Photomovement in *Euglena*

11

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Abstract

Motile microorganisms such as the green *Euglena gracilis* use a number of external stimuli to orient in their environment. They respond to light with photophobic responses, photokinesis and phototaxis, all of which can result in accumulations of the organisms in suitable habitats. The light responses operate synergistically with gravitaxis, aerotaxis and other responses. Originally the microscopically obvious stigma was thought to be the photoreceptor, but later the paraxonemal body (PAB, paraflagellar body) has been identified as the light responsive organelle, located in the trailing flagellum inside the reservoir. The stigma can aid in light direction perception by shading the PAB periodically when the cell rotates helically in lateral light, but stigmaless mutants can also orient with respect to the light direction, and negative phototaxis does not need the presence of the stigma. The PAB is composed of dichroically oriented chromoproteins which is reflected in a pronounced polarotaxis in polarized light. There was a long debate about the potential photoreceptor molecule in *Euglena*, including carotenoids, flavins and rhodopsins. This discussion was terminated by the unambiguous proof that the photoreceptor is a 400 kDa photoactivated adenylyl cyclase (PAC) which consists of two α- and two β-subunits each. Each subunit possesses two BLUF (Blue Light receptor Using FAD) domains binding FAD, which harvest the light energy, and two adenylyl cyclases, which produce cAMP from ATP. The cAMP has been found to activate one of the five protein kinases found in *Euglena*

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S.D. Schwartzbach, S. Shigeoka (eds.), *Euglena: Biochemistry, Cell and Molecular Biology*,
Advances in Experimental Medicine and Biology 979, DOI 10.1007/978-3-319-54910-1_11

(PK.4). This enzyme in turn is thought to phosphorylate proteins inside the flagellum which result in a change in the flagellar beating pattern and thus a course correction of the cell. The involvements of PAC and protein kinase have been confirmed by RNA interference (RNAi). PAC is responsible for step-up photophobic responses as well as positive and negative phototaxis, but not for the step-down photophobic response, even though the action spectrum of this resembles those for the other two responses. Analysis of several colorless *Euglena* mutants and the closely related *Euglena longa* (formerly *Astasia longa*) confirms the results. Photokinesis shows a completely different action spectrum. Some other *Euglena* species, such as *E. sanguinea* and the gliding *E. mutabilis*, have been investigated, again showing totally different action spectra for phototaxis and photokinesis as well as step-up and step-down photophobic responses.

Keywords

Astasia • *Euglena longa* • *Euglena gracilis* • *Euglena mutabilis* • Flavin • Photoactivated adenylyl cyclase • Photokinesis • Photophobic reactions • Photoreceptor • Phototaxis • Protein kinase • Pterin • Sensory transduction

11.1 Introduction

Many unicellular microorganisms as well as cell colonies are motile and orient themselves with respect to external physical and chemical parameters in their environment such as temperature (Häder et al. 2014), pH, oxygen (Colombetti and Diehn 1978; Porterfield 1997), chemicals and pollutants (Govorunova and Sineshchekov 2005; Ozasa et al. 2013; Azizullah et al. 2014), mechanical stimuli (Mikolajczyk and Diehn 1979; Fenchel 2013), the magnetic field of the Earth (de Araujo et al. 1986; Kavaliers and Ossenkopp 1994) and even electrical currents (Umrath 1959; Votta and Jahn 1972; Kim 2013). Many swimming cells orient themselves in the gravity field of the Earth using a mechanism called gravitaxis (see Chap. 12) (Richter et al. 2002; Nasir 2014).

Photosynthetic organisms such as flagellates require the presence of light for energy harvesting, so that it is not astonishing that they orient with respect to light to guide their migrations (Häder 1979; Häder and Lebert 2009; Peacock and Kudela 2014). But also heterotrophic organisms use phototactic orientation for habitat selection (Hu et al. 2014). Motile flagellates often move toward the light source at low irradiances (positive phototaxis, Fig. 11.1a) (Liu and Häder 1994; Giometto et al. 2015). Since excessive radiation can be detrimental for the cells, many organisms move away from the light source at high irradiances (negative phototaxis, Fig. 11.1b) (Lenci et al. 1984; Josef et al. 2005; Ma et al. 2012). In some cases an orientation at a specific angle to the light direction (e.g. perpendicular to a light beam, Fig. 11.1c) has been found, a behavior called diaphototaxis (Häder and Lipson 1986; Nultsch and Häder 1988; Rhiel et al. 1988). This behavior enables the organisms to swim horizontally at constant optimal light intensity.

In addition, many cells show other light-induced behavioral movement responses. Upon a sudden decrease in light intensity they show a step-down photophobic response which may be a stop, a change in swimming direction or a reversal of movement (Govorunova et al. 2004; Lenci

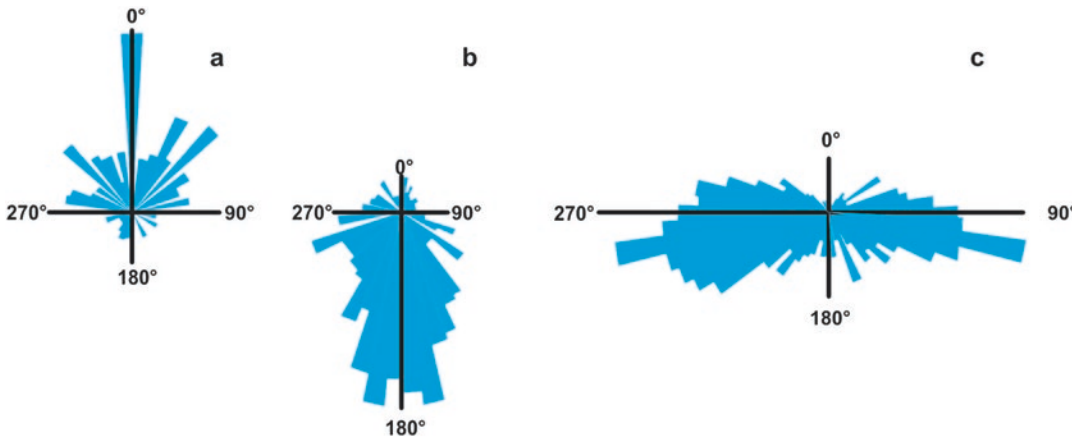

Fig. 11.1 (**a**) Positive phototaxis in *E. gracilis* strain Z swimming in a horizontal cuvette with white light at 10 W m^{-2} impinging from 0°. Tracks of swimming cells were recorded by automatic image analysis and the angles deviating from 0° (direction toward the light source) were binned in 64 sectors. The circular histogram shows the percentage of tracks in each angular sector. (**b**) Negative phototaxis in *E. gracilis* strain Z swimming in a horizontal cuvette with white light at 100 W m^{-2} impinging from 0° (modified from (Lebert and Häder 2000). (**c**) Diaphototaxis in the colorless *E. gracilis* strain FB swimming in a horizontal cuvette with white light at 1000 W m^{-2} impinging from 0° redrawn from (Lebert and Häder 1997)

et al. 2012). Imagine cells swimming in a horizontal container which is covered by a black lid with a square opening in the center irradiated by low intensity light from above. Cells swimming in the shade may enter the irradiated zone without a response, but, if they try to leave it they undergo a step-down photophobic response at the light/dark boundary. This behavior will result in an accumulation of cells in the irradiated area over time. This reaction is exploited in the so-called light trap method used to quantify photophobic responses (Nultsch and Häder 1979). Likewise, a sudden increase in the ambient light intensity may result in a step-up photophobic response which is elicited by a sudden increase in light intensity which would occur when an organism enters a irradiated area from a shaded one (Doughty and Diehn 1984; Ntefidou et al. 2003b; Takeda et al. 2013). This usually occurs at high light intensities. In this case cells trying to cross the border from the shaded area into the brightly lit zone will undergo a step-up photophobic response while they do not react when leaving the light field. This behavior results in a depletion of cells in the lit zone and an accumulation in the dark. Photophobic responses in *Euglena* have also been studied by the observation of individual cells embedded in a small agar chamber (Shimmen 1981).

A dependence of the swimming speed on the ambient irradiance is called photokinesis (Zhenan and Shouyu 1983; Melkonian et al. 1986; Iwatsuki 1992). This phenomenon can also result in accumulations of cells in certain areas (Häder 1987a). Imagine cells swimming fast in light but slow in the shaded area or even stopping (positive photokinesis); these cells will accumulate in the shaded area. This has been observed e.g. in the ciliate *Stentor coeruleus* (Iwatsuki 1992). Also the opposite has been found: cells swimming fast in the dark and slower in light will accumulate in the irradiated field (negative photokinesis). Another mechanism for cell accumulation is phototaxis of cells toward a light field irradiated by a strong light source such as a laser beam as shown for *Euglena* (Itoh and Tamura 2008).

While phototaxis has been studied in many flagellates to some extent only a few were investigated in detail, such as the Chlorophyte *Chlamydomonas reinhardtii* (Sineshchekov et al. 2002; Schmidt et al. 2006; Inaba et al. 2014). *Euglena* has been established as a model system for biochemical and behavioral studies, signal

transduction and molecular biology of the photoreceptor (Iseki et al. 2002; Wolken 2012; Masuda 2013; Ozasa et al. 2014; Giometto et al. 2015).

In the past, the movement and orientation of motile microorganisms was recorded and quantified by manual and video techniques (Colombetti et al. 1982), but are nowadays usually quantified using real-time image analysis and computerized cell tracking (Häder and Lebert 2000; Häder 2003).

