Chapter 25

Phototaxis Photoreceptor in *Euglena gracilis*

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Introduction

Many motile microorganisms, such as flagellates and ciliates, optimize their position in the water column by motile responses controlled by external stimuli. The photosynthetic, unicellular flagellate *Euglena gracilis* primarily uses light (Lebert and Häder 2000, Lebert 2001) and gravity (Häder et al 2003) for this purpose.

In addition to photokinesis, which is a steady-state dependence of the swimming velocity on the irradiance (Wolken and Shin 1958), the cells show photophobic responses upon a sudden step-down or step-up in the actinic light intensity (Doughty and Diehn 1984). At low light intensities (below $10\,W\,m^{-2}$) the cells show a directed movement toward the light source (positive phototaxis) and swim away from the light source at higher intensities (negative phototaxis) (Häder and Lebert 1985). In the water column, these antagonistic reactions to light and gravity control the vertical position of the cells in the water column (Häder and Griebenow 1988).

In contrast to many other light-responsive, eukaryotic, unicellular algae, neither is the stigma of *Euglena gracilis* organized in a quarter wavelength stack (Kreimer 1994), nor is it the photoreceptor for phototactic orientation. The receptor is believed to be located in the paraflagellar body, also called the paraxonemal body (PAB) (Andersen et al 1991). This organelle is located on the emerging flagellum, inside an invagination of the front end, at the position where the short flagellum merges. The photoreceptor pigments are oriented dichroically. In polarized light *Euglena* cells orient at an angle of about 30° clockwise to the electrical vector (Häder 1987). The dichroic orientation is further supported by the

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quasi-crystalline structure of the PAB seen in electron microscopy (Piccinni and Mammi 1978).

All published action spectra for phototaxis in *E. gracilis* show two major maxima in the UV-A and blue regions of the spectrum (see review by Lebert 2001). No sensitivity could be found above 520 nm. Based on these results as well as spectroscopic and biochemical data, most authors assume the photoreceptor molecules to be flavins and pterins (Brodhun and Häder 1990). Biochemical analysis of the PAB revealed four major proteins with molecular masses between 27 and 33 kDa carrying pterins and flavins as shown by their fluorescence spectra (Brodhun and Häder 1990, Häder and Brodhun 1991). In contrast, Gualtieri (1993) claimed that the phototaxis photoreceptor in *E. gracilis* is a rhodopsin.

Photoreceptor for Step-Up Photophobic Responses in *Euglena*

The controversy about the nature of the photoreceptor was ultimately solved by the molecular genetic analysis published in a highly recognized paper by Iseki et al (2002). The authors succeeded in isolating and identifying a flavoprotein photoactivated adenylyl cyclase (PAC). PAC is a member of a novel blue-light receptor family consisting of two PACα and two PACβ subunits with molecular weights of 105 (1019 amino acids) and 90 kDa (859 amino acids), respectively. Each subunit contains a tandem repeat of a FAD-binding domain and an adjacent adenylyl cyclase catalytic domain (Iseki et al 2002). The two genes have been sequenced and the overall similarity between PACα and PACβ is 72% at the nucleotide level. The location of the photoreceptor proteins in the PAB has been demonstrated by indirect immunofluorescence staining using polyclonal antibodies. Sequence alignment of the two flavin binding domains with genes from several bacteria and cyanobacteria revealed the presence of a BLUF (blue light receptor using flavins) domain.

Fluorescence excitation spectra of the photoreceptor pigments resembled the action spectrum for photophobic responses in *E. gracilis*. Excitation of the photoreceptor protein in vitro by UV/blue light (peaks at 370 and 450 nm) resulted in enhanced cAMP production by the cyclase. This secondary messenger is thought to control the flagellar beat pattern and trigger step-up photophobic responses.

Introducing double-stranded mRNA (dsRNA) of PACα or PACβ or both into the cells by electroporation resulted in RNA interference (RNAi) and completely blocked the step-up photophobic response. In addition, after RNAi the PAB could not be seen in the cells by Nomarski interference microscopy or autofluorescence, and also Northern blots did not show the gene products of PACα and PACβ in RNAi-treated cells. But RNAi of PACα or PACβ did not impair the step-down photophobic response (Iseki et al 2002), indicating that the PAC gene products are not the photoreceptor for the latter reaction. This is

consistent with the different action spectrum of the step-up responses (Matsunaga et al 1998).

Mutant Analysis

The colorless relative of *Euglena*, *Astasia longa*, also shows step-up (but not step-down) photophobic responses. PCR and subsequent sequencing indicated that *Astasia* possesses the PAC genes that are 95% homologous to those of *Euglena* (Ntefidou et al 2003a). In addition, several colorless mutants of *E. gracilis* have been sequenced. The mutants fall into two groups, one being closely related with *E. gracilis* and the other with *A. longa* (M Ntefidou, personal communication). Despite the differences, RNAi using dsRNA of PACα or PACβ or both inhibited step-up photophobic responses in all strains but not step-down responses in those strains that show this response (Table 1). This result clearly indicates that the step-down photophobic response is mediated by a different photoreceptor than the step-up response.

In *Astasia* no PAB could be found using autofluorescence of the organelle (Lebert and Häder 1997). Likewise, this method did not reveal the presence of PABs in the strains 1F, 9F and st$^-$, while small PABs could be detected in some cells of the strain FB. These findings raise the question where the photoreceptor for photophobic responses is located. Using confocal microscopy and indirect immunofluorescence with polyclonal antibodies against PACα showed that at least *Astasia* and the stigmaless strain lack the PAB while this method indicated the presence of PABs in the other strains. However, this technique clearly demonstrated that, in most strains, the PACα gene product occurs along the entire length of the flagellum. This indicate that, in contrast to the previous assumption, the photoreceptor for photophobic reactions is not located in the PAB (this is at least proven for the PAB-lacking strains), but on the flagellum.

