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Identification of 11-*cis* and all-*trans*-retinal in the photoreceptive organelle of a flagellate green alga

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Isolation of intact photoreceptive organelles (eyespot apparatuses) involved in blue-light mediated photoresponses in a flagellate green alga (*Spermatozopsis similis*) allowed for the first time the identification of both 11-*cis*- and all-*trans*-retinal in a plant cell. Both isomers were identified by HPLC analysis in conjunction with UV spectra. Additionally, reconstitution of a distinct absorption band, centered around 540 nm, was achieved by addition of exogenous 9-*cis*-retinal to bleached, isolated eyespot apparatuses.

Retinal; Phototaxis; Photoreceptive organelle; Eyespot; Green algae

1. INTRODUCTION

Flagellate green algae use a highly specialized mechanism – including a directional antenna (i.e. the eyespot apparatus), periodic modulation of the signal and signal amplification by quarter-wave interference reflection of light – to perform oriented movements with respect to the intensity and direction of light (phototaxis) [1,2]. An increasing body of evidence, however, suggests that at the molecular level photoreception and signal transduction in phototaxis of green algae involves common paths of the visual cycle and phototransduction chain of vertebrates. Fast electrical responses similar to the early and late receptor potentials in vision have been detected in the plasma membrane region of the eyespot apparatus [3–5]. Involvement of G-proteins and parts of the cGMP-mediated phototransduction chain have been implicated in phototaxis of *Chlamydomonas* [6,7]. Based on the restoration of phototactic behaviour in mutants by additions of exogenous retinal and retinal analogues, 11-*cis* retinal has been proposed as the photoreceptor pigment of *Clamydomonas* [8–10]. In extracts of these mutants, however, only all-*trans* retinal has been identified after induction of carotenoid biosynthesis [11]. We report here the presence of both 11-*cis*- and all-*trans*-retinal in isolated photoreceptive organelles of the green alga *Spermatozopis similis*.

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2. MATERIALS AND METHODS

Culture conditions, isolation and electron microscopy of intact eyespot apparatuses of *Spermatozopsis similis* Preisig et Melkonian (strain no. B 1.85) were as in [12]. For dark adaptation, cells were kept in darkness at 4°C for at least 30 min prior to isolation. All steps were carried out at 4°C under red light and dim room light. Pigments were extracted according to [13] and kept under N_2 at −20°C. Standard retinaloximes were formed using the same method. Aliquots (20 μl) were applied to a LiChrospher Si-100 HPLC column (particle size 5 μm, 4.6 × 250 mm) and eluted at a constant flow rate of 1.5 ml/min. Separation was carried out using the following gradient system: 5 min 80% solvent A (7% diethylether in *n*-hexane containing 0.075% ethanol) and 20% solvent B (1% ethanol in diethylether) increasing to 40% solvent B within 5 min followed by an immediate step to 100% solvent B (8 min). The absorbance of the eluate was recorded at 360 nm with a 1090A Hewlett Packard diode array detector, allowing the detection of spectra between 240 nm and 600 nm with a 1 nm resolution during elution. Retinal (all-*trans*, 9-*cis*, 13-*cis*) standards were obtained from Sigma. 11-*cis*-Retinal was prepared according to [13]. Bleaching and reconstitution experiments were done in 50 mM MES-KOH (pH 6.4). Bleaching was carried out under continuous stirring at room temperature with a projector (Leitz/Prado Universal; 230 Wm^{-2}) equipped with an UV cut-off filter (<440 nm; Hoya). Difference spectra were recorded against unbleached samples at 20°C in stirred cuvettes with a Shimadzu UV 260. Final ethanol concentrations did not exceed 0.5%.

3. RESULTS

With an estimated number of about 10^5 molecules/cell the phototaxis photoreceptor is undetectable against the bulk of photosynthetic pigments in whole cell extracts [1,8,14,15]. We therefore used isolated eyespot apparatuses of *S. similis* which retain all essential structural components of this complex photoreceptive organelle (Fig. 1; for a detailed analysis of this preparation see [12]) for identification of retinals.