11.2 The Organisms

Euglena gracilis is a photosynthetic unicellular flagellate (Buetow 1968a), but it can also live heterotrophically (Sumida et al. 2007). The size ranges between 50 µm and 80 µm length and 8 µm to 12 µm width (Buetow 1968b). Since the cell does not have a rigid cell wall its form is highly flexible ranging from almost spherical to an elongated spindle (Mikolajczyk and Diehn 1976, 1978; Mikolajczyk and Kuznicki 1981; Murray 1981). The cell body is covered with a pellicle which consists of longitudinal interlocked stripes which can slip with respect to one another (Suzaki and Williamson 1986; Leander et al. 2001), and the surface is covered with slime (Diskus 1955). The stripes are composed of the claudin-like, four-trans-membrane protein IP39 which forms linear arrays by a trimeric unit repeat which are similar to tight junctions (Capaldo et al. 2014). At the front end there is a bottle-like 5 µm × 10 µm invagination, called reservoir (Fig. 11.2), which allows pinocytotic uptake of external material (Kivic and Vesk 1974a; Bouck 2012; Omodeo 2013). A system of contractile vacuoles (one main vacuole with several accessory vacuoles) is associated with the reservoir, believed to be involved in osmoregulation (Rosati et al. 1996; Komsic-Buchmann and Becker 2012). At the base of the reservoir two flagella originate in basal bodies, but only one of them extends as a trailing flagellum from the reservoir while the other one reaches up to the paraxonemal body (PAB, also called paraflagellar body) (Tollin 1973; Piccinni and Mammi 1978), located on the long flagellum half way between the bottom and the opening of the invagination, called pharynx (Frey-Wyssling and Mühlethaler 1960; Kisielewska et al. 2015). The PAB is a small organelle (1 µm × 0.7 µm × 0.7 µm) (Wolken 1977; Omodeo 1980; Rosati et al. 1991) (Fig. 11.2). A paraxonemal rod (PAR) inside the flagellum connects the PAB to the cell matrix (Banchetti et al. 1980; Hyams 1982; Verni et al. 1992; Karnkowska et al. 2015). It consists of several proteins like in trypanosomes, the function of which is still unclear (Hyams 1982; Leedale 1982; Ngô and Bouck 1998). The membrane covering the PAB was found to be structurally specialized when observed with the freeze-fracture technique. There are large-sized (10–14 nm) intramembrane particles (Robenek and Melkonian 1983). The side next to the stigma is closely attached to the plasmalemma of the reservoir. Inside the cytoplasm, in close vicinity of the reservoir and next to the PAB it possesses a red structure, the stigma or eyespot which is so obvious that it was detected by early microscopists (Pringsheim 1937; Wolken 1956). This structure gave the flagellate its

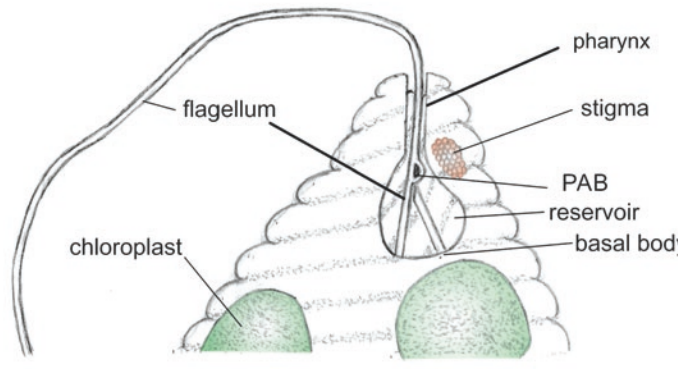

Fig. 11.2 Front end of a *Euglena* cell showing the reservoir and the stigma as well as chloroplasts. Inside the reservoir two flagella originate from basal bodies. One is short and touches the paraxonemal body (PAB) located on the long emerging flagellum (drawn by Dr. Maria Häder)

German name "Augentierchen" (small eye animal) and was considered to be the site of the photoreceptor (Cypionka 2010). The stigma consists of a number of globules (200–300 nm diameter) filled with carotenoids (Strother and Wolken 1960; Sperling et al. 1973; Benedetti et al. 1976; Heelis et al. 1979, 1980; Osafune and Schiff 1980; James et al. 1992). The main carotenoid in *E. gracilis* is antheraxanthin (80%) (Krinsky and Goldsmith 1960). Application of streptomycin to dark-bleached cultures of *Euglena* hampered the carotenoid synthesis, and electron micrographs showed a decrease in the number of stigma vesicles while no effect on the PAB was detected. Phototaxis decreased and finally disappeared after 5 weeks (Ferrara and Banchetti 1976). This does not mean that the photoreceptor is located within the stigma, but that its role as a shading device is compromised. In contrast to other algal groups, such as Chlorophytes, the stigma in *Euglena* is not enclosed within the chloroplast (Kivic and Vesk 1974b; Kronestedt and Walles 1975). Also in contrast to green algae, the globules are not organized in a rigid structure (Walne and Arnott 1967; Dodge 1969; Kivic and Vesk 1972a; b; Kreimer and Melkonian 1990). Therefore it does not function as an interference reflector device which is found in many stigmata of Chlorophytes such as *Chlamydomonas* (Kreimer 1994). Even though the stigma does not harbor the photoreceptor, as suggested by earlier authors (France 1909; Fong and Schiff 1978, 1979), it seems to be involved in photoperception, functioning as a screening device which in lateral light casts a periodic shadow on the photoreceptor (the PAB, see below) as the cell rotates around its long axis when it is propelled by its long flagellum in a forward direction (Barsanti et al. 2012). For comparison, we also shortly discuss several mutant strains of *Euglena gracilis*, some other species in the genus *Euglena* as well as the close relative *E. longa* (formerly *Astasia longa*) (Poniewozik 2014).

11.3 The Paraxonemal Body (PAB)

The PAB shows a paracrystalline structure (Kivic and Vesk 1972a; Forreiter and Wagner 2012). Wolken (1977) proposed a model of packed rods in a helical pattern based on optical diffraction patterns, and Piccinni and Mammi (1978) found monoclinic or slightly hexagonal cell units with the principal axes a = 8.9 nm, b = 7.7 nm, c = 8.3 nm und ß = 110°. This structure can be interpreted as an I-type 3-D crystal (Michel 1990), which is a stacks of 2-D crystal arrays stabilized by hydrophobic interactions between the planes and hydrophilic interactions with the surrounding aqueous environment. More than 100 layers of the 2-D crystals are thought to form the observed structure of the PAB (Gualtieri 1993b). 2-D type crystals have been found e.g. in the purple membrane in *Halobacterium salinarium*, with a very high concentration of bacteriorhodopsin to harvest light energy (Oesterhelt 1998) or in photosynthetic membranes of higher plants (Toporik et al. 2012) and purple bacteria (Sznee et al. 2014).

11.4 Photoresponses

As indicated above *Euglena gracilis* shows three types of photoresponses: both positive and negative phototaxis, step-up and step-down photophobic responses and photokinesis.

11.4.1 Phototaxis

In contrast to photokinesis and photophobic responses phototaxis depends on the direction of the impinging light. Phototaxis in motile microorganisms has been known for more than a century (France 1908, 1909). At low irradiances the flagellates move toward the light source (positive phototaxis) which can be shown both in horizontal or vertical observation chambers. At higher irradiances the cells switch to negative phototaxis and swim away from the light source (Häder et al. 1981). In *Euglena*, the threshold for the change from positive to negative phototaxis is found between 10 and 100 W m^{-2}, but it depends on other external factors (Häder 1987b, 1998) as well as whether the cells swim in a horizontal or vertical cuvette. In the latter case the cells orient simultaneously with respect to light and gravity (see below). At intermediate light intensities

some of the cells show positive others negative phototaxis. As the ecological consequence of this behavior, *Euglena* cells accumulate at a certain depth in the water column at which the impinging sun light has been attenuated to about 30 W m^{-2} (Häder and Griebenow 1988). A similar behavior was found in the freshwater *E. proxima* (Hasle 1950). In comparison, the green flagellate *Chlamydomonas* is much more sensitive than *Euglena* and has a threshold for positive phototaxis at 0.001 W m^{-2} (Feinleib and Curry 1971). This reflects the fact that *Chlamydomonas* is often found much deeper in the water column with less light availability than *Euglena*.

The mechanism of light direction detection was debated for a long time. Some researchers held the notion that oriented movement depends on a series of photophobic responses (Jennings 1906; Mast 1911): When an organism experiences a decrease in irradiance it might make a turn or reverse the direction of movement. When this strategy is repeated over time the organism will move up the light gradient and thus show a positive phototaxis. The same reasoning holds for negative phototaxis being based on consecutive step-up photophobic responses. This hypothesis is based on a two-instant mechanism (Feinleib 1975): The photoreceptor takes readings of the ambient irradiance and compares it to a previous measurement. This orientation mechanism has also been described as "biased random walk" (Hill and Vincent 1993; Hill and Häder 1997), which is also found in chemotaxis of bacteria (Wadhams and Armitage 2004). This theory implies that the photoreceptors for phototaxis and photophobic responses are identical. For *Euglena gracilis* the hypothesis that light direction detection is based on a series of repetitive photophobic responses can be rejected at least for negative phototaxis, since in this flagellate the threshold for negative phototaxis is much lower than that for the step-up photophobic response. We will also see below that the molecular analysis of the photoreceptor showed that the step-down photophobic response in *Euglena* is not mediated by the same photoreceptor which drives phototaxis.