Table 1. Occurrence of step-up and step-down photophobic responses in *Euglena gracilis* (E.g.) and *Astasia longa* (A.l.) and its inhibition by RNAi

DsRNA	E.g. wt	E.g. dark grown	E.g. 1F	E.g. 9F	E.g. FB	E.g. st$^-$	A.l.
Step-up phobic responses							
Control	+	+	+	+	+	+	+
PACα	–	–	–	–	–	–	–
PACβ	–	–	–	–	–	–	–
PACα and PACβ	–	–	–	–	–	–	–
Step-down phobic responses							
Control	+	+	–	–	–	+	–
PACα	+	+	–	–	–	+	–
PACβ	+	+	–	–	–	+	–
PACα and PACβ	+	+	–	–	–	+	–

Wt, wild type; st$^-$, stigmaless.

PAC Involvement in Phototaxis

As indicated above, wild-type (green and dark grown) *Euglena* cells show positive or negative phototaxis depending on the irradiance. None of the other strains show normal phototaxis, but some perform a diaphototaxis (swimming perpendicular to the incident light beam) at high fluence rates (Lebert and Häder 1997). Since the action spectrum for phototaxis is similar to that for photophobic responses (Häder and Reinecke 1991), it might be possible that the same or similar photoreceptor might be responsible for the phototaxis.

To tackle this question, RNAi was used again and phototactic orientation was determined at both high and low fluence rates using an automatic, real-time tracking system (Häder and Lebert 2000). In fact, both positive and negative phototaxis was eliminated after RNAi using PACα, PACβ or both as templates (Ntefidou et al 2003b). Neither the swimming velocity nor the form factor of the cells were affected by this treatment. It is interesting to note that the inhibition of light-dependent orientation lasted more than two months and at least double as long as the impairment of step-up photophobic responses by the same treatment. The latter result could mean that phototaxis is mediated by a slightly different photoreceptor protein from the same family than that involved in photophobic responses, or that different concentrations of the gene product are needed for the different reactions. In any case it is obvious that the inhibitory dsRNA is carried to the daughter cells after cell division over many generations and only dilutes out after a considerable time. Since only cells that possess a PAB show phototactic orientation, it could be speculated that the PAC photoreceptor responsible for phototaxis may be located in the PAB rather than on the flagellum. But why do some of the mutants, which do possess a PAB, not show phototactic orientation? One interesting observation is that the mutant 1F of *E. gracilis* does not show flavin fluorescence of PAB preparations (Häder and Lebert 1998). This may indicate that while the PAB is produced it does not contain a functional PAC with a bound FAD.

The Role of Pterins in Phototaxis

Biochemical and spectroscopic data indicate that pterins may be involved in phototaxis of *E. gracilis* (Brodhun and Häder 1990, 1993, 1995, Häder and Brodhun 1991, Lebert and Häder 1997). The hypothesis was that pterins operate as antenna pigments that transfer the energy absorbed to flavins, which in turn perform the primary photochemical reaction. Pterin biosynthesis starts from GTP and is mediated by a key enzyme, GTP cyclohydrolase I. In contrast, the flavin pathway is controlled by GTP cyclohydrolase II. GTP cyclohydrolase I has been found in many organisms including bacteria, fungi and mammals (Auerbach et al 2000). In *Escherichia coli* it is a homodecamer with 247 kDa. The genetic sequence and the protein structure are known. It has dimensions of 65×100 Å

and contains 10 active sites with one zinc ion per active site (Auerbach et al 2000). The enzyme was also found in *E. gracilis* and has been sequenced. We are grateful to J. Maier (Tübingen) who donated us the gene in form of a plasmid.

We used the sequence to produce dsRNA and to insert it into *E. gracilis* by electroporation. The result was a complete inhibition of both positive and negative phototaxis by RNAi, which again lasted for many generations. As with RNAi of PAC, neither swimming velocity nor cell form was affected. But, very surprisingly, neither step-up nor step-down photophobic reactions were impaired. These results can be interpreted by a number of options. First, the energy absorbed by only flavins is not sufficient to drive phototaxis but to mediate photophobic responses. The PAC photoreceptor for step-up (but not step-down) photophobic responses could be different from that responsible for phototaxis and the latter needs the cooperation with pterins. The final option is that pterins are needed for the correct assembly of the PAB. If true, the latter assumption would support the hypothesis that the PAC photoreceptor responsible for phototaxis is different and located in a different place from that involved in step-up photophobic responses.

Summary and Conclusions

Photoactivated adenylyl cyclase (PAC) is a tetrameric 390 kDa protein which controls step-up photophobic (but not step-down) responses in *E. gracilis* wild-type and phototaxis mutant strains as well as in the colorless relative *A. longa*. This novel class of photoreceptor pigments was also found to mediate positive and negative phototaxis. While the original hypothesis that PAC is located in the paraxonemal body (PAB) seems to hold for phototaxis, it cannot be true for photophobic responses in several mutants and *Astasia* that lack a PAB. Instead the location of PAC along the entire length of the flagellum was shown by confocal immunofluorescence microscopy. This indicates that there is a whole family of related PAC proteins in *Euglena* and its relatives. This is confirmed by a sequence analysis of PAC genes in the mutants that revealed considerable divergence between the strains. It remains to be explored whether or not step-down photophobic responses, the action spectrum of which is different from that for step-up responses, is mediated by still another PAC photoreceptor. While the involvement of pterins in phototaxis was assumed and is now proven by RNAi, it was a surprise that the presence of pterins is not mandatory for step-up photophobic responses.

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