Protoplasma (1996) 191: 172–177

PROTOPLASMA

Microspectrofluorometry of the autofluorescent flagellum in phototactic brown algal zoids

Hiroshi Kawai[1,*], **Shago Nakamura**[2], **Mamoru Mimuro**[3], **Masaki Furuya**[4], and **Masakatsu Watanabe**[3]

[1] Kobe University Research Center for Inland Seas, Rokkodai, Kobe, [2] Department of Environmental Biology and Chemistry, Faculty of Science, Toyama University, Toyama, [3] National Institute for Basic Biology, Okazaki, and [4] Advanced Research Laboratory, Hitachi Ltd., Saitama

Received July 30, 1995
Accepted November 4, 1995

Dedicated to Professors Masakazu Tatewaki and Tadao Yoshida on the occasion of their academic retirement

Summary. Posterior flagella of zoids of *Scytosiphon lomentaria* and *Chorda filum* (Phaeophyceae, Chromophyta) were isolated and subjected to microspectrofluorometry using a high sensitivity microspectrofluorometer in order to characterize the flagellar autofluorescent substances. Vigorous agitation in a hypertonic medium yielded preparations of largely intact flagella retaining detectable green flagellar autofluorescence. Under 380–425 nm excitation light, maximum emission of flagellar autofluorescence was observed at 495 nm, whereas under 400–440 nm excitation light fluorescence shifted to around 510 nm. Comparison of these spectra with the fluorescence spectra of 4′,5′-cyclic FMN isolated from fertile thalli of *S. lomentaria*, and of 6-carboxypterin suggested that two (or more) different fluorescent substances (presumably a flavin and a pterin) are present in the flagella.

Keywords: *Chorda filum*; Flagellar fluorescence; Flavin; Microspectrofluorometry; Phototaxis; *Scytosiphon lomentaria*.

Abbreviations: DTT dithiothreitol; FMN flavin mononucleotide; HEPES N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]); PEG polyethylene glycol; PFB paraflagellar body; Tris tris(hydroxymethyl)aminomethane.

Introduction

In brown algal flagellate cells (zoids), the (intraplastid) stigma and proximal swelling of the posterior flagellum have been thought to be involved in phototactic photoreception, because these features are observed only in those cells that show phototaxis (Müller et al. 1987; Kawai 1988, 1992). However, the function of these structures has not been studied in detail. Recently, the brown algal stigma was shown to act as a concave mirror reflecting and focusing light onto the flagellar swelling (Kawai et al. 1990, Kreimer et al. 1991). This result, and the presence of electron dense material localized in the flagellar swelling (Dodge 1973, Moestrup 1982, Kawai et al. 1990, Kreimer 1994) are indirect evidences that the flagellar swelling is the true photoreceptive site for phototaxis in brown algal flagellate cells.

Furthermore, phototactic flagellate cells of brown algae are reported to show green autofluorescence only in posterior flagella, associated with the stigma and flagellar swelling (Müller et al. 1987, Kawai 1988), whereas non-phototactic cells lack a stigma, flagellar swelling, and flagellar autofluorescence. Therefore the autofluorescent substance is also considered to be involved in photoreception. In order to identify the autofluorescent substance, Yamano et al. (1993) isolated that dominant green fluorescent substances from entire mature thalli, including abundant zoids still retained in zoidangia, of the brown alga *Scytosiphon lomentaria* and identified 4′,5′-cyclic FMN (flavin mononucleotide) for the first time as a natural product. They also noted a similar but chemically much more unstable compound in the extract that is considered to be a precursor or a decomposed product of 4′,5′-cyclic FMN, but its chemical identi-

* Correspondence and reprints: Kobe University Research Center for Inland Seas, Rokkodai, Kobe 657, Japan.

ty has not been clarified yet. The concentrations of these substances were considerably higher in mature plants containing abundant zoids (with flagellar fluorescence) than in immature thalli. Therefore they concluded that the substances may correspond to the autofluorescent substances localized in the posterior flagella.