Alternatively, the organism detects the light direction with a more sophisticated sensor than just a light-intensity measuring photoreceptor. This is based on a one-instant mechanism. E.g., some ciliates posses a complicated optical organelle which like a primitive eye, is capable of discerning the direction of the impinging light (Omodeo 1975; Selbach et al. 1999).

Buder devised an ingenious experiment to tackle the question whether phototaxis is controlled by an orientation along a spatial gradient of light or the result of a directional movement with respect to the light direction. He passed light through a biconvex lens so that he produced a converging light beam. The cells swam toward the light source, passed through the focal point and continued to swim toward the light source even though the light intensity decreased with the distance from the focal point (Buder 1919).

In negative phototaxis the rear end of the cell with its chloroplasts casts a shadow on the photoreceptor. Circular histograms of the swimming directions indicate that a course correction is initiated when the cell deviates by more than 25° from the light direction (Häder et al. 1986) indicating that in this position the rear end does no longer shade the photoreceptor.

When exposed to two perpendicular light beams, at low fluence rates which induce positive phototaxis, the cells do not orient on the mathematical resultant of the two vectors but orient themselves with respect to either light beam (Häder 1993). At equal light intensities approximately half of the population swims toward one light source and the remainder toward the other light source. When the intensity of one of the light beams exceeds that of the other by more than 10%, almost all cells move in the direction of the stronger light beam (Lebert and Häder 2000). In contrast, at high fluence rates which induce negative phototaxis the cells orient on the resultant away from the two perpendicularly impinging light beams. When the cells orient with respect to light they are simultaneously exposed to the gravity field of the Earth unless they are under zero-g conditions such as on a satellite or on the International Space Station (ISS). In a vertical

cuvette they show a gravitactic orientation. Shortly after inoculation into new medium the cells swim downward. In contrast, older cells (>10 days) swim upward. When they simultaneously perceive a (horizontal) light beam perpendicular to the gravitational field of the Earth the cells swim on an intermediate track on the resultant; the angular deviation from the vertical depends on the irradiance of the light beam (Kessler et al. 1992). In order to estimate the effect of the simultaneously operating gravitaxis on the precision of phototaxis a space experiment was carried out on a sounding rocket flight inside a TEXUS rocket (Kühnel-Kratz et al. 1993). As expected, under microgravity (μg) conditions the precision of phototaxis was higher than in the 1-g control but the inversion from positive to negative phototaxis was the same for a sample taking shortly after landing of the rocket as in the control cells which had been exposed to 1 g all the time. In addition, phototactic orientation was reached faster at μg than at 1-g conditions (Häder 1997).

The mechanism of changing movement direction is closely linked to the rotation of the cell and the position of the flagellum. While swimming, a cell rotates at about 1 Hz around its long axis describing a cone with a ca. 15° opening angle at the front end. The flagellum is more or less parallel to the long axis. When a low intensity light beam (which induces a positive phototaxis) impinges perpendicular to the long axis, the flagellum swings out at an angle from the long axis, when during rotation the flagellum is oriented away from the light beam. This impulse triggers a small angular turn of the front end toward the light source. This is also seen in the output of a computer simulation (Häder 1993). This course correction is repeated as long as the long axis is not yet aligned with the light direction. When the flagellum is oriented away from the light direction, the stigma intercepts the light path onto the PAB photoreceptor (Häder 1998). Thus it modulates the light intensity seen by the PAB with the frequency of the cell's rotation with a minimum when the flagellum is opposed to the light direction. This was regarded as a proof for the shading hypothesis (Clayton 1964) and seemed to confirm the notion that phototaxis is brought about by a repetitive step-down photophobic response. For negative phototaxis this process has to be just the opposite with the additional shading from the rear end with its chloroplasts.

In order to determine the angle of the course correction a circular cuvette was rotated by an electric motor in a strong lateral light beam (30 klx) which induced negative phototaxis (Häder et al. 1986). The moving cells were recorded from below using a dark red monitoring light (>690 nm) which does not induce a visible movement response. When the cuvette was rotated at a low angular velocity (<20°/s) the cells still showed negative phototaxis but the mean swimming direction was shifted in the direction of the rotation. At higher rotational speeds the precision of orientation decreased and the swimming was more random. This indicates that the cells were capable of course corrections of up to 20°/s. Since the time for one rotation is about 1 s, the cells reorient by about 20° per rotation.

However, some mutants of *E. gracilis* lack a stigma but still show negative phototaxis, but no positive phototaxis (Vavra 1962; Checcucci et al. 1976; Häder 1993). In contrast, mutants which lack both PAB and stigma neither show positive nor negative phototaxis (Pringsheim 1948; Vavra 1962; Lebert and Häder 1997). Though many mutants are known lacking stigma, PAB and/or chloroplasts (Schiff et al. 1971, 1980; Shneyour and Avron 1975; Falke et al. 1997), only a few have been analyzed regarding phototaxis (Lebert and Häder 1997). These mutants have not been induced but occurred spontaneously and were isolated by accident. In one investigation, three of the studied mutants were stable, while one included some revertants which may be due to the fact that *Euglena* is polyploid.

Euglena longa, a close relative of *E. gracilis*, possesses a stigma but no PAB and does not show phototaxis (Suzaki and Williamson 1983; Mikolajczyk 1984a, 1984b) but step-up photophobic responses, while step-down responses were not observed, indicating that separate receptors exist for step-up and step-down photophobic responses. This was later confirmed by the molecular biological identification for the photoreceptor in *Euglena* (Iseki et al. 2002). All these

findings indicate that the PAB is the true photoreceptor in *E. gracilis* and that the stigma only has an accessory role (at least in positive phototaxis). Thus, the classical shading hypothesis had to be discarded.

As indicated above, earlier electron microscopic analyses have revealed a quasicrystalline structure within the PAB (Kivic and Vesk 1972b). Could that mean that the photoreceptor molecules are dichroically oriented in a specific pattern with reference to the long axis and the location of the stigma? In order to answer this question, experiments have been carried out with polarized light impinging from above onto a horizontal swimming chamber with *E. gracilis* (Bound and Tollin 1967; Creutz and Diehn 1976; Häder 1987b). The assumption is that light is only absorbed when the absorption vector is perpendicular to the light direction. When the electric dipole of the polarized light was oriented in a specific direction, the cells swam at an angle of about 30° clockwise from the electric dipole (Fig. 11.3a). This behavior was confirmed and also seen in a computer simulation which assumes the same orientation of the photoreceptor molecules as indicated in the electron micrographs (Häder 1993). Further experiments with polarized light in all three dimensions revealed the orientation of the absorption vectors in 3D with respect to the cell axes (Häder 1987b). Tracing the cells in a vertical cuvette with polarized light impinging from above indicated that the absorption vectors of the photoreceptor pigments are dichroically oriented predominantly 60° counterclockwise from the flagellar plane (when one looks onto the front end of the cell, Fig. 11.3b) (Häder 1987b). These results were confirmed in a mathematical model for the signal received by the dichroic photoreceptor in *E. gracilis* when irradiated by polarized light (Hill and Plumpton 2000). Diehn assumed that *Euglena* has two perpendicularly oriented photoreceptor systems responsible for positive and negative phototaxis, respectively (Diehn 1969c). During the revolution of the cell around its long axis a dichroically oriented photoreceptor has two positions with maximal absorption for light hitting perpendicularly to the swimming direction. This could explain the diaphototactic orientation found in the 1F mutant which does not have a screening

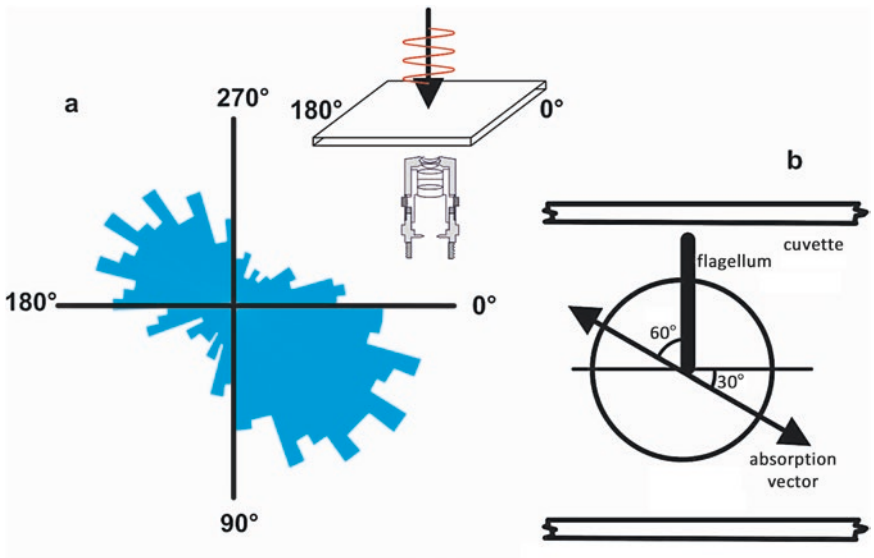

Fig. 11.3 (**a**) *Euglena* cells swimming in a horizontal cuvette orient themselves 30° clockwise from the electric vector of a polarized light beam swinging in a plane 0–180° impinging from above (*inset*), (**b**) absorption vectors of the photoreceptor pigments are dichroically oriented predominantly 60° counterclockwise from the flagellar plane when one looks onto the front end of the cell. Redrawn after (Häder 1987b)

stigma. The 1F mutant lacks chloroplasts, stigma and PAB. It shows only diaphototaxis and neither positive or negative phototaxis. In the wild type cells one of the absorption maxima is excluded by the presence of the stigma, so that the cells orient only in one direction. In negative phototaxis the rear end of the cell assumes the role of a shading device and the presence of the stigma is not required for negative phototaxis.

