The photoreceptor protein of *Euglena gracilis*

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Abstract We isolated the photoactive protein E*rh*, isolated from the photoreceptor of the unicellular photosynthetic flagellate *Euglena gracilis*. It is a 27 kDa protein with a photocycle resembling that of sensory rhodopsin, but with at least one stable intermediate. We recorded the absorption spectrum of the parent form of this protein both under native form and in the presence of hydroxylamine and sodium borohydride, and the fluorescence spectra of both the parent and intermediate forms. We suggest that E*rh* is a rhodopsin-like protein and propose a simple photocycle. This protein shows optical bistability, without thermal deactivation.

Key words: Rhodopsin-like protein; Photoreceptor; Optical bistability; Photocycle; *Euglena gracilis*

1. Introduction

Since the turn of the century the photosynthetic and photosensitive flagellate *Euglena* has provided an intriguing subject for photo-biological studies. This flagellate dwells in shallow natural ponds, and uses sunlight as a source of energy and information. Its chloroplasts are the energy supplying devices, whereas a simple but sophisticated system is used as a light detector. Two flagella are inserted in the sub-apical invagination of the cell termed the reservoir. The eyespot, composed of red-orange pigment granules, lies in the adjacent cytoplasm. Only one flagellum emerges from the cell and consists of an axoneme, a paraxial rod running parallel to it, and a photoreceptor near its base [1].

As the cell rotates while swimming, the eyespot comes between the light source and the photoreceptor, thus modulating the light that reaches it, and regulating the steering of the locomotor flagellum [2,3]. The frequency of the rotation is 2 Hz [4].

This configuration of eyespot, photoreceptor and flagellum represents a simple but complete visual system. The photoreceptor is a three-dimensional natural crystal of about $1\times0.7\times0.7$ μm. This crystal is made up of a single protein and can be interpreted as a three-dimensional crystal of type I [5], i.e. a stack of two-dimensional crystal protein layers characterized by in-plane hydrophobic interactions and held together by hydrophilic interactions as confirmed on the basis of cryo-fractured images [6].

Other examples of naturally occurring crystalline light-detecting organelles are the bacteriorhodopsin of *Halobacterium halobium*, the reaction centers of photosystem II in the photosynthetic membranes of green plants, and the photosynthetic membranes of *Rhodopseudomonas viridis* and related purple bacteria [7]. Among these, the photoreceptor of *Euglena* acquires a special meaning since it is the only crystal of a photodetecting protein consisting of about 100 layers [6]. Moreover, Barsanti et al. [8] reported the presence of a photochromic pigment in the photoreceptor of *Euglena gracilis*, which undergoes repeated and reversible fluorescence changes with a determinate kinetics.

What about the protein that constitutes the photoreceptor? Gualtieri and coworkers [9] and James and coworkers [10] measured the absorption spectrum of a single *Euglena* paraflagellar photoreceptor. Due to the great similarity between these spectra and the absorption curve of rhodopsin α-band centered at 510 nm, both research groups suggested a pigment such as a rhodopsin-like protein as the photoreceptive protein in *Euglena*. Inhibition of the photoreceptor formation by means of nicotine was obtained by Barsanti et al. in 1992 [11]; retinal extraction from intact cells was successively achieved by the same group [12].

In this paper we report the purification of the protein that forms the photoreceptor of *Euglena*.

2. Materials and methods

2.1. Cultures

Cultures of *E. gracilis* strain Z cells (Sammlung von Algen Kulturen Göttingen, 1224-5/25) were grown axenically in Cramer–Myers medium 0.025 M in sodium acetate (pH 6.8) [13], under constant temperature (24°C) and continuous illumination (2×10^2 μmol photons m^{-2} s^{-1}).

2.2. Isolation of photoreceptors

The isolation solution was prepared as follows: Triton X-100 was dissolved in a HEPES-buffered solution (100 mM HEPES–KOH, pH 7.00; 20 mM PIPES; 10 mM EDTA; 50 mM sucrose; 1 mM dithiothreitol; 7.5% v/v glycerol) to a final detergent concentration of 4% v/v, filtered and added to cells (4:1) previously suspended in 100 mM HEPES buffer (pH 7.00). Cells were washed with the isolation solution once a week for a month; cells were then broken using an Ashcroft Duralife (USA) pressure homogenizer. A preliminary centrifugation at $500\times g$ eliminated the unbroken cells. The supernatant was collected and layered on a 60% Percoll gradient (Pharmacia, Germany) (all percentages expressed as w/v) in a Corex tube (Du Pont, Germany). Gradient was developed for 30 min at $20\,000\times g$. The resulting three fractions were washed free of Percoll by resuspension and repeated centrifugation at $40\,000\times g$ for 15 min. The fraction at 1.2 g/l density proved to be the fraction containing the photoreceptors.

