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Riboflavin-binding sites associated with flagella of *Euglena*: A candidate for blue-light photoreceptor?

A. Nebenführ[1], A. Schäfer[1], P. Galland[2], H. Senger[2], and R. Hertel[1]*

[1] Institut für Biologie III der Universität, Schänzlestrasse 1, W–7800 Freiburg i. Br. and
[2] Fachbereich Biologie/Botanik, Universität Marburg, Lahnberge, W–3550 Marburg, Federal Republic of Germany

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Abstract. The hypothesis was tested that reversible riboflavin (RF)-binding sites are part of the photoreceptor in *Euglena gracilis*. Published evidence shows that the phototactic stimulus – with a flavin-type action spectrum – is perceived at the paraflagellar body (PFB). Flagella with PFBs were isolated from *Euglena gracilis* by a combined cold and Ca^{2+} shock. Saturable binding of [^{14}C]RF was demonstrated with such preparations, in the oxidized state as well as under reducing conditions in the presence of dithionite. Affinities for RF were high: K_D (oxidized) = 0.08 μM, and K_D (reduced) = 0.7 μM. Flavin mononucleotide and flavin adenine dinucleotide showed lower binding affinities. The in vitro RF binding per unit of protein was enriched approximately tenfold in the flagellar preparations when compared with homogenates of whole cells. The number of (reduced) binding sites per entire flagellum was determined to be in the order of 10^6. This number is in line with published estimates of chromophores bound in or at the PFB.

Key words: Blue-light – *Euglena* – Flagellum – Flavin binding – Photoreceptor – Riboflavin

Introduction

Well-defined blue-light photoresponses have been described for many organisms, but the relevant photoreceptor molecules are still unknown. The action spectra resemble the absorption spectra of flavins or carotenoids (Galland and Senger 1988). In some cases – e.g. *Phycomyces* phototropism (Presti et al. 1977) – the involvement of carotenoids can be ruled out; thus, flavins are considered to be the most likely photoreceptors.

Most models of blue-light perception assume that the flavin chromophore is covalently or tightly bound to the protein moiety of the photoreceptor in the plasma membrane. In higher plants, however, the concentration of such tightly bound flavins seems to be too low to account for the observed physiological sensitivity (Hertel 1980). At the same time, the concentration of free cellular flavins (mostly riboflavin, RF) is relatively high, usually in the micromolar range. Hertel (1980) therefore proposed that the photoreceptor consists of a membrane protein and a "loosely" bound chromophore (RF) that is in exchange with a free, cytosolic pool. Indeed, a specific and reversible binding of RF to membrane preparations of *Cucurbita, Zea* (Hertel et al. 1980) and *Phycomyces* (Dohrmann 1983) has been observed.

The model receives support from the work of Fritz et al. (1989) on *Neurospora* mutants deficient in RF biosynthesis. Low concentrations (0.08 μM) of RF added to the medium allowed hyphal growth, but photoresponses – here, phase shifts of the circadian clock – were still suppressed. Light sensitivity developed only at higher RF concentrations, above 0.14 μM in the medium; it further increased with increasing extracellular RF concentrations up to 20 μM, corresponding to approx. 8 μM intracellular RF. Several chemical RF analogs were tested that cannot be converted to flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD), or covalently attached to proteins. These analogs showed essentially the same "photo-sensitizing" effect as did RF. Analogs, however, that could not penetrate the cell surface were not able to support any light response. Fritz et al. (1989) therefore concluded that "free cellular RF acts as the photoreceptor for light-induced phase shifts of the circadian conidiation rhythm in *Neurospora*".

Up to the present, no causal biochemical connection between a reversible binding of RF and any photophysiological response has been shown. It is possible, however, to approach this question indirectly by using an organism in which the subcellular localization of the photoreceptor is precisely known, and testing whether in vitro RF-binding sites are particularly enriched in this location, e.g. in a specific organelle.

In the case of *Euglena gracilis*, the photoreceptor is located in or at the paraflagellar body (PFB) of the major flagellum (see Kuznicki et al. 1990). The stigma ("eye-

* To whom correspondence should be addressed

Abbreviations: FAD = flavin adenine dinucleotide; FMN = flavin mononucleotide; PFB = paraflagellar body; RF = riboflavin

spot") is excluded as the photoreceiving organelle because cells bleached with streptomycin lose their chloroplast and stigma, but nevertheless retain phototaxis (Checcucci et al. 1976). Further experiments have shown that when a laser microbeam is directed only to the area of the PFB, and not to other parts of the *Euglena* cell, there occurs a violent stroke reaction of the flagellum (Colombetti et al. 1982).