Euglena shows a strong circadian rhythm which affects orientational responses (Verworn 1889; Bruce and Pittendrigh 1956; Tollin and Robinson 1969; Edmunds 1984; Petersen-Mahrt et al. 1994) as well as other reactions such as cell division and cellular cAMP concentration (Bruce and Pittendrigh 1958; Feldman and Bruce 1972; Bruce 1973; Bünning 1973; Carre et al. 1989; Lebert et al. 1999). This may also be a reason that several authors found different results in their investigations of *Euglena* phototaxis. Also culture conditions and age strongly affect phototactic orientation (Häder et al. 1987).

11.4.2 Photophobic Responses

Photophobic responses (also called phobic reactions or shock responses) were first reported by Engelmann (1883). The older literature has been reviewed (Haupt 1959; Feinleib and Curry 1967; Diehn 1973; Nultsch 1975; Nultsch and Häder 1979). Photophobic responses are elicited by rather sudden changes in irradiance. If the change occurs over a longer time period, the organisms adapt to the new condition without showing a phobic response. The phobic response occurs only when the change in irradiance exceeds a species-specific discrimination threshold (Clayton 1959), which can be as low as a few percent (Nultsch and Häder 1970). In *Euglena* the response is characterized by a stop and tumble which can last a few seconds; after this the cell resumes swimming in a new direction (Doughty 1991). High speed cinemicrography showed that the cells respond with the turning toward the dorsal side when exposed to a sudden increase in light intensity (Diehn et al. 1975). In this organism step-down photophobic responses occur at low irradiances and step-up responses at higher irradiances (Diehn 1969c, 1973). Ecologically this behavior can be interpreted as helping the organism to prevent swimming from a low-irradiance region into an even darker shadow or from a high-irradiance region into excessive light. Very low irradiances may not be sufficient for photosynthesis and very high irradiances may be detrimental for the cells. The action spectra for the step-up and step-down photophobic responses in *Euglena* differ slightly but show a resemblance to a flavin chromophore (Diehn 1969a; Barghigiani et al. 1979b; Walne et al. 1984).

11.4.3 Photokinesis

As described above, photokinesis describes the dependency of the swimming speed on the light intensity. This behavior was first described almost 140 years ago (Strasburger 1878). It can result in an accumulation of organisms in lighted or shaded areas (see above). In *Euglena* a not very pronounced positive photokinesis was described (Wolken and Shin 1958). The increase in velocity saturates at 300 lx white light. However, a 10–15 min adaptation period is needed before the velocity reaches a new steady state (Mast 1911) which might indicate that metabolic processes are involved producing higher energy. The increase in swimming speed seems to be due to an increased flagellar beating frequency (Ascoli 1975). However, also the non-photosynthetic close relative *E. longa* has been found to show positive photokinesis (Mast 1911).

In *Euglena* the action spectrum of this response is still under debate. Some researchers found an effect in red light and proposed the involvement of the photosynthetic pigments such as chlorophyll *b* and β-carotene (Mast 1911; Ascoli 1975). Other researchers claim a strong effect of blue light (Nultsch and Throm 1975). Therefore it is not decided whether photokinesis depends on photosynthesis or is controlled by a blue light receptor (Haupt 1959).

11.5 The Photoreceptor(s)

Before molecular biology tools became available the question for the photoreceptor involved in a light-dependent responses such as phototaxis was tackled using action spectroscopy (Foster 2001). Using narrow band color filters (such as interference line filters) the organism is exposed to a selected wavelength band and the response is quantified at increasing irradiances (Foster 2001). This is repeated for all relevant wavelengths. Next the inverse of the required irradiance for a certain response (e.g. 50%) is plotted versus the wavelength giving an action spectrum or in other words the efficacy of the actinic light in dependence of the wavelength. The form of the action spectrum is compared with the absorption spectrum of a presumed photoreceptor. In reality things can be more complex e.g. by the presence of shading pigments. In order to avoid this complication, the method of threshold action spectra is used which are constructed again by plotting the wavelength-dependent response versus log light intensity (Foster and Smyth 1980). The reciprocals of the threshold intensity at which no reaction occurs, obtained by the linear interpolation of the response-intensity plots, should indicate the properties of the photoreceptor molecule.

11.5.1 Earlier Results and Hypotheses

Most flagellates have sensitivity in the UV/blue-green range (300–550 nm) of the spectrum. But there are exceptions: The action spectrum for phototaxis in *Chlamydomonas reinhardii* extends up to 600 nm (Foster and Smyth 1980; Johnson et al. 1991). In *Ochromonas danica* (Mast 1914) and *Peridinium gatunense* (Häder and Liu 1991) the spectrum even extends into the red region of the spectrum. These diverse spectral sensitivities probably indicate that phototaxis has evolved in multiple parallel events (Kivic and Walne 1983).

Early action spectra for photoaccumulation of green, dark-bleached and streptomycin-treated colorless *Euglena*, all of which possess a PAB, have been published by Checcucci et al. (1976). These spectra indicate the presence of a flavin-type photoreceptor. These measurements have been performed with the "phototaxigraph" (Lindes et al. 1965; Diehn and Tollin 1966; Checcucci et al. 1975). This instrument records the density of cells in a light trap and this is interpreted as a quantification of phototaxis when cells are attracted from the outside of the trap by light scattered from cells already inside the trap. However, as we have seen above, photoaccumulations can be brought about by several photoresponses including phototaxis. Another action spectrum for phototaxis was published by Gössel (1957).

Light microscopic images of the front end of *Euglena gracilis* show the reservoir with the two flagellar bases. Where they join a distinct body which represents the PAB can be seen (Fig. 11.4a). When excited with monochromatic light at 440 nm this spot shows a distinct blue fluorescence with a peak at about 520 nm (Fig. 11.4b). The red background fluorescence is derived from the chlorophyll in the cells. The flagella can be isolated with the PAB still attached (Gualtieri et al. 1986; Brodhun and Häder 1990) after the cells have been osmotically swelled in order to open the reservoir (Fig. 11.4c). Scanning electron micrographs of isolated flagella also show the PAB still attached to the flagellum (Brodhun et al. 1994). In large scale isolation experiments the chromoproteins of the PABs can be separated. SDS gel electrophoresis and isoelectric focusing shows the presence of four major proteins which are lacking in *Euglena longa* (which does not have a PAB) (Brodhun and Häder 1995a; Häder 1998). When excited, all four chromoproteins showed fluorescence emission spectra which were interpreted as representing flavins and pterins (Ghetti et al. 1985; Brodhun and Häder 1990; Häder 1991; Lebert 2001). Three proteins (M_r 27, 27.5. 31.6) contained pterins and the other one a flavin (M_r 33.5) (Lebert and Häder 2000). The excitation spectrum strongly resembled the action spectrum for phototaxis confirming the notion that a flavoprotein might be the photoreceptor for this response in *E. gracilis* (Brodhun and Häder 1990, 1995a). In addition, microspectrofluorometric studies of the PAB confirmed the presence of a flavin chromophore which showed

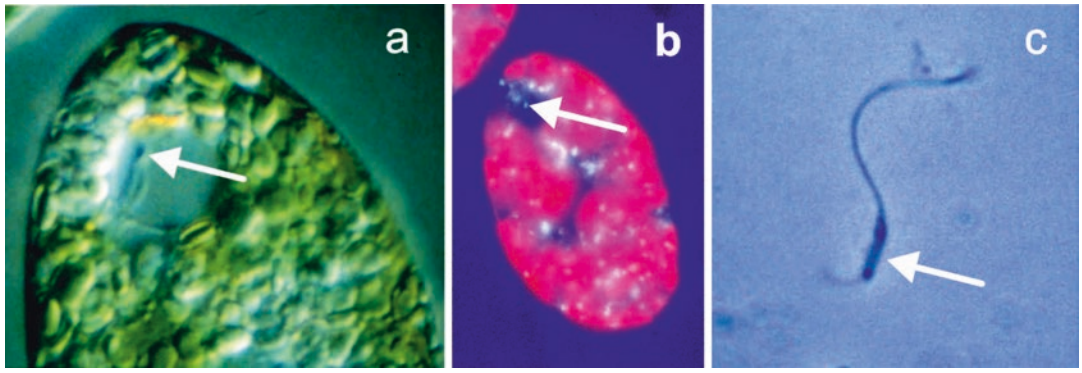

Fig. 11.4 Light microscopic photograph (**a**) and fluorescence microscopic image (**b**) of *Euglena gracilis* with the PAB (*arrows*). The chloroplasts show a red fluorescence and the blue spot in the reservoir indicates the PAB. The two flagellar bases are also seen in (**a**). (**c**) Isolated flagellum with PAB attached

an emission at 520 nm when excited between 400 and 500 nm (Benedetti and Checcucci 1975; Benedetti and Lenci 1977; Schmidt et al. 1990; Sineshchekov et al. 1994b). This interpretation is further strengthened by the fact that the fluorescence emission is strongly polarized, which has to be expected from the paracrystalline structure of the PAB (Sineshchekov et al. 1994b). The PAB fluorescence quantum yield is rather low (0.005 as compared to solubilized riboflavin ~0.25) (Ghetti et al. 1985). This indicates a strong coupling of the photoreceptor molecules to the signal transduction chain. Only when the transduction chain is saturated by excessive light or the coupling of the photoperception to the subsequent steps in the transduction chain is disturbed the fluorescence yield increases. The action spectrum for phototaxis in the green strain Z of *E. gracilis* extends to about 500 nm (Fig. 11.5a). Also when the chloroplasts and chlorophyll are removed by cultivating the cells in an organic medium in the dark, the action spectrum of these colorless strains still has the same shape (Häder and Reinecke 1991) consistent with a flavin chromophore.