Brown algal flagellar autofluorescence has yet only been studied on whole cell suspensions. The fluorescence emission spectrum was reported to have a maximum at 530 nm when excited at 450 nm in *Giffordia mitchelliae* and *Sphacelaria rigidula* (Müller et al. 1987) or 518 nm when excited at 440 nm in *Scytosiphon lomentaria* (Kawai 1988). Based on these fluorescence spectra, both authors suggested that the autofluorescent substance is a flavin or its derivative. However, in order to confirm that the autofluorescence showing the above-mentioned maxima truly originates from posterior flagella and not from some other substances in the cytoplasm, microspectrofluorometry of flagella is necessary. For such measurements, it is essential to isolate flagella from the cell body without seriously damaging the autofluorescent substances. Methods for isolating flagella that have been reported for green flagellates (Witman et al. 1972, 1978) have not been applied to brown algal zoids. Therefore, this paper aimed to establish a method to detach intact flagella from the cell bodies while retaining flagellar autofluorescence, and to measure the fluorescence spectra of isolated flagella by high sensitivity microspectrofluorometry in order to characterize the flagellar autofluorescent substance(s) in brown algal zoids.

Material and methods

Isolation of flagella

Fertile thalli of *Chorda filum* (L.) Stackhouse and *Scytosiphon lomentaria* (Lyngb.) Link were collected at Oshoro, Hokkaido Prefecture and Takahama, Fukui Prefecture, Japan, respectively. The thalli were washed in filtered sea water several times, immersed in sterilized sea water, and illuminated with white fluorescent light to stimulate the release of zoids. Freshly released, actively swimming zoids were harvested using their photoaccumulation responses.

The zoids have two different types of flagella (anterior hairy and posterior smooth) laterally inserted to the cell body. In order to isolate the flagella from the cell bodies, we tried the following three methods. (1) Agitation shock method (Auclair and Siegel 1966): Swimming cells were suspended in ice-cold hypertonic sea water (filtered sea water supplemented with 0.5 M NaCl and 2% polyethylene glycol (PEG) 20,000). Flagella were physically detached from the cell body by vigorous agitation shocks given to the suspension using a vortex mixer for 5 min. (2) pH-shock method (Witman et al. 1972): Swimming cells were subjected to pH-shock by reducing the pH from 7.2 to 4.5 with 0.5 N acetic acid in a solution containing 10 mM Tris-HCl and 5% sucrose. (3) Dibucaine method (Witman et al. 1978): Swimming cells were treated with dibucaine (up to 4.2 mM) in a solution containing 10 mM HEPES-NaOH (pH 7.4), 5 mM $MgSO_4$, 1 mM DTT and 4% sucrose. Then cell bodies were removed by centrifugation at 3,000 **g** for 5 min for 3–4 times until any remaining cell bodies become unrecognizable under light microscopy. The supernatant containing isolated flagella was centrifuged at 25,400 **g** for 60 min to obtain a dense fraction of isolated flagella (mixture of anterior and posterior flagella). The fraction was immediately frozen at lower than –20 °C for stock and subjected to microspectrofluorometry at the Application Technology Department, Techno-Research Laboratory, Hitachi Ltd. at Katsuta-shi, Ibaraki.

Microspectrofluorometry

The fluorescence emission of individual isolated flagella was measured using a Nikon EFD2 epifluorescence microscope equipped with a Hitachi U-6500 microscope spectrometer (Hitachi Ltd). Measurements were made under two different excitation modes, V (violet)-excitation mode (380–425 nm excitation filter, 430 nm dichroic mirror, 450 nm barrier filter) and BV (blue-violet)-excitation mode (400–440 nm excitation filter, 455 nm dichroic mirror, 470 nm barrier filter), illuminated with an Osram HBO 100 W/2 mercury lamp. Pinholes used for microspectrofluorometrical measurements provided observation fields 0.3 μm or 0.5 μm in diameter at the sample position. The spectral sensitivity of the fluorescence detector system, composed of a diffraction grating and a photodiode array, was calibrated using a calibration program under a standard light source.

