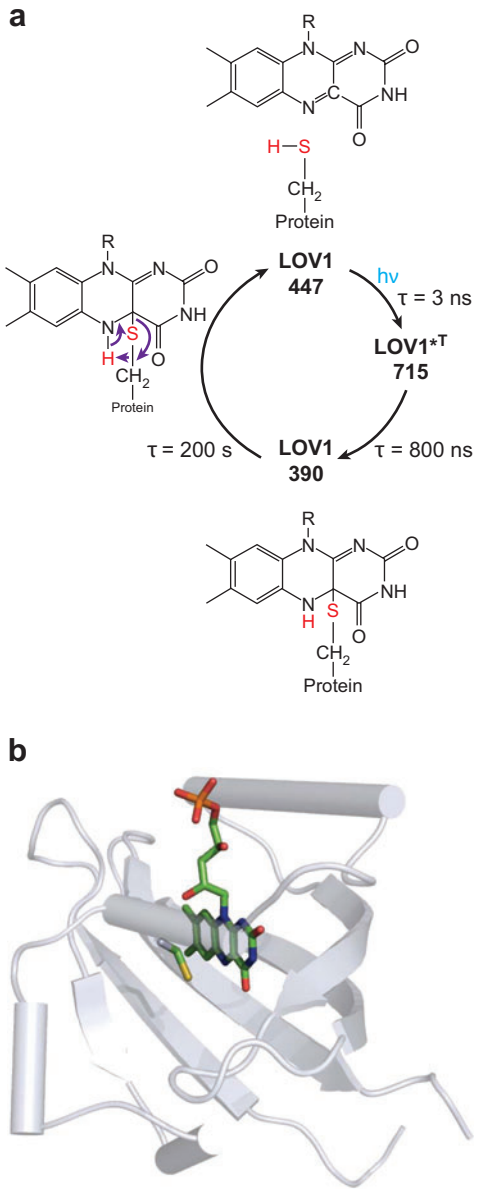


**Figure 8**

(*a*) Simplified representation of the phototropin (Phot)-LOV1 photocycle from *Chlamydomonas reinhardtii* (54). $LOV1^{*T}$ is the excited triplet state. (*b*) *Chlamydomonas* Phot-LOV1 with flavin mononucleotide (FMN) and the reactive cysteine (*yellow*). Protein Data Bank id: 1n9I. Reproduced with permission from *Biophysical Journal*.

## NEOCHROME

Photoorientation of the giant chloroplast in *Mougeotea* has been known for a long time to be regulated by light (81). Experiments using microbeams and polarized light implied that the gradient of a phytochrome in Pfr

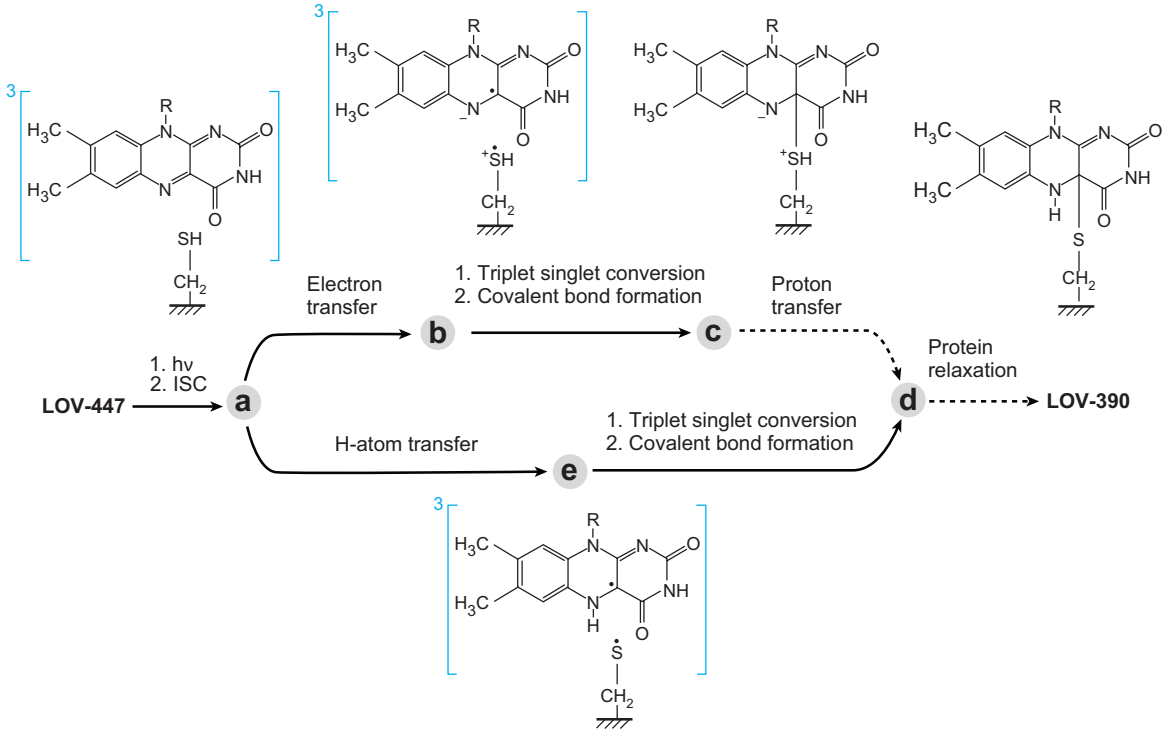


**Figure 9**

Suggested reaction pathway and intermediates of photoadduct (LOV-390) formation in wild-type LOV domains. Reaction steps that are inhibited at t < 80° K are shown by dashed arrows. Reprinted with permission from Reference 77. Copyright 2004 American Chemical Society.