Another confirmation for a flavin to be the chromophoric group in the photoreceptor for phototaxis was obtained by feeding *E. gracilis* over several generations with roseoflavin. This molecule is incorporated as a chromophore into the photoreceptor instead of the original flavin. Roseoflavin has an absorption peak at 500 nm and the spectrum extends up to 600 nm (Fig. 11.5a) (Häder 1998; Häder and Lebert 1998). When untreated cells were exposed to actinic light >550 nm produced by a cut-off filter they did not show phototactic orientation since the action spectrum extends only to ~500 nm (Fig. 11.5b). However, the roseoflavin-treated cells showed a clear phototaxis at wavelengths >550 nm (Fig. 11.5c). But it is of interest that the population displayed both positive and negative phototaxis. This can be easily explained by the fact that the carotenoids in the shading stigma do not absorb in this wavelength range, so that the cell cannot distinguish between light coming from the stigma side and the opposite direction.

When exposed to ultraviolet radiation the four proteins isolated by FPLC from the PAB were damaged and their amounts were significantly reduced (Brodhun and Häder 1993, 1995b). Both fluorescence excitation and emission spectra of the isolated PAB proteins decreased upon exposure to ultraviolet radiation (Häder and Brodhun 1991). As a result, exposure to solar or artificial UV-B radiation impaired phototaxis (Häder 1985, 1986; Häder and Häder 1988). However, this inhibition was not specific; chlorophyll content, photosynthetic oxygen production and motility were likewise affected (Gerber and Häder 1995; Richter et al. 2007). A polychromatic action spectrum of the inhibition confirmed that the high-energy UV-B radiation was most effective (Gerber et al. 1996). Filtering out the

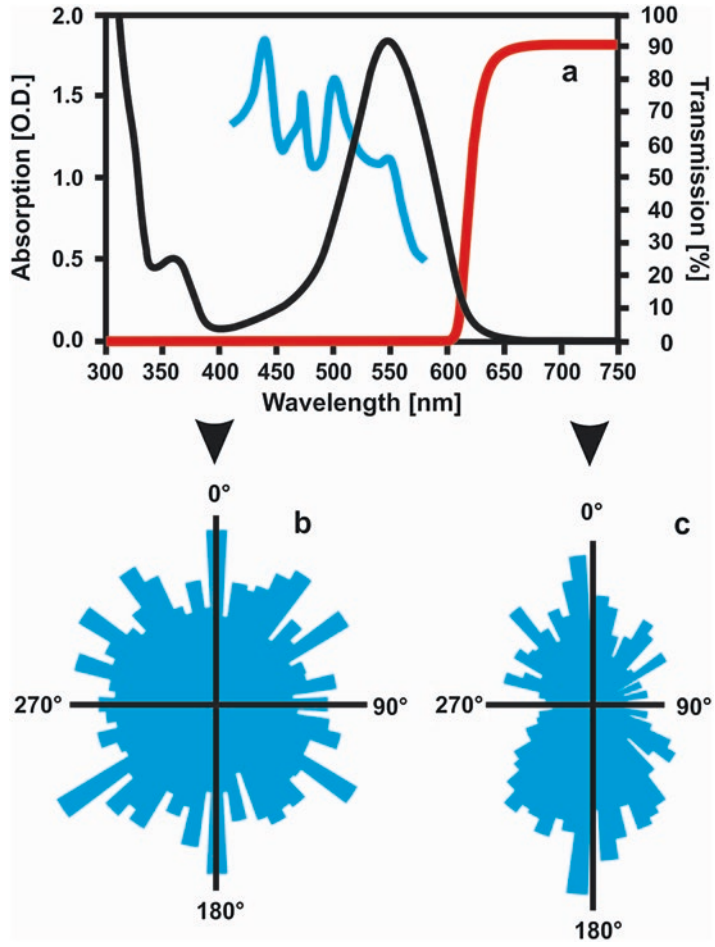

Fig. 11.5 (**a**) Action spectrum for phototaxis in *E. gracilis* (*blue line*), after (Häder and Reinecke 1991), absorption spectrum of roseoflavin (*black line*) and transmission spectrum of the OG 570 cut-off filter (*red line*). Histograms of phototactic orientation in wild type *E. gracilis* grown in medium (**b**) and cells grown in the presence of roseoflavin (**c**) when exposed to actinic light filtered by OG 570 cut-off filter. Redrawn after (Häder and Lebert 1998)

UV-B wavelength band from solar radiation in a field study provided some protection from excessive radiation: bleaching and immobility occurred later than in unfiltered sunlight (Gerber and Häder 1993).

Earlier work using flavin quenchers, such as KI, $MnCl_2$ and NaN_3 which are well known as effective quenchers of the electronically excited state of flavins, were effective in inhibiting the negative phototaxis in the organism (Colombetti et al. 1982; Lenci et al. 1983) but not the step-up photophobic reaction (Mikolajczyk and Diehn 1975). Neither KCN, a general metabolic inhibitor, nor KCl affected phototaxis. Both KI and $MnCl_2$ clearly quenched the fluorescence of 1 mM aqueous solutions of riboflavin.

Galland and coworkers suggested pterins as a possible UV-absorbing chromophore involved in phototactic photoperception of *Euglena gracilis* (Galland and Senger 1988a, b). Using the Okazaki large spectrograph and a computerized video motion analysis, Matsunaga and coworkers found that in addition to UV-A and blue light the action spectrum for photophobic responses of *E. gracilis* extended well into the UV-B with peaks at 270 nm (step-down response) and 280 nm (step-up response), respectively (Matsunaga et al. 1998) which they attributed to the combined action of 6-biopterin and FAD. In addition, fluorescence and emission spectra of the isolated PAB proteins indicated that pterins might also be involved (Galland et al. 1990). The colorless mutant 1F of *Euglena gracilis* does not possess flavins, as indicated by the fluorescence emission spectrum, but it shows the pterin emission band centered around 525 nm (Häder and Lebert

1998). This result indicates that the diaphototaxis found in the 1F and other mutants is not mediated by a flavin but by a pterin.

The separated flavoproteins had an apparent molecular mass of about 33,500 and the pterin-binding protein 27,000 (Brodhun and Häder 1990). Sineshchekov and coworkers suggested that there is an energy transfer from the pterins to the flavins, which can be disrupted by solubilization of the PAB (Sineshchekov et al. 1994a; Lebert and Häder 2000). It is assumed that the pterins are located on the outside of the PAB while the flavins are inside (Häder and Lebert 1998).

In order to confirm that flavins could be involved as chromophoric groups of photoreceptor pigments for phototaxis binding studies with [^{3}H]-labeled riboflavin were carried out (Brodhun et al. 1994). Nebenführ et al. also showed riboflavin-binding sites associated with flagella of *E.gracilis* (Nebenführ et al. 1991). Also Neumann isolated a riboflavin-binding protein from the flagella of *E. gracilis* (Neumann and Hertel 1994).

High and saturable binding was found in *Euglena* flagella with, but also without, attached PABs. In contrast, *Astasia* did not show any binding activity.

To be exhaustive, it should be mentioned that carotenoids have also been discussed as photoreceptors for phototaxis in *Euglena* (Bendix 1960; Wolken 1960, 1977; Batra and Tollin 1964; Bensasson 1975; Gualtieri 1993a; b). In fact carotenoids, especially in the form of rhodopsins (Walne et al. 1998), have been found to be involved in photoorientation in many organisms such as *Chlamydomonas*, *Halobacterium* or *Paramecium* (Nakaoka et al. 1991; Govorunova et al. 2004; Kim et al. 2009). Gualtieri and others, based on theoretical considerations, absorption spectroscopy and gas chromatography-mass spectrometry, proposed that the photoreceptor pigment for *Euglena* phototaxis is a rhodopsin (James et al. 1992; Barsanti et al. 1993a; Gualtieri 2001). This hypothetical rhodopsin was thought to undergo a photocycle which has been analyzed by fluorescence emission spectroscopy (Evangelista et al. 2003). Hydoxylamine reacts with free and opsin-bound retinal. It was found to block the formation of the PAB and impaired photoaccumulation of the cells in the phototaxigraph (Barsanti et al. 1993b). Application of nicotine, an effective inhibitor of carotenoid biosynthesis blocked the biosynthesis of retinal by inhibiting the formation of the cyclohexylidin ring. In *Euglena* it prevented the formation of the PAB and impaired the accumulation of cells in a light field, interpreted as the result of phototaxis (Barsanti et al. 1992). However, when this experiment was repeated by growing *E. gracilis* cells up to 4 months at the highest possible concentration of nicotine (4 mM) the cells survived and neither positive nor negative phototaxis was impaired (Häder and Lebert 1998), indicating that retinal is not likely the chromophoric group of the photoreceptor. It should be mentioned that the accumulation in a light field can be brought about by a number of photoresponses such as phobic reactions or photokinesis (see Sects. 11.4.2 and 11.4.3). In fact, a photoaccumulation of *Euglena* was found in red light fields (Checcucci et al. 1974). This behavior was interpreted as an aerotactic attraction to the photosynthetically produced oxygen by organisms inside the light field. So the underlying mechanism in the experiment by Barsanti et al. might have been inhibited by nicotine, but it was not phototaxis.