Fluorescence spectra of 4′,5′-cyclic FMN isolated from fertile thalli of *Scytosiphon lomentaria* (Yamano et al. 1993) were measured with a Hitachi 850 spectrofluorometer (Hitachi). Flavin tetralactate and 6-carboxypterin (Wako Pure Chemical Industries, Osaka) were used as standards for fluorescence spectra. The spectral sensitivity of the apparatus was numerically corrected after transferring the digitized data to a microcomputer. Simulation of the fluorescence spectrum was carried out by a method based on least-square fit procedures (Mimuro et al. 1988).

Results

Flagellar isolation

Figure 1 A and B shows epifluorescence photomicrographs of intact zoids of *Scytosiphon lomentaria*; they showed reddish orange fluorescence of chloroplasts and green autofluorescence of posterior flagella. The image for *Chorda filum* zoids was essentially the same as for *S. lomentaria*. Flagella isolated by the agitation shock method were almost intact and the flagellar fluorescence could be observed under epifluorescence microscopy (Fig. 2 A, B). Isolated flagella preparation included both fluorescent straight flagella (posterior flagella) and undulated non-fluorescent flagella (anterior flagella), however, ca. 75% (counted from several micrographs of isolated flagella, n = 728) of total isolated flagella were posterior flagella. Freshly detached posterior flagella often showed brighter fluorescence at one end suggesting

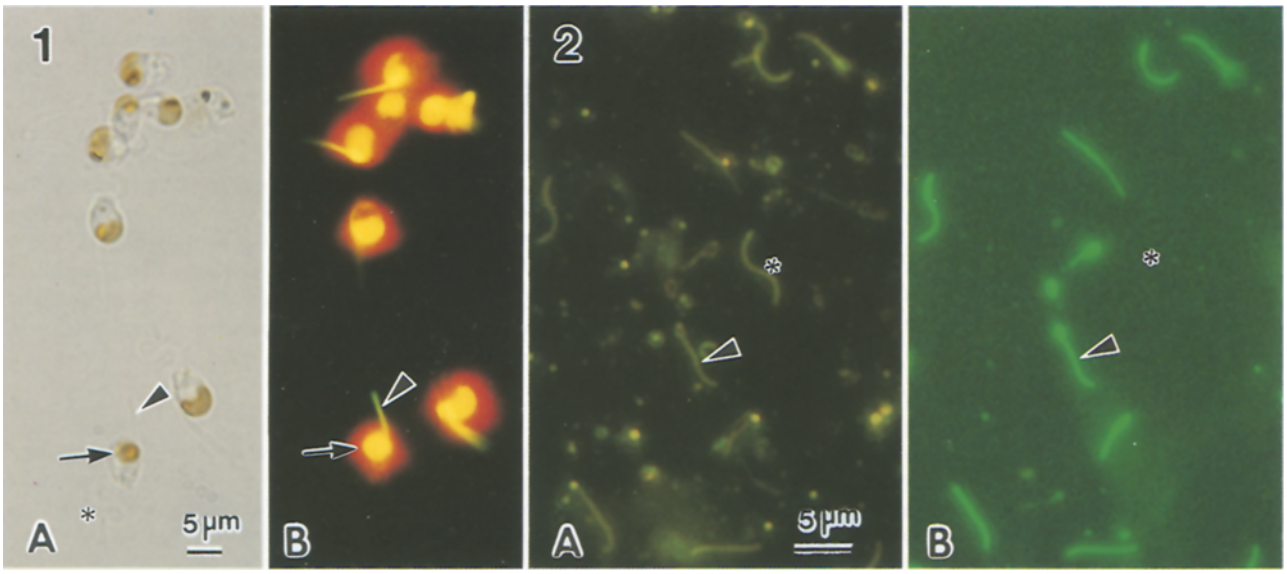

Fig. 1 A, B. Autofluorescence of chloroplasts and posterior flagella in *Scytosiphon lomentaria*. **A** Bright-field image; **B** epifluorescence micrograph under blue-violet (400–440 nm) excitation light. Arrow, chloroplast (with reddish-orange autofluorescence in **B**); arrowhead, posterior flagellum (with green autofluorescence in **B**); asterisk, anterior flagellum (without fluorescence in **B**)