The most abundant pigments present in extracts of

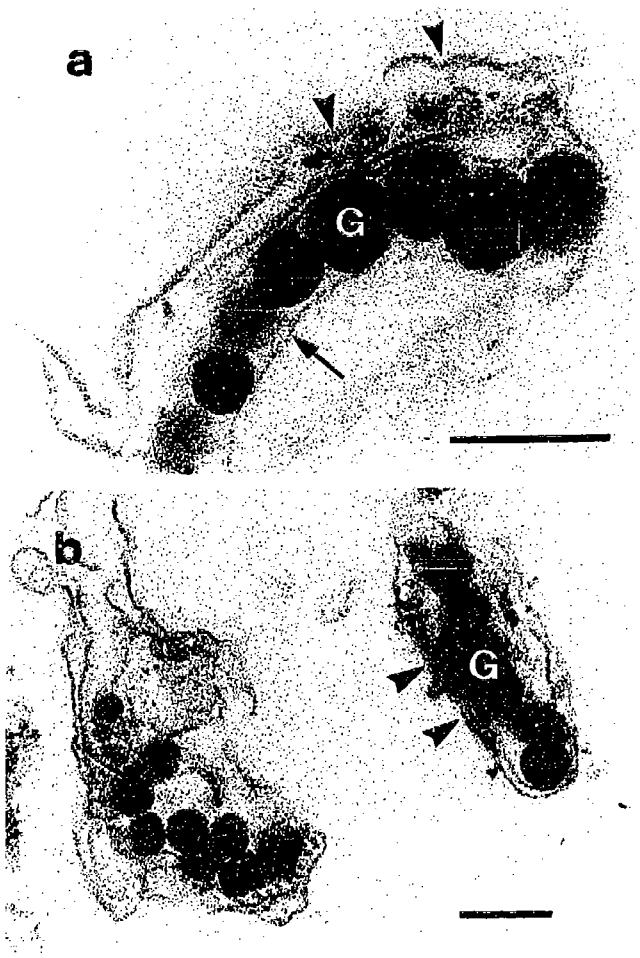

Fig. 1a,b. Ultrastructure of isolated eyespot apparatuses from *Spermatozopsis similis* showing structural integrity of this photoreceptive cell organelle. G = carotenoid-rich lipid globules forming the eyespot plate. Arrowheads = plasma membrane and chloroplast envelope overlying the lipid globules. Small arrow = thylakoid subtending the lipid globule layer. Scale bars = 0.2 μm.

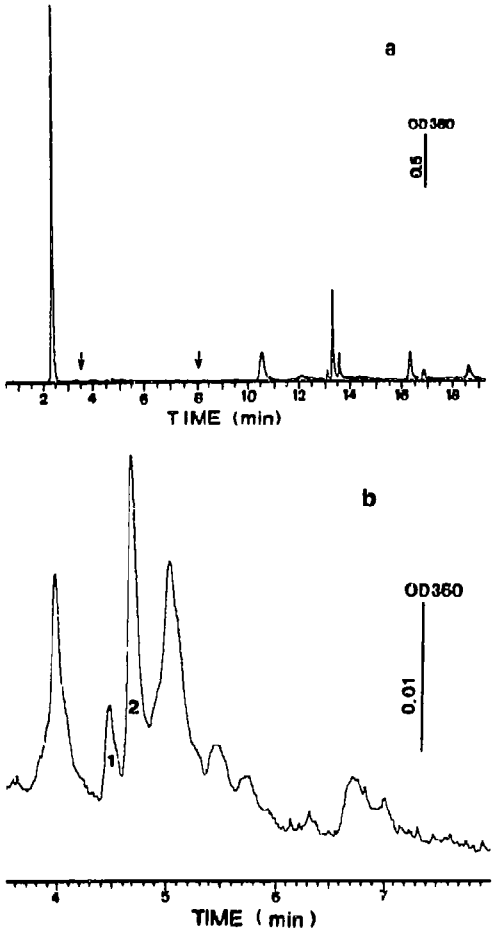

Fig. 2. HPLC analysis of extract from isolated eyespot apparatuses: (a) Total chromatogram of extract; (b) Region shown in arrows in (a). Detail of total chromatogram at higher sensitivity. Numbers indicate peaks coinciding with retention times of standard *syn*-11-*cis*-retinaloxime (1) and *syn*-all-*trans*-retinaloxime (2).