11.5.2 Molecular Biology of the Photoreceptor for Phototaxis and the Step-Up Photophobic Response

All the discussions and speculations about the photoreceptor for photomovement in *E. gracilis* have been resolved by the molecular biological identification of the chromoproteins involved (Iseki et al. 2002). Iseki and coworkers succeeded in isolating sufficient quantities of PABs for biochemical analysis by separating the photoreceptors from the flagella followed by subcellular fractionation using a sucrose density gradient centrifugation. They separated a 400-kDa protein that binds flavins from the isolated PAB preparations by liquid chromatography. The protein is

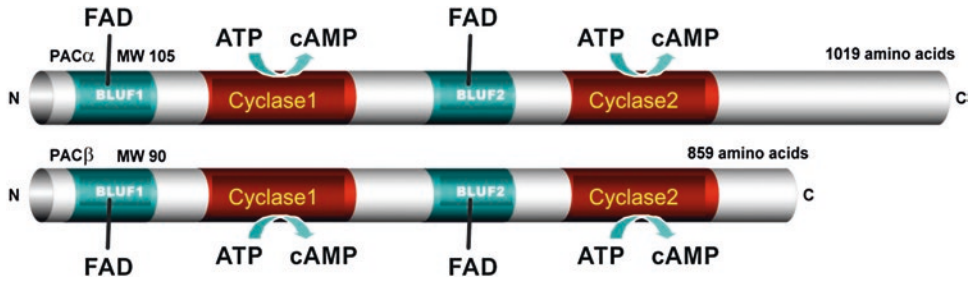

Fig. 11.6 The PACα and PACβ gene products from *E. gracilis* have two FAD binding sites (BLUF1 and BLUF2) and two adenylyl cyclases each

composed of four subunits: two subunits (named PACα) which consist of 1019 amino acids each with a molecular weight of 105 kDa and two more subunits (named PACβ) which consist of 859 amino acids each with a molecular weight of 90 kDa. The α and β subunits are similar to each other. Each of the four subunits has two flavin-binding BLUF (*B*lue *L*ight receptor *U*sing *F*AD) domains and two adenylyl cyclase catalytic domains (Fig. 11.6). The 400-kDa flavoprotein purified from the PAB preparations showed adenylyl cyclase activity that was induced by blue-light irradiation and was accordingly named photoactivated adenylyl cyclase (PAC). The photoactivation of PAC was extensively studied by Yoshikawa et al. (2005). PAC activity was dependent on both the photon fluence rate and the duration of irradiation, between which reciprocity held well within the range of 2–50 μmol m^{-2} s^{-1} (a total fluence of 1200 μmol m^{-2}). Intermittent irradiation also activated PAC in a photon fluence-dependent manner irrespective of the cycle periods, which implies that the increase of PAC activity occurred only during the light period and that elevated PAC activity decreased within 100 ms after the irradiation had stopped (Yoshikawa et al. 2005). Such a sharp switching property of PAC is suitable to be used as a tool to control various cellular processes by light, i.e., optogenetics. In fact, attempts to optogenetically control cAMP levels in *Aplysia* neurons (Nagahama et al. 2007), *Xenopus* oocytes, cultured mammalian cells, *Drosophila* brains (Schröder-Lang et al. 2007), and *Caenorhabditis* neurons (Stierl et al. 2011; Weissenberger et al. 2011) have been reported.

The biological functions of PAC in *Euglena* cells were examined using RNAi knockdown of the genes. When the double stranded RNAs encoding PACα (the 105-kDa subunit) and/or PACβ (the 90-kDa subunit) were introduced into *Euglena* cells, they significantly suppressed the gene expression of PAC, which resulted in the loss of the step-up photophobic response but not the step-down photophobic response (Iseki et al. 2002). Ntefidou et al. (2003b) found that the RNAi knockdown of PAC also effectively suppressed both the positive and negative phototaxis of *Euglena*. Inhibition of either PACα or of PACβ completely blocked negative phototaxis. Knockout of both genes had the same effect. Obviously both genes are required for a functioning phototaxis. From these observations PAC was concluded to act as a sensor for the step-up photophobic response and phototaxis in *Euglena* (Häder et al. 2005; Lüdtke and Häder 2007). However, it is important to emphasize that PAC does not control the step-down photophobic response.

The photoactivation mechanism of PAC still remains to be elucidated mainly due to difficulties in obtaining sufficient amounts of the protein for X-ray crystallography or vibrational spectroscopy. All attempts to express the full-length PAC subunits in *Escherichia coli* have so far failed. Ntefidou et al. (2006) succeeded in expressing PAC in a soluble form in insect cells, but neither crystallization nor spectroscopic analysis using the expressed protein has yet been reported. In contrast, when only the second BLUF domain of PAC (F2) was expressed in *E. coli*, a part of the expressed protein could be collected in a soluble

form that binds flavins (Ito et al. 2005). Although most of the expressed protein collected was insoluble, sufficient amounts of soluble, flavin-binding protein were recovered by refolding the insoluble protein with added flavins after denaturation by guanidine hydrochloride. The recombinant F2 protein showed a photocycle between a dark state and a slightly red-shifted signaling state similar to other bacterial BLUF domains (Ito et al. 2005). The quantum efficiency for the phototransformation of PACαF2 (0.28–0.32) is higher than that of PACβF2 (0.06–0.08), whereas the half-life for the dark relaxation of PACαF2 (34–44 s) is longer than that of PACβF2 (3–6 s) (Ito et al. 2010). Such photocycle features of PACαF2 and PACβF2 indicates different sensitivities for the photoactivation of PACα and PACβ, which may contribute to the wide range of light sensitivity in *Euglena* photobehavioral responses. Iwata et al. (2011) examined the photoreaction of PACαF2 using FTIR spectroscopy and found broad positive peaks in the difference spectrum at the 2900–2400 cm^{-1} region, which were attributed to the O–H stretching vibration of tyrosine, providing direct evidence for the light-induced switching of the hydrogen bond network in the BLUF domain. Single molecule fluorescence spectroscopy was also applied to PACαF2 and native PAC purified from *Euglena* (Fujiyoshi et al. 2011). The fluorescence from a single PACαF2 molecule measured at 1.5 K decreased in one step to background levels, whereas a single PAC molecule bleached in several steps, indicating the involvement of an energy transfer between FADs in the single PAC molecule. Fujiyoshi et al. (2011) also observed reversible spectral jumps of fluorescence from single molecules, which were attributed to a structural change around the hydrogen bonds at the FAD-binding site because the Q514A mutation of PACαF2 suppressed these spectral jumps.

11.6 Mutants and Related Organisms

There are a number of mutants of *E. gracilis* (Lebert and Häder 1997) as well as the close relative *Euglena longa*, formerly known as *Astasia longa* (Krause 2008). All these mutants lack chlorophyll and photosynthesis and consequently live heterotrophically. Table 11.1 summarizes some of the characteristics of these strains. With the exception of *E. longa* all mutants have some form of phototaxis (diaphototaxis and/or negative phototaxis). In addition to the wild type, the FB and 9F mutants have a PAB. The FB mutant is of interest since some cells have a stigma. This mutant may not consist of a uniform population and may contain some revertant cells, which might explain the presence of a stigma in some cells. All mutants are capable of step-up photophobic responses, but only the wild type strain Z shows step-down responses. All mutants and *E. longa* lack positive phototaxis, but all mutants show diaphototaxis at high light intensities and the FB mutant also shows a negative phototaxis component (Lebert and Häder 1997). All strains in this group display both positive and negative gravitaxis.

Even though some of these mutants do not have a PAB, PCR showed the presence of the PACα gene in all mutants but not in *E. longa* (Ntefidou and Häder 2005). However, in the latter organism a largely modified PAC was found, which was dubbed AlPACα (at that time the organisms was still known under the name *Astasia*) (Ntefidou et al. 2003a). The amino acid sequences of the first BLUF domain of PACα and PACβ from the wild type and the mutant strains 9F, FB, 1F and st^- of *E. gracilis* as well as AlPACα and AlPACβ from *E. longa* show a homology of between 43 and 91% (Ntefidou and Häder 2005). The BLUF1 and BLUF2 subunits of the PAC proteins resemble the N-terminal end of the AppA flavoprotein found in the purple bacterium *Rhodobacter sphaeroides*, which also binds FAD (Gomelsky and Kaplan 1995). In this bacterium the photoreceptor regulates the expression of photosynthesis genes (Gomelsky and Kaplan 1998; Masuda and Bauer 2002). The catalytic domain of class III adenylyl cyclases is found in many organisms from bacteria to protists, fungi, trypanosomes, insects and mammals (Koumura et al. 2004). It is interesting to note that the adenylyl cyclase in the *Euglena* PAC genes are more closely related to those found in bacteria than those in eukaryotes including the

Table 11.1 Physiological characteristics of the wild type *E. gracilis* (Eg), the 1F, 9F and FB mutants and the colorless *E. longa* (El, formerly *Astasia longa*). PAB paraxonemal body, dia. Diaphototaxis, pos. and neg. positive and negative phototaxis, PAC photoactivated cyclase, its presence is confirmed by PCR

Strain	Chloroplasts	Stigma	PAB	Phototaxis	Step-up	Step-down	PACα (PCR)
Eg w^+	+	+	+	pos./neg.	+	+	+
Eg 1F	–	–	–	dia.	+	–	+
Eg 9F	–	–	+	dia.	+	–	+
Eg FB	–	(+)	±	dia./neg.	+	–	+
El w^+	–	–	–	–	+	–	AlPACα