Fig. 2 A, B. Isolated flagella of *Scytosiphon lomentaria*. **A** Dark field image; **B** epifluorescence photomicrograph under BV (blue-violet, 400–440 nm) excitation light. Arrowhead, posterior flagellum (with green fluorescence in **B**); asterisk, anterior flagellum (without fluorescence in **B**)

the existence of a flagellar swelling (Fig. 2 B). Isolated flagella tended to become short and swollen when held at room temperature. In contrast, flagella isolated by the pH-shock method or the dibucaine method became shorter and swelled immediately after detachment, and the fluorescence of posterior flagella was no longer visible under epifluorescence microscopy (photographs not shown).

Chorda filum zoids are larger than those of *Scytosiphon lomentaria* (9–11 µm and 7–8 µm in length, respectively); this permitted us to obtain more intact flagella from *C. filum*, because gentler centrifugation was sufficient to remove cell debris during the isolation procedure. *Chorda filum* flagella generally gave better results in the microspectrofluorometry, presumably a consequence of the mild treatment as well as the larger cell size, which was more suitable for observation. Therefore, in the following experiments we preferentially used *C. filum* flagella.

Microspectrofluorometry

Figure 3 shows average emission spectra of single flagellar swellings (n = 30) of *C. filum* excited under the BV excitation mode (Fig. 3 A; 400–440 nm excitation light) and the V excitation mode (Fig. 3 B; 380–425 nm). They showed different maximum peaks depending on the excitation light conditions. Under the V excitation mode including UVA, maximum emission was observed at 495 nm, whereas under the BV excitation mode it shifted to around 510 nm. The blunt peak near 680 nm in Fig. 3 A results from chlorophylls of chloroplast fragments contaminating the preparation. Preliminary measurements on single flagella of *S. lomentaria* gave essentially the same results. Emission spectra of the more distal parts of single flagella could not be measured, because they were too feeble under 0.3 and 0.5 µm pinholes and could not be discriminated due to a low signal-to-noise ratio.

Fluorescence spectra of 4′,5′-cyclic FMN and flavin tetralactate (Fig. 4) were very similar to each other with the maximum around 523 nm in sea water, hypertonic sea water containing 0.5 M NaCl and 2% PEG, and 10 mM phosphate buffer (pH 6). The emission maximum of 6-carboxypterin was blue-shifted; with the maximum at 448 nm in phosphate buffer, and at 458 nm in sea water or hypertonic sea water. Difference spectra of emission spectra of *Chorda* flagellar autofluorescence (Fig. 5 A, B corresponding Fig. 3 A, B) and 4′,5′-cyclic FMN (Fig. 5 A, B corre-