2.3. Retinal extraction

The extraction was performed on the fraction containing the photoreceptors, which was obtained from Percoll gradient. The sample was treated with a solution of 6 M formaldehyde in 10% (v/v) isopropanol,

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and then extracted with a mixture of dichloromethane and hexane; successive column chromatography separation and HPLC purification were performed according to the procedure of Gualtieri et al. [12].

2.4. Column chromatography and gel filtration

Isolated, purified photoreceptors were solubilized by incubating them in Milli-Q water until no fluorescence emission was detectable any more (about 1 h). This extract was filtered and applied to an anion exchange chromatographic column (20 cm long, 16 mm diameter) of DEAE-52 (Whatman) equilibrated with 20 mM Tris–HCl, pH 7.4. The column was then washed with one column volume of this buffer, and eluted with a NaCl gradient (0–0.5 M) in Tris–HCl buffer. The eluted fractions were analyzed using absorption spectroscopy, and the fractions showing an absorption band around 510 nm were pooled and subjected to gel filtration using a pre-packed Superose 12 column (Pharmacia). One milliliter fractions were collected by eluting with NH_4HCO_3 20 mM and Tris–HCl 20 mM.

2.5. Reaction of Erh with hydroxylamine and sodium borohydride

Hydroxylamine (100 mM) reaction was carried out at pH 7.0 in 20 mM Tris–HCl pH 7.0. Samples were illuminated in the spectrometer at 490 nm for 15 min. Sodium borohydride reaction was carried out by adding an excess of powder under alkaline conditions.

2.6. Fluorescence microscopy

A Zeiss Axioplan fluorescence microscope (Germany) equipped with an epifluorescence system, a 100× (N.A. 1.3) planapochromatic objective, and a 100 W Mercury lamp was used. Fluorescence images from *Euglena* cells were acquired with the following filter combinations: a UV-blue set (8 nm band pass excitation filter, 365 nm; chromatic beam splitter, 395 nm; barrier filter, 397 nm; 800 $\mu W/cm^2$), and a blue-violet set (8 nm band pass excitation filter, 436 nm; chromatic beam splitter, 460 nm; barrier filter, 470 nm; 1100 $\mu W/cm^2$). Irradiances were measured with a HandHeld Optical Power Meter Model 840.

2.7. Photography

Fluorescence photographs were taken with a Minolta X-100 camera mounted on the Zeiss Axioplan microscope, and recorded on Kodak Ektachrome 100 ASA color film.

2.8. Spectroscopy

Absorption spectra were recorded using a Jasco 7850 spectrometer. Fluorescence spectra were recorded using a Perkin Elmer LS 50 B fluorimeter.

3. Results and discussion

The photoreceptor isolation procedure developed by Gualtieri et al. [14] involved protrusion of the reservoir and flagellar bases. This procedure produced pure fractions of flagella with their photoreceptors still attached, but a relatively low yield of isolated photoreceptors since the solution employed destabilized the structure of the photoreceptors, thereby precluding subsequent critical biochemical and structural characterization.

By adopting the isolation procedure presented here a high yield of photoreceptors was very easily obtained, with their structure very well preserved. Fig. 1 shows an image of a fraction obtained from the Percoll gradient (density: 1.2 g/l). This fraction, upon photo-excitation at 365 nm and successively at 436 nm, reveals a very high content of green optically bistable photoreceptors. No other fluorescent objects but the isolated photoreceptors are visible in the microscopic field. From this sample, retinal was extracted according to the procedure of Gualtieri et al. [12]. No other chromophore was detected in the sample.

Incubation in the isolation solution leads to the extraction of all the soluble proteins and cellular structures, with the exception of the photoreceptor and the pellicle [15]. The