Flavin-type autofluorescence has been reported to occur in the PFB region of intact, living *Euglena* (Benedetti and Checcucci 1975) and of isolated flagella (Schmidt et al. 1990). In addition, flavoproteins have been demonstrated in flagellar preparations by Brodhun and Häder (1990). It should be mentioned that pterins are discussed as possible auxiliary photoreceptors at the flagellar base (Brodhun and Häder 1990; Galland et al. 1990; Schmidt et al. 1990).

Gualtieri et al. (1986) devised a method to isolate the major flagellum of *Euglena gracilis* with the PFB still attached. If RF-binding sites are actually associated with photoreception they should be found in high concentration in such preparations. The experiments reported here test this prediction.

Materials and methods

Radiochemicals and chemicals. [2-^{14}C]Riboflavin (2 GBq · mmole^{-1}) was purchased from Amersham Buchler (Braunschweig, FRG); α/β-octylglucoside from Sigma (München, FRG) and from Biomol (Hamburg, FRG). Mouse monoclonal anti-α-tubulin antibodies, clone DM1A, and anti-mouse IgG, alkaline-phosphatase conjugate, were purchased from Sigma where the other materials for gels and blotting were also obtained.

All other chemicals were obtained from Merck (Darmstadt, FRG) and Roth (Karlsruhe, FRG).

Growth conditions. A culture of *Euglena gracilis,* strain Z Pringsheim, obtained from the culture collection in Göttingen, FRG (Schlösser 1982) was inoculated in 2 or 5 l carboys containing culture medium described by Beale et al. (1981). The cells were grown as described by Galland et al. (1990) in a controlled culture room at 22° C under continuous illumination from fluorescent lamps (3 W · m^{-2}). A current of air was filtered through several stages of sterile cotton or glass-wool filters, and bubbled through the cultures. The growth period lasted some 8 d until the cultures had reached a density of approx. 10^6 cells · ml^{-1}.

Isolation procedure. The flagella were isolated according to the method of Gualtieri et al. (1986) with modifications by Galland et al. (1990). In a first low-speed centrifugation (1000 · *g* for 10 min) at room temperature, the cells were pelleted and then resuspended at a titer of about 10^7 cells per ml. (Higher accelerations or chilling cause premature deflagellation.) All subsequent steps were carried out at 4° C or on ice.

Calcium chloride was added to a final concentration of 100 mM to detach the flagella which were then separated by a second low-speed centrifugation (1000 · *g* for 10 min at 4° C) to sediment the cells, and finally pelleted at 22 000 · *g* for 20 min.

The flagella were resuspended in 2 ml binding-assay buffer (10 mM Tris-HCl, 5 mM $MgSO_4$, pH 7.5), blended in a glass homogenizer and frozen at –80° C for later tests. Freezing did not affect the binding capacity. Cells were homogenized by grinding them to powder under liquid N_2 in a mortar.

In one experiment (see Fig. 4), the cell homogenate was prepared by treating the material, frozen in liquid N_2, for 6 · 30 s in a "Mikro-Dismembrator II" (from Braun, Melsungen, FRG).

At all stages of the isolation, cells and-or flagella were counted under a light microscope (Type III RS; Zeiss, Oberkochen, FRG). The final yield was low, usually about 5% of the flagella present in the original culture.

Binding assay. The binding test – modified according to Hertel et al. (1980) – was routinely performed in 1.5-ml micro centrifuge tubes (Eppendorf, Hamburg, FRG) under normal laboratory light. Test buffer (10 mM Tris-HCl, 5 mM $MgSO_4$, pH 7.5, with 0.1 or 0.2% octylglucoside), [^{14}C]RF (often $5 \cdot 10^{-8}$ M) and varying amounts of unlabelled RF were added as specified. The detergent was added to facilitate ligand access. If the binding properties of reduced RF were to be investigated, 5 mM Na-dithionite was added quickly from 125 mM dithionite dissolved in H_2O immediately before use. The assay was started with the addition of 100 µl of the *Euglena* material. The final volume was 0.5 ml per test.

After an incubation period of 10 min on ice, the total volume was diluted in 5 ml of 10 mM Tris-HCl (pH 7.5) and immediately poured over glass-fiber filters (Whatman GF/B; Bender und Hobein, Freiburg, FRG) into a vacuum flask; the filters had been preincubated in 0.3% polyethyleneimine (PEI) for 1 h (filter test modified after Thein and Michalke 1988). To avoid unspecific attachment of radioactivity to the filters they were rinsed with another 5 ml of 10 mM Tris-HCl (pH 7.5). The PEI-coated filters, where the protein-RF-complex is retained, were clear of liquid about 10 s after dilution, and were then transferred to scintillation fluid (Ultima Gold from Beckmann Co, München, FRG) and counted in a Beckmann Liquid Scintillation Counter.