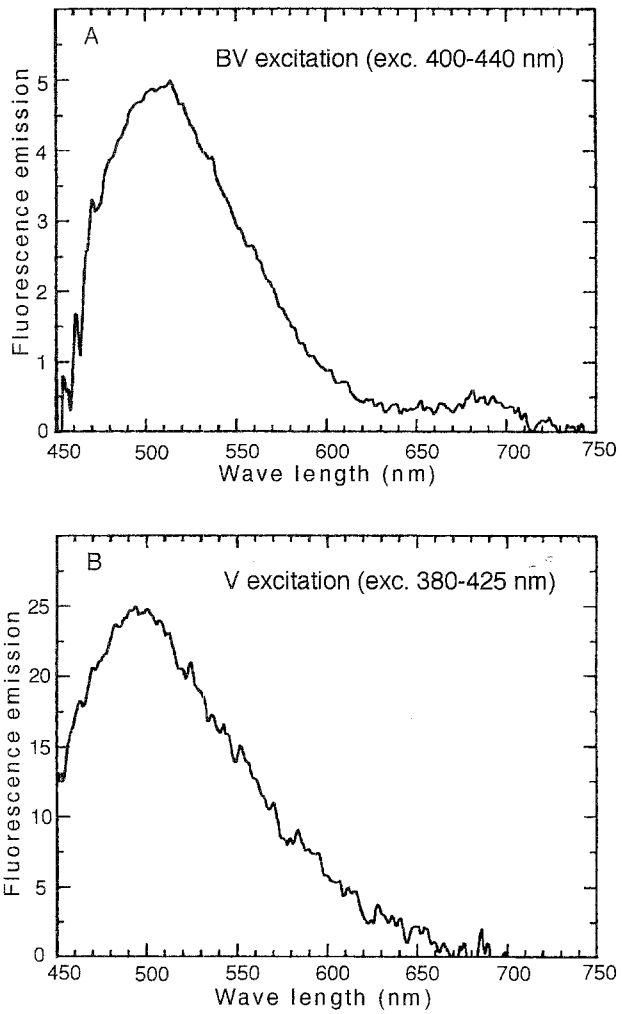

Fig. 3. Emission spectra of flagellar autofluorescence measured at flagellar swelling in *Chorda filum*. **A** Excited under BV (blue-violet, 400–440 nm) excitation mode; **B** excited under V (violet, 380–425 nm) excitation mode. Spectral sensitivity of the apparatus was numerically corrected

sponding Fig. 4) suggested the presence of a second substance having a maximum emission near 490 nm.

Discussion

Flagella were detached from cells either by chemical or physical shock procedures; however, the former resulted in the bleaching of fluorescence. In contrast, the physical (vigorous agitation) shock procedure gave better results and isolated flagella were intact enough for the application of fluorometry. However, this method induced a secondary effect on the flagellate cells; they tended to become roundish with the flagella coalesced to the cell body surface when vigorously vibrated or centrifuged. Thus difficulties still

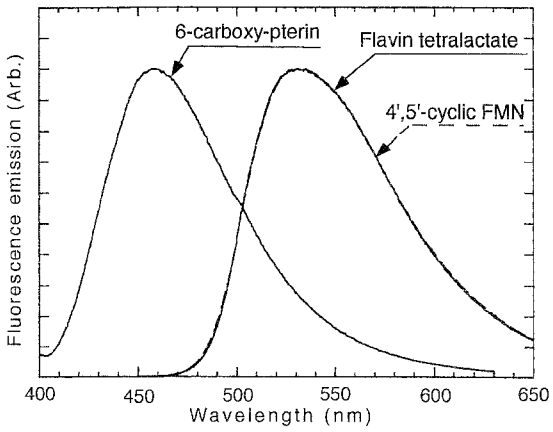

Fig. 4. Emission spectra of 4′,5′-cyclic FMN, flavin tetralactate, and 6-carboxypterin in sea water