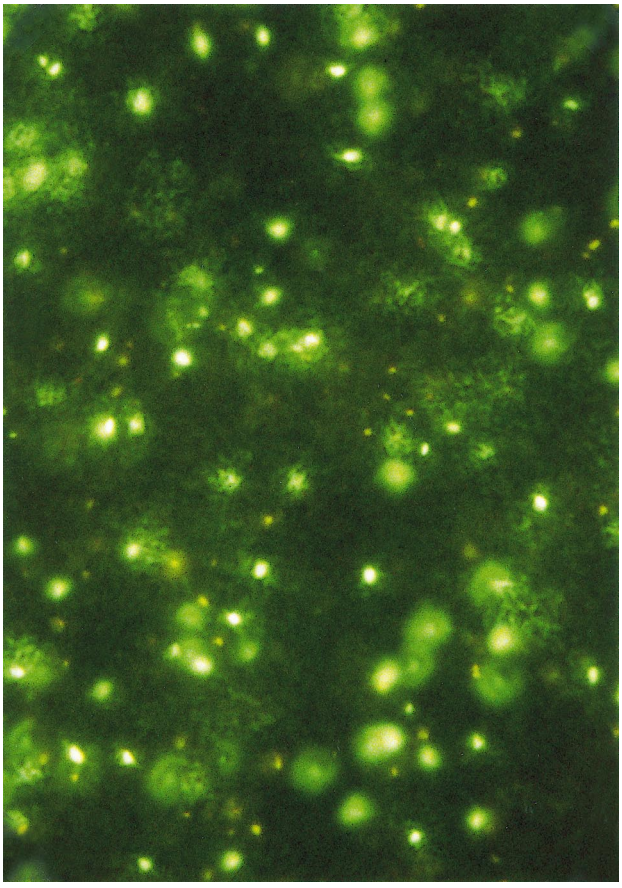

Fig. 1. Fluorescence micrograph of the fraction of photoreceptors obtained from the Percoll gradient. The photoreceptors are the green fluorescent spots. Scale bar, 5 μm.

three-dimensional crystalline organization of the photoreceptor and its high protein content would explain the lack of effect of a detergent like Triton X-100 on the photoreceptor of *Euglena*. Moreover, by transmission electron microscope and optical microscope inspection, the photoreceptor structure seems to be strengthened (data not shown). This is probably due to the bonding of detergent molecules by hydrophobic interactions to hydrophobic segment of the photoreceptor proteins [16,17].

Solubilization of photoreceptors was obtained by re-suspension in de-ionized Milli-Q water. The complete dissolution of the photoreceptor crystals was achieved after 1 h incubation. Under fluorescence microscopy with the two excitation wavelengths that induce optical bistability the sample showed no emission. Possibly the hydrophilic interactions between photoreceptor layers are loosened in this ion-free solution leading to dissolution of the crystalline structure. This aqueous extract was loaded onto an anion exchange chromatographic column to separate the different proteins. The eluted fractions were analyzed using absorption spectroscopy. Three fractions showing an absorption band around 510 nm were pooled and subjected to gel filtration to separate single proteins.

Upon SDS–PAGE one of the collected fractions showed a single band with a molecular weight of 27 kDa (Fig. 2), very similar to that of other rhodopsin-like proteins already identified in other taxonomic groups. In fact, we can consider *Euglena* to be evolutionarily located in the middle between

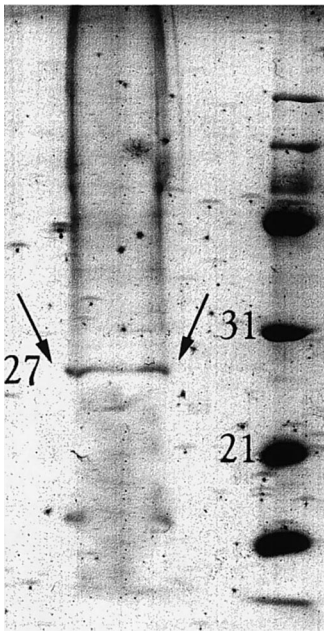

Fig. 2. Coomassie-stained SDS–PAGE gel of a fraction obtained from gel filtration. The putative rhodopsin-like protein is the 27 kDa protein indicated by the arrows.

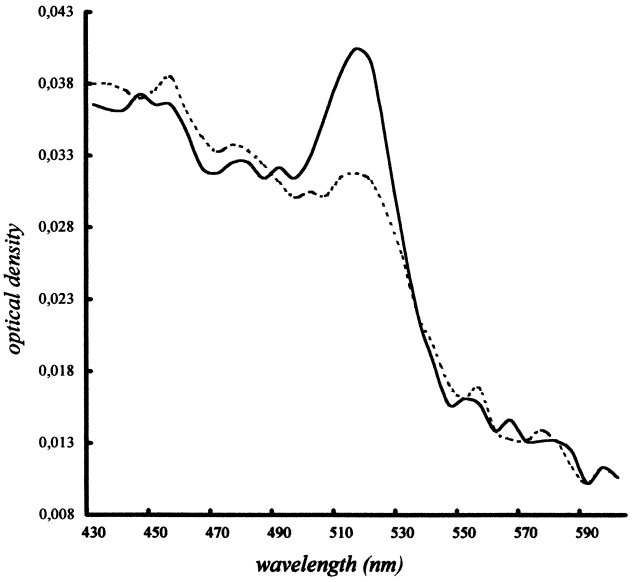

Fig. 3. Absorption spectrum of the 27 kDa native protein showing the band centered at 515 nm (solid line). After treatment with hydroxylamine (NH_2OH), the band undergoes dramatic fading (dashed line).