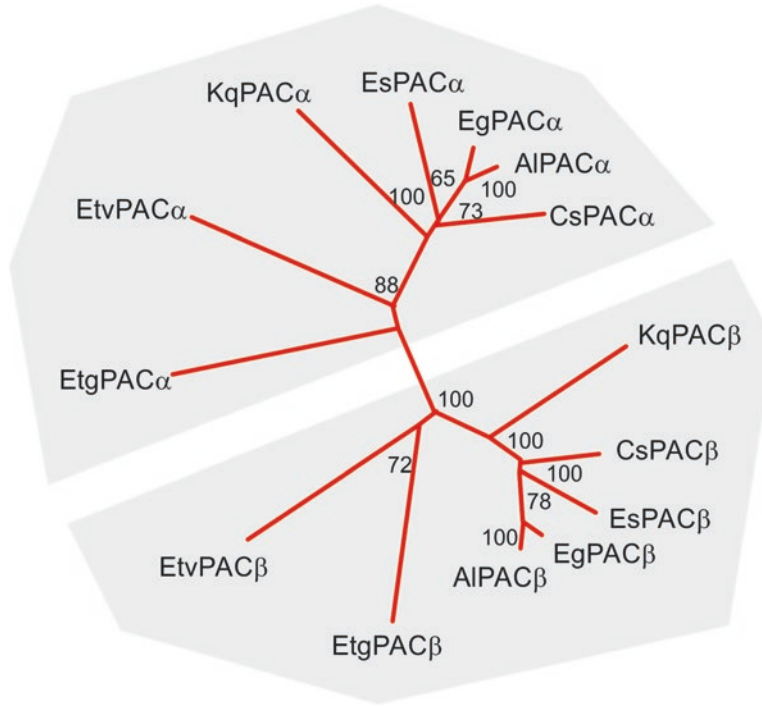

Fig. 11.7 Phylogenetic tree of the PAC proteins in the phototrophic euglenoids *Euglena gracilis* (Eg), *Euglena stellata* (Es), *Colacium sideropus* (Cs), *Eutreptia viridis* (Etv), *Eutreptiella gymnastica* (Etg) as well as the heterotrophic *Khawkinea quartana* (Kq) and *Astasia longa* (Al, new name *Euglena longa*) constructed by the neighbor-joining method using the Clustal X program (Thompson et al. 1997). Redrawn after (Koumura et al. 2004)

trypanosomes. This fact supports the notion that *Euglena* has obtained the gene by secondary endosymbiosis, which is also reflected in the fact that the chloroplasts are covered by a triple membrane. In order to understand the evolution of PAC, protein sequences were compared from several euglenoids by reverse transcriptase-polymerase chain reaction (RT-PCR) including the photosynthetic *Euglena stellata, Colacium sideropus, Eutreptia viridis, Eutreptiella gymnastica* and the heterotrophic *Khawkinea quartana*, but not the phagotrophic *Petalomonas cantuscygni* (Fig. 11.7). Based on these findings the evolutionary tree of PAC starts from a common ancestor. From this the trypanosomes received the trypanosome-type adenylyl cyclases. The other line leads from the common ancestor to phagotrophic euglenoids such as *Petalomonas cantuscygni* (Koumura et al. 2004). The phototrophic euglenoids developed after acquisition of chloroplasts by secondary endosymbiosis which also transferred the PAC genes. The osmotrophic euglenoids such as *Astasia* and *Khawkinea* lost the chloroplasts again but kept the PAC genes.

Confocal immunofluorescence was used to find the localization of PAC proteins in the wild type and mutant strains. It is interesting to note that the flagella of all strains contain PAC gene products even though the fluorescence is much lower than that of the PABs. Furthermore, the

RNAi knockdown of PAC also suppressed the step-up photophobic responses in the wild type and all studied mutant strains as well as in *E. longa* (Ntefidou et al. 2003a). In contrast, only the wild-type strain Z showed step-down photophobic responses which could not be eliminated by RNAi against PAC.

Recently, PAC-like genes were found in genome sequences of a sulfur bacterium *Beggiatoa* sp. (Ryu et al. 2010; Stierl et al. 2011) and a free-living amoeba *Naegleria gruberi* (Fritz-Laylin et al. 2010). Both of them have a BLUF domain and a cyclase domain showing high similarity to the C-terminal half of PACα and PACβ, which implies that they represent an ancestral form of PAC.

11.7 Signal Transduction Chain

The signal transduction chain in photomovement of *Euglena* is still hypothetical. Diehn and Tollin have applied a number of inhibitors and uncouplers of photosynthetic phosphorylation and concluded that the main energy source for phototaxis is photophosphorylation (Diehn and Tollin 1967). However, these experiments were carried out with the so-called phototaxigraph and the observed results probably reflect the reduced motility of the cells due to impaired ATP production since they are in the dark until they enter the light field. In addition, this assumption is ruled out since bleached cells which lack photosynthesis show phototaxis.

Some researchers have speculated about a coupling of the flavin photoreceptor to the signal transduction chain via a cytochrome, but no experimental evidence is available (Fong and Schiff 1978; Gualtieri 1993b). Based on studies with ionophores, inhibitors, on channel blockers, various pH and ion concentrations, Doughty and Diehn proposed a mechanism for the step-down photophobic response (Diehn 1969b; Barghigiani et al. 1979a; Doughty and Diehn 1979, 1982, 1983, 1984) where upon irradiation the PAB is supposed to modulate the activity of a hypothetical NA^+/K^+ exchange pump in the flagellar membrane. This increases the intraflagellar sodium concentration which in turn opens sodium-controlled calcium channels allowing the influx of calcium. The increased calcium concentration finally results in a change in the flagellar beating pattern. However, experiments manipulating the external Ca^{2+} concentration indicated that a Ca^{2+} influx from the medium into the flagellar space is not essential for phototaxis (Meyer and Hildebrand 1988). In a theoretical study, Bovee and Jahn (1972) assumed that the PAB has piezoelectric properties. Upon irradiation it discharges and alters the position or shape of the flagellum and thus the swimming direction. Also Froehlich and Diehn (1974) suggested an electrical type of stimulus transduction by a flavin receptor pigment embedded in a lipid matrix. However, all drugs which impair the photophobic responses did not affect phototaxis: neither the application of ouabain, a specific inhibitor of the Na^+/K^+ exchange pump, nor gallopamin hydrochloride, an organic calcium channel blocker, affected the phototactic orientation in *Euglena* (Häder et al. 1987). In contrast, heavy metal ions (lead, copper, cadmium and mercury) strongly impaired phototactic orientation (Stallwitz 1992; Stallwitz and Häder 1993). The application of triphenylmethyl phosphonium ion ($TPMP^+$) which is a lipophilic membrane-penetrating cation specifically inhibited positive phototaxis and reversed it to negative phototaxis, shifting the transition from positive to negative phototaxis to lower light intensities. These findings indicate that phototaxis might be controlled by a proton or cation gradient across the membrane (Colombetti et al. 1982). Therefore a number of researchers proposed that the membrane potential might be involved (Simons 1981; Harz et al. 1992). In fact, injecting negative electric pulses as well as changing the ionic environment of the cells (Ca^{2+} and Mg^{2+}) changed the flagellar beating pattern (Nichols and Rikmenspoel 1977, 1978, 1980; Nichols et al. 1980; Tamponnet et al. 1988). But exposing swimming cells to an electric field had no effect on phototactic orientation in *Euglena* (Häder et al. 1987). Since *Euglena* shows very sensitive behavioral reactions to heavy metals and other pollutants at very low concentrations it is employed in bioassays based on computerized,

on-line analysis of motility and orientation (Tahedl and Häder 2001; Häder 2004; Ahmed and Häder 2011; Azizullah et al. 2013).

The application of caffeine, an inhibitor of the phosphodiesterase, reversed the negative phototaxis at high irradiances (1000 W m^{-2}) into a positive one (Richter et al. 2006). Ammonium ions specifically enhance step-down photophobic responses, as well as L-methionine-DL-sulfoximine, an inhibitor of ammonium assimilation (Matsunaga et al. 1999). In contrast, cycloheximide, an inhibitor of eukaryotic protein synthesis, impaired the step-down photophobic response and enhanced the step-up reaction, which was interpreted as suggesting that newly synthesized proteins are specific for the photoperception and signal transduction of the step-down photophobic response.

The photoreceptor for the step-up photophobic response and phototaxis has been revealed to be PAC, a light-dependent enzyme that produces cAMP upon blue light irradiation (Iseki et al. 2002; Ntefidou et al. 2003b). Intracellular cAMP levels in *Euglena* remarkably increased within 1 s after the onset of irradiation and then returned to the original level, corresponding well with the kinetics of the step-up photophobic response (Yoshikawa et al. 2005). Two possibilities had been postulated regarding the downstream signaling pathway from cAMP to the photobehavioral responses (Watanabe and Iseki 2005). One is that cAMP opens cyclic nucleotide-gated channels to facilitate an influx of Ca^{2+} that may modulate flagellar motility. The other is that cAMP activates a protein kinase A that may phosphorylate flagellar proteins to change the mode of flagellar beating. The latter seemed more plausible than the former because a catalytic subunit of a protein kinase had been cloned from *Euglena* (Kiriyama et al. 1999). Daiker et al. (2011) found that staurosporine, a protein kinase inhibitor, considerably blocked phototaxis as well as gravitaxis at low concentrations. Using PCR, five different kinases from *Euglena* were cloned. The blockage of only one of the kinases (PK.4) by RNAi suppressed both gravitaxis and phototaxis (Fig. 11.8), which suggested that PK.4 is the downstream component of cAMP signaling in both gravitaxis and phototaxis (Daiker et al. 2011). A hypothetical signaling cascade from PAC to a flagellar apparatus during step-up photophobic response and phototaxis in *Euglena* is summarized in Fig. 11.9. The photoreceptor molecule consisting of two PACα