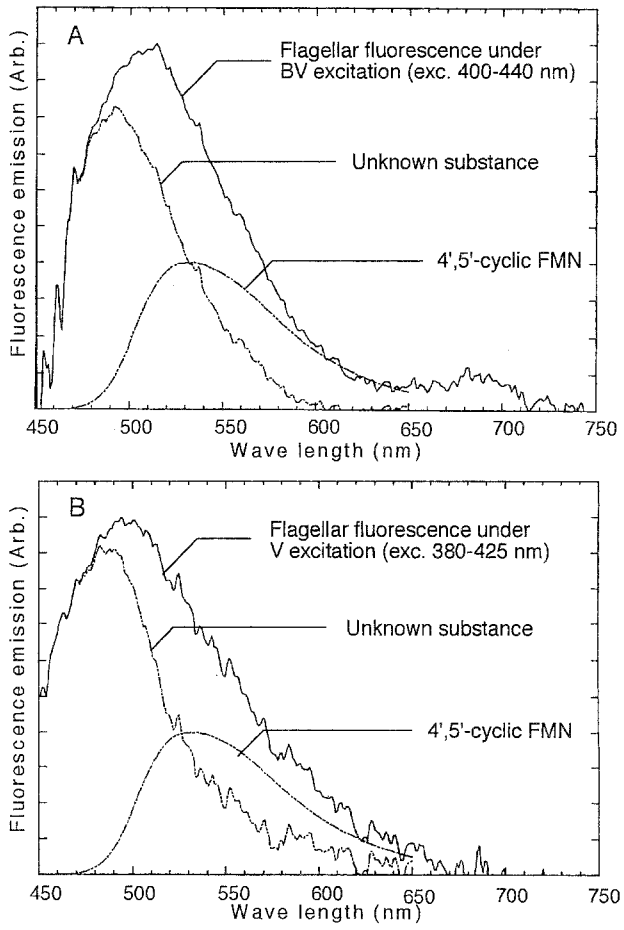

Fig. 5. Difference spectra of emission spectra of *Chorda* flagellar autofluorescence, **A** under BV (blue-violet) and **B** under V (violet) excitation mode (corresponding to Fig. 3 A, B) and 4′,5′-cyclic FMN (corresponding to Fig. 4). Note occurrence of a second emission spectrum with maximum near 490 nm

remain in collecting a large quantity of intact isolated flagella for further photometrical and biochemical analysis. Our preparation tended to contain more posterior flagella than anterior ones. This may reflect their structural differences that anterior flagella have tubular mastigonemes while posterior flagella are smooth and they have slightly different sedimentation coefficients.

The fluorescence emission spectrum (max. = 510 nm) of isolated *Chorda filum* flagella excited by blue light resembles that of cell suspensions of *Giffordia mitchelliae* and *Sphacelaria rigidura* (max. = 530 nm with excitation at 440 nm; Müller et al. 1987) and *Scytosiphon lomentaria* (max. = 518 nm with excitation at 450 nm; Kawai 1988), but did not exactly agree. This may be attributed to the fact that the emission spectra of whole cells were influenced by the fluorescent substances in the cell bodies, or because the present measurement was only conducted on flagellar swellings whereas the measurements on cell suspensions also include fluorescence from other part(s) of flagella (i.e., middle to terminal parts) as well as whole cell bodies.

The emission spectrum of *C. filum* flagellar autofluorescence was not identical to either of the flavin compounds examined, 4′,5′-cyclic FMN derived from *S. lomentaria* mature thalli or commercial flavin tetralactate. The shift of fluorescence maxima under different excitation wavelengths (Fig. 3 A, B) as well as the differences between spectra of flagellar autofluorescence and 4′,5′-cyclic FMN (Fig. 5 A, B) suggest the presence of at least a second fluorescent substance having an emission peak near 490 nm. Since pterin shows an emission maximum at a wavelength shorter than that of flavin (458 nm in 6-carboxypterin and ca. 475 nm in xanthopterin; Schmidt et al. 1990), it is a probable candidate for the second compound. This result that multiple fluorescent substances were included in the flagella was similar to the fluorescence spectrum of *Euglena gracilis* (Euglenophyceae) in which a similar green autofluorescent substance has been shown to localize in the paraflagellar body (PFB, or flagellar rod) on longer motile flagella (Benedetti and Checcucci 1975, Benedetti and Lenci 1977, Ghetti et al. 1985, Galland et al. 1990, Schmidt et al. 1990, Sineshchekov et al. 1994). Recent studies by Galland et al. (1990) and Schmidt et al. (1990) suggested the presence of a flavin as well as a pterin in the PFB of euglenoids. The fact that the emission peaks were different between *E. gracilis* and *C. filum* might be attributed to difference in the binding to proteins in cells. Pterin is shown to be bound to protein in *E. gracilis* PFB (Brodhun and Häder 1990), which may have slightly shifted the emission peak from that of free pterin. The same tendency can be expected for flavin, that is flavo-protein.