eyespot apparatuses were carotenoids, mainly β-carotene, and unknown xanthophylls (retention times: 2.1–2.5 and >10 min; Fig. 2a). Several peaks in the elution range of standard *syn*-retinaloximes (4.3–5.2 min) were detectable at a higher sensitivity (Fig. 2b). In all preparations (n=5), the retention times of two of them coincide with those of *syn*-11-*cis*- (peak 1) and *syn*-all-*trans*-retinaloxime (peak 2). The absorption spectra of these peaks between 300 and 500 nm are shown in Fig. 3. The average absorption maxima (345 and 358 nm) and the fine structure of the spectra closely match that of the *syn*-oximes of 11-*cis*-retinal (peak 1, Fig. 3a) and all-*trans*-retinal (peak 2, Fig. 3b). The absorbance ratio (peak 2:peak 1) at 360 nm varies between 1.3 and 2.4 in different preparations.

Upon prolonged irradiation of isolated eyespot apparatuses a successive decrease in the whole absorption range of the carotenoids accompanied by a smaller increase in absorption <390 nm was observed by difference spectroscopy (irradiated vs. non-irradiated eyespot apparatuses; Fig. 4a). The addition of exogenous 9-*cis*-retinal to bleached and non-bleached preparations led,

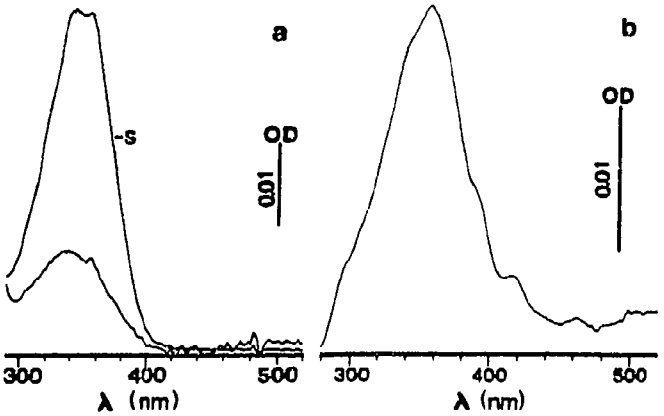

Fig. 3. Spectra of peaks (a) 1 and (b) 2 from HPLC-analysis (Fig. 2). For comparison the spectrum of *syn*-11-*cis*-retinaloxime (S) is included.