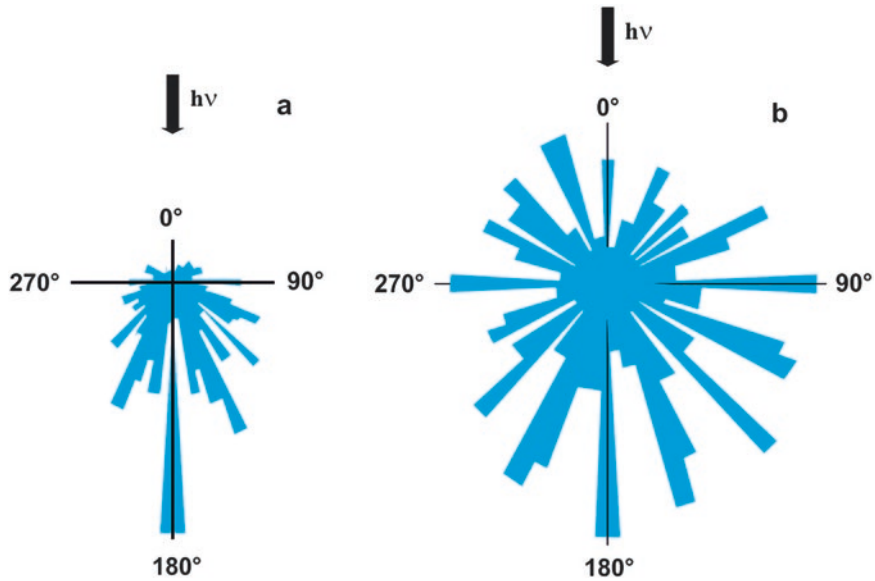

Fig. 11.8 Inhibition of negative phototaxis by RNAi against protein kinase A PK.4 25 days after RNA knockdown (**b**). Control (**a**). Redrawn after (Daiker et al. 2011)

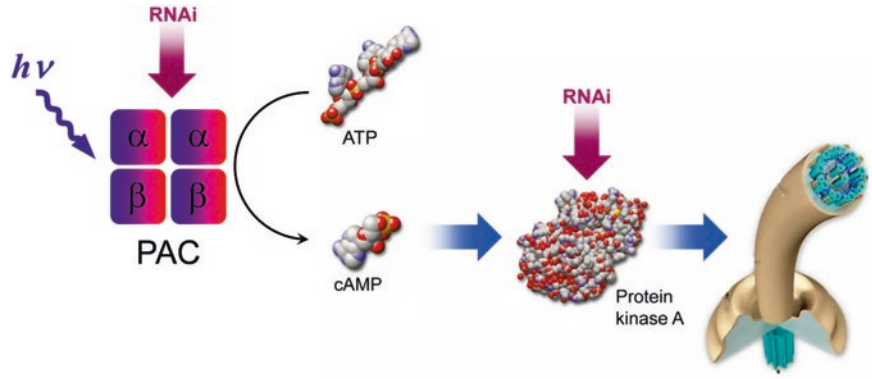

Fig. 11.9 Assumed signal transduction chain for phototaxis and step-down photophobic responses in *E. gracilis*. After light activation of the photoreceptor molecule consisting of two PACα and PACβ subunits, the adenylyl cyclase domain produces cAMP from ATP which is believed to activate a protein kinase A inside the flagellum. The resulting phosphorylation of one or several proteins within the flagellum causes a change in the flagellar activity

and PACβ subunits is activated by light absorbed by FAD bound to the BLUF domains. This activates the adenylyl cyclase domain which produces cAMP from ATP. cAMP in turn is believed to activate a protein kinase A inside the flagellum. The resulting phosphorylation of one or several proteins within the flagellum causes a change in the flagellar activity.

11.8 Other *Euglena* Species

In contrast to *E. gracilis*, the green *Euglena mutabilis* does not possess flagella, but moves in a gliding fashion. The cells contain both a stigma and a PAB, which, however, differ in shape and size from the organelles found in *E. gracilis*. When exposed to lateral light the cells show positive phototaxis (Häder and Melkonian 1983). They swing left and right, as if to scan the light direction, and move in the direction of the light source. The precision of orientation increases with the light intensity up to 100 lx and then decreases again. Negative phototaxis was not observed. The action spectrum is completely different from that in *E. gracilis* as it has a number of peaks in the blue and green range of the spectrum but extends well into the red (Fig. 11.10). It can only be speculated about the nature of the photoreceptor. The peaks in the blue region might be due to the action of a flavin and the long wavelength sensitivity cold be due to the action of photosynthetic pigments as in the case of desmids (Wenderoth and Häder 1979). Since the degree of phototaxis is higher in white light than at any individual wavelength, regardless of the fluence rate, it could be speculated that phototaxis in this organism depends on the interaction of more than one photoreceptor. Since the cells do not rotate during locomotion a periodic shading mechanism can be excluded for the light direction perception.

E. mutabilis also shows step-up and step-down photophobic responses (Melkonian et al. 1986). When a cell moves in the light and enters a shaded area it bends away from the shade. By repeated responses it can maneuver along the dark/light boundary. The same behavior is found when a cell glides in a dark area and suddenly hits a bright area; the sudden increase in light intensity induces a step-up photophobic response and the cell turns away from the bright area.

Photokinesis has also been observed in *E. mutabilis* (Melkonian et al. 1986). In darkness less than 10% of the cell population are motile. The percentage increases when exposed to light at fluence rates >20 W m^{-2} (ca. 4000 lx) white light and reaches about 100% between 50 and 100 W m^{-2} when recorded 10 min after the onset of light. The action spectra for photokinesis as well as step-up and step-down resemble each other and also that of phototaxis.

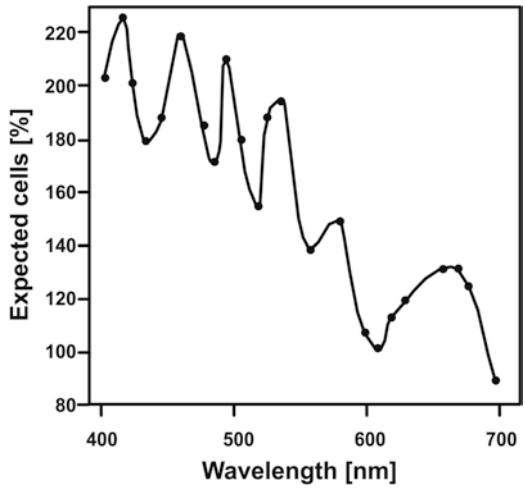

Fig. 11.10 Action spectrum of positive phototaxis in *E. mutabilis* based on fluence-rate response curves. Abscissa, wavelength in nm; ordinate, fraction of cells moving toward the light source within a sector ±30° as percentage of the fraction expected in this sector in a randomly oriented population. Redrawn after (Häder and Melkonian 1983)

The red colored freshwater *Euglena sanguinea* can be occasionally found in the neuston (top layer) of ponds (Gojdics 1939). The color is due to a high concentration of carotenoids such as β-carotene, astaxanthin-diester and diadinoxanthin. The cells possess flagella and orient precisely using positive phototaxis; negative phototaxis has not been observed even at irradiances of 600 klx, which is far in excess of solar radiation (Gerber and Häder 1994). The sensitivity to light is rather low as compared with *E. gracilis* and reaches a plateau at about 10 klx. Further work on this interesting organism was hampered by the fact that nobody has succeeded in cultivating this flagellate.

11.9 Conclusions and Future Directions

The mechanism for photoperception of phototaxis has been revealed by the finding that the photoreceptor is located in the paraxonemal body inside the trailing flagellum inside the reservoir. The PAB has a dichroic structure which is reflected in the polarotaxis in polarized light. The stigma aids in light direction perception by casting a shadow on the PAB when the cell rotates in lateral light during forward locomotion, however it is not indispensable as shown by the fact that stigmaless mutants are capable of a (modified) phototaxis. The long search for the molecular identity of the photoreceptor molecules has been terminated by the molecular biological identification of a photoactivated adenylyl cyclase (PAC) consisting of two α- and β-subunits each. Upon light activation these enzymes produce cAMP from ATP which has been found to activate a specific protein kinase (PK.4). The latter enzyme is thought to phosphorylate proteins inside the flagellum which result in a reorientation and course correction of the swimming path.

While the step-up photophobic response and both positive and negative phototaxis are mediated by PAC, the receptor for the step-down photophobic reaction has not yet been identified but proven not to be PAC. Also the photoreceptor for photokinesis and those for phototaxis in the gliding *E. mutabilis* as well as the red colored *E. sanguinea* still need to be revealed having completely different action spectra extending into the red region of the spectrum. Spectrofluorometric analysis has indicated an additional role for pterins in the photoperception of *E. gracilis*. Their role and location are not yet completely resolved. While the location of PAC inside the PAB (and also in the flagellum outside the reservoir) was confirmed by confocal immunofluorescence, the location of the protein kinase needs to be determined. It is also not clear if further elements are involved in the sensory transduction chain. In addition, the cooperation with the other responses to environmental stimuli has to be elucidated including gravitaxis which uses the same protein kinase (PK.4) but operates with a different adenylyl cyclase. The proteins which control the bending of the trailing flagellum as well as their molecular action have not been characterized.

Acknowledgements The authors thank their long-time coworkers Peter Richter, Maria Ntefidou and Sebastian Strauch, who have critically read this manuscript. The financial support for the underlying work for this review by DFG, DLR, BMBF and JSPS is gratefully acknowledged.

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