The presence of similar flagellar autofluorescent substances also has been confirmed in various algal systematic groups, e.g., Euglenophyceae, Prymnesiophyceae (= Haptophyceae), Phaeophyceae, Synurophyceae, Chrysophyceae, and Xanthophyceae (Müller et al. 1987, Kawai and Inouye 1989, Kawai 1992, Kreimer 1994). Those flagellar autofluorescent substances are grouped into three types according to the differences in their localization in the cells (Kawai and Inouye 1989, Kawai 1992): (1) Euglenoid type – autofluorescent substance located in a part (paraflagellar body, PFB) of a long locomotive flagellum; (2) prymnesiophycean type – located only in proximal part of one of the two morphologically indistinguishable flagella; (3) core chromophyte type – located throughout the entire (usually shorter) posterior flagellum, associated with a proximal flagellar swelling. Undoubtedly Euglenophyceae, containing chlorophyll *a* and *b* as photosynthetic pigments, are phylogenetically very different from the latter two (i.e., Prymnesiophyceae and all other chromophytes). Prymnesiophyceae and other chromophytes (core chromophytes) have been believed to be relatively closely related, sharing many basic characters (e.g., nature of chlorophylls, presence of periplastidal ER suggesting the endosymbiosis of eukaryotic algae). Chromophytes and euglenophytes are considered to be phylogenetically distant, and their photoreceptors are likely to have evolved independently.

Action spectra for phototactic responses in brown algal flagellate cells (Kawai et al. 1990, 1991; Kawai 1992) are reported to show a typical pattern for the so-called near-UV/blue-light response, with the maxima around 420 and 460 nm (Watanabe 1995). It is noteworthy that *Euglena*, which also has green autofluorescent substances in the paraflagellar body, shows a similar near-UV/blue-light type of action spectrum for phototaxis (maxima at 370, 440, and 470 nm; Watanabe 1995) as brown algae. This contrasts with the blue/green light type of action spectra in phototactic responses of Chlorophyceae (e.g., *Chlamydomonas* phototaxis, maxima at 443, 503 nm; Watanabe 1995), in which retinal-rhodopsins are suggested to act as photoreceptive pigments, instead of flavins and pterins (Foster et al. 1984; Galland and Senger 1988a, b; for review, see Kreimer 1994).

Acknowledgements

We are grateful to Dr. Eric Henry for critically reading and improving this manuscript and Dr. Haruhisa Shirahama for providing 4′,5′-cyclic FMN. We are also grateful to Ms. Masae Miyagawa and staff members of Application Technology Department, Techno-Research Laboratory, Hitachi Ltd. for the use of their microspectrophotometer (U-6500) and Dr. Akira Nagatani, Riken Institute, for the use of their microspectrophotometer (U-6000, Hitachi Ltd.) for the preliminary measurements. M.M. acknowledges the Ciba-Geigy Foundation (Japan) for the Promotion of Science for financial aid.

References

Auclair W, Siegel BW (1966) Cilia regeneration in the sea urchin embryo: evidence for a pool of ciliary proteins. Science 154: 913–915

Benedetti PA, Checcucci A (1975) Paraflagellar body (PFB) pigments studied by fluorescence microscopy in *Euglena gracilis*. Plant Sci Lett 4: 47–51

– Lenci F (1977) In vivo microspectrofluorometry of photoreceptor pigments in *Euglena gracilis*. Photochem Photobiol 26: 315–318

Brodhun B, Häder D-P (1990) Photoreceptor proteins and pigments in the paraflagellar body of the flagellate *Euglena gracilis*. Photochem Photobiol 51: 865–870

Dodge JD (1973) The fine structure of algal cells. Academic Press, London

Foster KW, Saranak J, Patel N, Zarilli G, Okabe M, Kline T, Nakanishi K (1984) A rhodopsin is the functional photoreceptor for phototaxis in the unicellular eukaryote *Chlamydomonas*. Nature 311: 756–759

Galland P, Senger H (1988a) The role of flavins as photoreceptors. J Photochem Photobiol B 1: 277–294