Archaebacteria and Chlorophyceae [18]. In the former group rhodopsin-like proteins were purified and sequenced; sensory rhodopsin I has a molecular weight of 25 kDa [19], and sensory rhodopsin II has a molecular weight of 23 kDa [20]. In the algae another rhodopsin-like protein (chlamyopsin) with a molecular weight of 30 kDa was sequenced as well [21].

Spectroscopically, the extracted protein showed a well defined absorption band centered at 515 nm (Fig. 3, solid line). This spectrum can be superimposed on the 'in vivo' absorption spectrum recorded on the photoreceptor by Gualtieri et al. [9], and by James et al. [10], and on the absorption spectrum of sensory rhodopsin II from *Natronobacterium pharaonis* [22]. Although rhodopsin-like proteins can tune their absorption spectrum from 380 to 640 nm [23], the distribution of λ_{max} of their absorption curves is centered around 515 nm [24]. No other putative photoreceptive pigments show an absorption maximum at that wavelength. The extracted protein does not fit the Dartnal nomogram [24], but shares this characteristic with other rhodopsin-like proteins, such as the sensory rhodopsin II from *N. pharaonis* [18].

To confirm the hypothesis that the extracted protein was a rhodopsin-like protein, we treated this protein with both hydroxylamine (NH_2OH) and sodium borohydride ($NaBH_4$). The amine reacts with all aldehydes in aqueous solutions to form oximes, which are stable compounds. In the case of visual pigments, NH_2OH combines with retinal molecules released in the bleaching of rhodopsin or displaces the retinal from opsin to form oximes in the light and also in the dark [25]. The effect of NH_2OH on our protein was a dramatic fading of the absorption band centered at 515 nm (Fig. 3, dashed line). This clearly indicates that the chromophore is an aldehyde, possibly the extracted retinal. No other bands (i.e. oxime band) were detectable due to the high Triton X-100 absorbance and the scattering band that hides the absorption region of the oxime. The observed lack of effect of NH_2OH on *Euglena* photoreceptor by James et al. [13] during the cell's life cycle could be explained by the much higher rigidity of the three-dimensional crystalline structure of the photoreceptor in comparison with the native extracted protein. This rigidity would limit the protein conformational changes, thus hindering the accessibility of water and, therefore, hydroxylamine to the Schiff base. We also employed $NaBH_4$, which is known to react rapidly with bleached rhodopsin in solution by reducing the retinylidene linkage. The effect we observed was comparable to that of NH_2OH, namely the extinction of the 515 nm absorption band (data not shown).

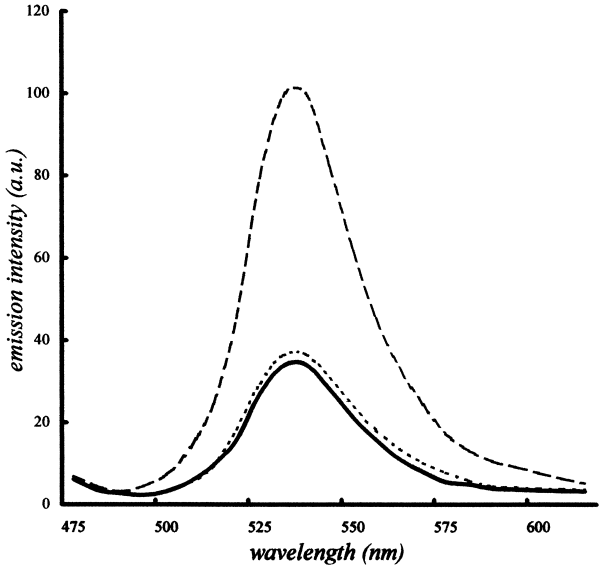

Fig. 4. Fluorescence emission spectra of the 27 kDa native protein. Only a faint emission around 530 nm is initially present (solid line); upon excitation at 365 nm, the protein undergoes an increase of its emission (dashed line). Further 1 min excitation at 435 nm restores the previous lower value of fluorescence (dotted line).