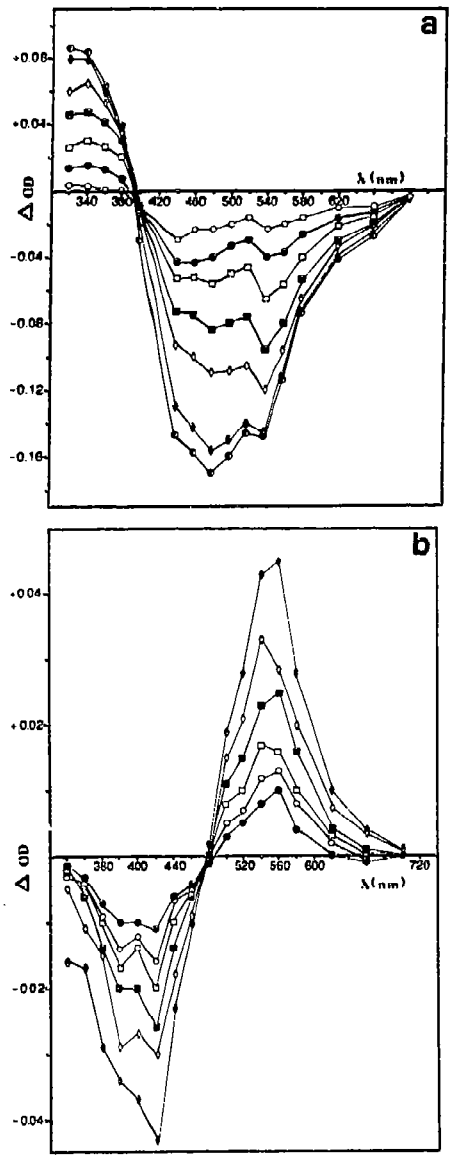

Fig. 4. (a) Absorbance difference of bleached minus unbleached isolated eyespot apparatuses after 30 (○), 45 (●), 60 (□), 75 (■), 90 (◇), 120 (◆) and 135 (◐) min of bleaching. (b) Absorbance difference of bleached minus unbleached eyespot apparatuses reconstituted with 22 μM 9-*cis*-retinal. For clarity only the absorbance difference of a spectrum taken 20 min after addition of retinal is shown. Spectra were recorded 120 (●), 180 (○), 220 (□), 280 (■), 350 (◇) and 460 (◆) min after addition of 9-*cis*-retinal.

after rapid shifts, to a slow reconstitution (starting 20–40 min after addition of retinal) of a distinct absorption band centered around 540 nm in the difference spectrum (Fig. 4b). Simultaneously we observed a similar decrease in the absorption range of free 9-*cis* retinal (Fig. 4b). The isosbestic point at ~480 nm indicates the formation of a single species. In parallel experiments using the unbleached sample (against buffer) only a small absorption decrease over the entire absorption range due to oxidation of pigments was observed (not shown).

4. DISCUSSION

According to our knowledge the experiments described in this study demonstrate for the first time the presence of 2 retinoids of the classical vertebrate visual cycle (11-*cis*- and all-*trans*-retinal) in isolated eyespot apparatuses of a flagellate green alga. The use of purified eyespot apparatuses allows the unequivocal assignment of these isomers to the photoreceptive organelle of phototaxis and is the basis for their detection against the bulk of pigments present in a photosynthesizing alga. The isomer identity was verified by: (i) the match of spectra, even in fine structure, and absorption maxima [16]; and (ii) coincidence of retention times with standards.

Nonspecific isomerization usually occurs after release of retinoids from their native environment by detergent or organic solvent extraction [17]. The procedure used is, however, known to retain the original isomeric configuration of retinal during extraction from biological material [13,18] and thus the presence of the two isomers is presumably no extraction artifact. This is also supported by the absence of other retinal isomers in our preparations. In the case of nonspecific isomerization a certain percentage of 9-*cis*- and 13-*cis*-retinal, originating either from all-*trans*- or 11-*cis*-retinal, is observed [17]. The variable ratios between all-*trans*- and 11-*cis*-retinal (1.3–2.4) that we observed may be explained by incomplete dark adaptation prior to isolation or by isomerization induced by dim light during isolation. In dark-adapted bacteriorhodopsin a 1:1 ratio between the two isomers 13-*cis*- and all-*trans*-retinal occurred, whereas after light adaptation only the all-*trans* isomer was found [19]. If similar phenomena apply also to algal retinals the previous demonstration of only all-*trans*-retinal in *Chlamydomonas* [11] could be explained. In order to induce retinoid biosynthesis the FN68 mutants of *C. reinhardtii*, blocked in carotenoid biosynthesis in darkness, were irradiated for 1 h with green light prior to extraction [11].

The high amount of carotenoids in the eyespot lipid globules precludes the direct spectroscopic identification of a retinal protein in these preparations. The slow reconstitution of a distinct absorption band centered arround 540 nm upon addition of exogenous 9-*cis*-retinal, which can also be detected as a shoulder in normal spectra of isolated eyespots [12], also indicates the presence of a retinal protein in our preparations. This absorption band covers the spectral range for which side bands in action spectra of positive phototaxis and the primary photoinduced potential difference have been reported [3].

Absorption maxima ranging from 350 nm to 580 nm [19,20] and reconstitution times varying from minutes to many hours, depending on the isomer and preparation used, are known for different retinal-based pigments [21-24]. Since only opsin- but not bacterio-opsin-

based pigments can be reconstituted by 9-*cis*-retinal [19,24], the reconstitution of the 540 nm band points to the presence of an opsin-like protein in the eyespot apparatus of *S. similis*. Although the opsin-like photoreceptor protein for green algal phototaxis awaits unequivocal identification and further characterization, preliminary evidence for its presence in *Chlamydomonas* has been obtained [11,25].

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