– – (1988b) The role of pterins in the photoreception and metabolism of plants. Photochem Photobiol 48: 811–820

– Keiner P, Dörnemann D, Senger H, Brodhun B, Häder D-P (1990) Pterin- and flavin-like fluorescence associated with isolated flagella of *Euglena*. Photochem Photobiol 51: 675–680

Ghetti F, Colombetti G, Lenci F, Campani E, Polacco E, Quaglia M (1985) Fluorescence of *Euglena gracilis* photoreceptor pigment: an in vivo microspectrofluorometric study. Photochem Photobiol 42: 29–33

Kawai H (1988) A flavin-like autofluorescent substance in the posterior flagellum of golden and brown algae. J Phycol 24: 114–117

– (1992) Green flagellar autofluorescence in brown algal swarmers and their phototactic responses. Bot Mag Tokyo 105: 171–184

– Inouye I (1989) Flagellar autofluorescence in forty-four chlorophyll *c*-containing algae. Phycologia 28: 222–227

– Müller DG, Fölster E, Häder D-P (1990) Phototactic responses in the gametes of the brown alga, *Ectocarpus siliculosus*. Planta 182: 292–297

– Kubota M, Kondo T, Watanabe M (1991) Action spectra for phototaxis in zoospores of the brown alga *Pseudochorda gracilis*. Protoplasma 161: 17–22

Kreimer G (1994) Cell biology of phototaxis in flagellate algae. Int Rev Cytol 148: 229–310

– Kawai H, Müller DG, Melkonian M (1991) Reflective properties of the stigma in male gametes of *Ectocarpus siliculosus* (Phaeophyceae) studied by confocal laser scanning microscopy. J Phycol 27: 268–276

Mimuro M, Yamazaki I, Itoh S, Tamai N, Satoh K (1988) Dynamic fluorescence properties of D_1-D_2-cytochrome b_{599} complex isolated from spinach chloroplasts: analysis by means of the time-resolved fluorescence spectra in picosecond time range. Biochim Biophys Acta 933: 478–486

Moestrup Ø (1982) Flagellar structure in algae: a review, with new observations particularly on the Chrysophyceae, Phaeophyceae (Fucophyceae), Eustigmatophyceae and *Reckertia*. Phycologia 21: 427–528

Müller DG, Maier I, Müller H (1987) Flagellum autofluorescence and photoaccumulation in heterokont algae. Photochem Photobiol 46: 1003–1008

Schmidt W, Galland P, Senger H, Furuya M (1990) Microspectrophotometry of *Euglena gracilis*. Pterin- and flavin-like fluorescence in the paraflagellar body. Planta 182: 375–381

Sineshchekov VA, Geiß D, Sineshchekov OA, Galland P, Senger H (1994) Fluorometric characterization of pigments associated with isolated flagella of *Euglena gracilis*: evidence for energy migration. J Photochem Photobiol 23: 225–237

Watanabe M (1995) Action spectroscopy: photomovement and photomorphogenesis spectra. In: Hoospool W, Song P-S (eds) CRC handbook of organic photochemistry and photobiology. CRC Press, Boca Raton, pp 1260–1272

Witman GB, Carlson K, Berliner J, Rosenbaum JL (1972) *Chlamydomonas* flagella. I. Isolation and electrophoretic analysis of microtubules, matrix, membranes, and mastigonemes. J Cell Biol 54: 507–539

– Plummer J, Sander G (1978) *Chlamydomonas* flagellar mutants lacking radial spokes and central tubules. Structure, composition, and function of specific axonemal components. J Cell Biol 76: 729–747

Yamano K, Saito H, Ogasawa Y, Fujii S, Yamada H, Shirahama H, Kawai H (1993) The autofluorescent substance in the posterior flagellum of swarmers of the brown alga *Scytosiphon lomentaria*. A possible photoreceptive pigment in phototaxis. In: 35th Symposium on the chemistry of natural products, Kyoto, 1993, pp 646–653