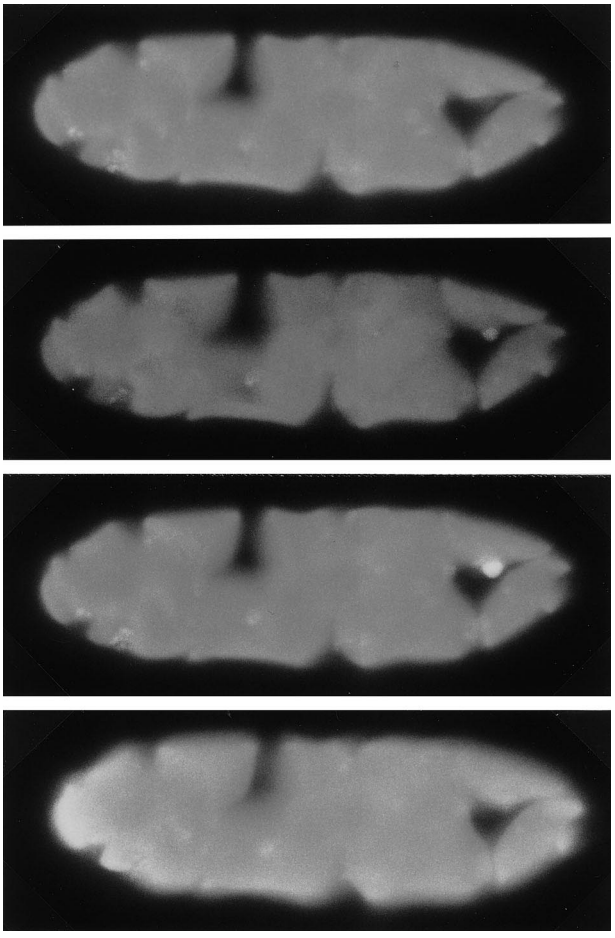

Fig. 5. 'In vivo' photocycle of *Euglena* photoreceptor: under the 436 nm excitation light the cell shows only the typical red emission of the chlorophyll and a faint or no emission in the photoreceptor area (top panel). Under the 365 nm excitation light the photoreceptor undergoes an increase of its emission from zero to a maximum in the green range (second panel). Subsequently, under 436 nm excitation light the organelle shows a bright green emission (third panel) that gradually fades (bottom panel). Scale bar, 5 μm.

We have tested the emission features of the protein extracted from the photoreceptor of *E. gracilis*. The protein shows only a faint emission around 530 nm (Fig. 4, solid line); however, upon 1 min excitation at 365 nm inside the fluorometer the protein undergoes an increase of its emission (Fig. 4, dashed line). Further 1 min excitation at 435 nm inside the fluorometer restores the previous lower value of fluorescence (Fig. 4, dotted line). This behavior reproduces exactly the 'in vivo' photoreceptor photocycle (Fig. 5). Hence, we tried to record the absorption spectrum of the stable intermediate, but we did not succeed because of the overlapping strong absorption of Triton X-100 and scattering by other material present in the sample.

A simple photocycle with stable intermediates can be hypothesized. The protein of *E. gracilis* photoreceptor possesses a bistable photocycle, i.e. the non-fluorescent parent form (first conformer) of its protein upon photo-excitation at 365 nm generates a green fluorescent stable intermediate (second conformer) that can be photochemically driven back to the parent form using excitation at 436 nm. The first conformer absorbs at 515 nm and does not fluoresce. The second conformer, which emits in the green range, should have an absorption band bathochromically shifted in comparison with the parent form, and absorb the incident light at 365 nm and 436 nm, respectively.

The presence of a fluorescent intermediate allows different possible interpretations. In our opinion, since E*rh* protein undergoes a natural three-dimensional crystallization to form the photoreceptor of *Euglena*, it must possess a quite peculiar structure, which possibly restricts retinal movements inside the binding pocket so as to highly influence protein fluorescence properties [26]. Although data exist on fluorescent intermediates of the photocycle of rhodopsin-like proteins under different experimental conditions [27–30], still this feature could seem atypical of those proteins. However, a detailed and comparative analysis of the spectroscopic properties (absorption and fluorescence) of the photoreceptor of *E. gracilis*, which are the same as the E*rh* protein [8], should exclude other photoreceptor proteins.

This is the first report on the identification of the photosensitive protein of the photoreceptor of *E. gracilis*. This protein, possibly a rhodopsin-like protein, possesses special characteristics, since it shows optical bistability, which does not undergo thermal deactivation, and can form three-dimensional crystals.

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