form bound at the cell periphery controls chloroplast orientation (35). Low levels of blue light mimicked this behavior, which led Haupt (35) to propose that a chimeric photoreceptor for blue and red light absorption mediates chloroplast movement. Suetsugu and coworkers (89) identified two genes in *Mougeotea* that encode hybrid proteins that resemble phytochrome 3 (phy3) from *Adiantum capillus veneris*. These hybrid proteins comprise two LOV domains, a kinase, and an N-terminal phytochrome domain (Phy-Phot-hybrid). The authors named these members of the recently discovered photoreceptor class neochrome1 and neochrome2 (MSNeo1 and MSNeo2). The recombinant proteins show typical phytochrome bilin binding and red/far-red–reversible photochromism but no FMN binding could be achieved. Both genes are able to rescue red light–induced chloroplast movement in *Adiantum* phy3 mutants, which indicates functional equivalence with respect to red light sensing. As phytochrome-phototropin chimeras, neochromes originally seemed to verify Haupt's old prediction exactly. However, this has now been called into question, because MsNeo cannot perceive blue light in planta. More likely, blue light–mediated chloroplast photoorientation is achieved by the phototropins MsPhot1 and MsPhot2 in this alga. More conventional phytochromes were identified in *Mesotenium* (McPhy1b) (U31283, U31284 in the NCBI Nucleotide Database), *Mougeotia scalaris* (MsPhy1) (Gb:S52048), and *Chara foetida* (Gb:X80291), but they were not studied functionally in any detail.

**EPR:** electron paramagnetic resonance

## AUREOCHROME

The most recent blue light receptor family to be discovered was functionally characterized in the stramenopile algae *Vaucheria frigida* (Xanthophyceae) and *Fucus distichus* (Phaeophyceae) (93). Two homologs were identified in *Vaucheria*; each has one basic-region/leucine-zipper (bZIP) domain and one LOV-sensing domain. The authors have named these chromoproteins AUREOCHROMEs (AUREO1 and AUREO2) because stramenopiles species are typically golden-yellow in color. AUREO1 binds FMN via its LOV domain and forms a 390 nm–absorbing form, indicative of formation of a cysteinyl adduct to the C(4a) carbon of the FMN upon blue light irradiation. The adduct decays to the ground state in approximately 5 min. The bZIP domain binds the target sequence TGACGT. AUREO1-target binding is strongly enhanced by blue light treatment, implying that AUREO1 functions as a blue light–regulated transcription factor. The function of AUREO1 as photoreceptor for light-induced branching (92) was elucidated through RNA interference (RNAi) experiments. RNAi of AUREO2 unexpectedly induces the formation of sex organ primordia instead of branches, implicating AUREO2 as a sub-switch to initiate development of a branch, but not a sex organ. AUREO sequences are also found in the genome of the marine diatom *Thalassiosira pseudonana* (Bacillariophyceae), but are not present in green plants. AUREOCHROME therefore represents a blue-light receptor of photosynthetic stramenopiles.

### FUTURE ISSUES

1. With respect to flavin-based photoreceptors such as phototropin (Phot) and phytochromes, the work in algae is lagging behind the work that has been done in higher plants. This is because no photoreceptor mutants are available in algae. This deficit can only be partially compensated for by the application of RNAi, because the reduction of the photoreceptor content is stage-dependent, a target of silencing, and not specific in cases where several genes with overlapping function occur in the alga. Only clean gene targeting can solve this problem and more effort should be invested into this goal. The microalga in which clean gene targeting is first possible will become the leading model species.
2. Algae are exceptionally rich in sensory photoreceptors and the study of their genomes, especially those of freshwater algae, will bring more light-gated enzymes to our knowledge.
3. The molecular analysis of the unusual algal sensory photoreceptors has only recently begun, but tight collaboration of biologists, biochemists, physicists, and theoreticians will guarantee fast progress.
4. Applications for light-activated enzymes that metabolize or produce universal metabolic key compounds will soon be discovered and will support science in other fields.
5. The challenge lies in the study of the biology and the biophysics in or on the living alga. Only these studies will allow the discovery of new natural treasures.

## DISCLOSURE STATEMENT

The author is not aware of any biases that might be perceived as affecting the objectivity of this review.

## ACKNOWLEDGMENTS

I thank all my coworkers who worked with great patience on the characterization of algal photoreceptors, especially during the years from 1985 to 1996 when the molecular identification was not in sight. I thank Tilo Mathes and Oliver Ernst for discussion during preparation of the manuscript and for sharing unpublished data. The author also gratefully acknowledges the continuous support by the Deutsche Forschungsgemeinschaft.



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**58. The first recording of electrical responses from a green alga, *Haematococcus pluvialis*.**

**59. Demonstrated convincingly that the pigmented spot of single-celled algae is the light sensitive organelle that controls behavioral light responses.**

**66. ChR1 is the first example of a light-gated ion channel; it causes inward directed photocurrents and can depolarize the cell enough to trigger secondary voltage-gated channels at high light intensities.**

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## Errata

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