'Saturable' or 'specific' [^{14}C]RF binding is defined as the difference in filter-bound radioactivity (in cpm) between a sample containing only radioactive RF and a parallel sample to which saturating amounts of unlabelled RF had been added. Binding is often expressed as % binding = saturably bound cpm · 100/total cpm in assay. This value per mg protein added to the assay, can be used to compare different preparations.

Routinely, assays were run in duplicate; the mean is presented. Table 1 illustrates the raw data and the unspecific binding: e.g. the duplicate samples for the left column were 85 and 75 cpm, or 13 and 35 cpm, respectively, and for the right column 212 and 214 cpm, or 14 and 13 cpm, respectively. This gives an impression of the accuracy of the binding tests. Most other data are presented as "specific (= saturable) binding".

Polyacrylamide gel electrophoresis, Western blots and protein determination. Samples from *Euglena* cells and flagella were separated on a 10% acrylamide sodium dodecyl sulfate gel according to Laemmli (1970). One half of the gel was then transferred to a semi-dry blotting apparatus (Biometra, Göttingen, FRG); the proteins were blotted electrophoretically to a nitrocellulose membrane (BA 83, 0.2 µm; Schleicher und Schüll, Dassel, FRG). Positive bands were detected using mouse monoclonal anti-α-tubulin antibodies (1/2000 in test) and enzyme-conjugated anti-mouse-IgG (1/3000), and the blot was stained with 5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt/nitro blue tetrazolium (BCJP/NBT). Western blotting and staining according to Harlow and Lane (1988).

For protein determination the Coomassie method (Spector 1978) was used.

Results

Riboflavin binding in flagellar preparations. The first aim was to detect and characterize saturable binding of [^{14}C]RF in flagellar material isolated according to Gualtieri et al. (1986). This procedure employs a calcium shock that causes the flagella to beat vigorously, thereby everting the anterior invagination in which the base of the flagellum is attached. This ejection shifts the point of

highest stress towards the flagellar base, just above the cell membrane. As a consequence, flagella break off below the PFB which remains attached to its flagellum. Thus, isolation of flagella by differential centrifugation will co-purify PFBs and separate them from the cell bodies. During the procedure an enrichment of flagellar structures and of tubulin (see below, Fig. 4) was consistently observed.

The flagellar structures counted under the light microscope were of very variable length. Either they broke off at different points outside the invagination, or they were broken during homogenization. Therefore, one has to assume that not all of the flagellar pieces counted – probably less than 50% – carried a PFB.

The results in Table 1 show that both oxidized and reduced RF can bind to the flagella isolated as described above. In the flagellar preparations, [^{14}C]RF binding was proportional to protein concentration, at least between 0 and 60 µg (data not shown).

Dithionite indeed acts as a reducing agent. The products of its oxidation, e.g. bisulfite, do not interfere with the test, since RF remains in the reduced state which is not attacked by bisulfite (Müller and Massey 1969). After 40 min incubation of the dithionite test mixture, reoxidation had occurred as seen by the reappearance of oxidized (yellow) RF.

The RF binding, both in the oxidized and the reduced state, was reversible since radioactivity could be displaced from the sites after the binding incubation, by adding a surplus of unlabelled RF to the dilution medium and leaving the diluted samples for 10 min (data not shown).

Saturation curves with increasing RF concentrations are presented in Fig. 1a. From these data and from the corresponding Scatchard plots (Fig. 1b), affinities and number of sites can be estimated. In the absence of dithionite, the number of "oxidized" binding sites (Fig. 1b, open symbols, intersection of the regression line with the abscissa: 3.2 pmol per assay, corresponding to 0.05 nmol · mg^{-1} protein), and their affinity towards the ligand (Fig. 1b, negative reciprocal of the slope, a $K_D = 0.07$ µM) were calculated. In another similar experiment these values were 0.8 pmol and 0.09 µM, respectively. In the presence of dithionite, the number of binding sites (Fig. 1b, closed symbols) was determined as 7 pmol

Table 1. [^{14}C]Riboflavin binding to a flagellar preparation from *Euglena* in the presence (reduced) and in the absence (oxidized) of dithionite. 40 µg protein or approx. 4 · 10^7 flagella were used in each sample. The radioactive ligand had a concentration of 4 · 10^{-8} M [^{14}C]RF (i.e. 1861 cpm); 3 · 10^{-5} M unlabelled RF was added to part of the samples as competitor. Each value is based on duplicate determinations

	Oxidized	Reduced
–	80 cpm	214 cpm
+ Unlabelled RF	24 cpm	14 cpm
Saturable binding	56 cpm	200 cpm
% bound/free RF	3.0	11
% bound/free · mg^{-1} protein	75.3	274

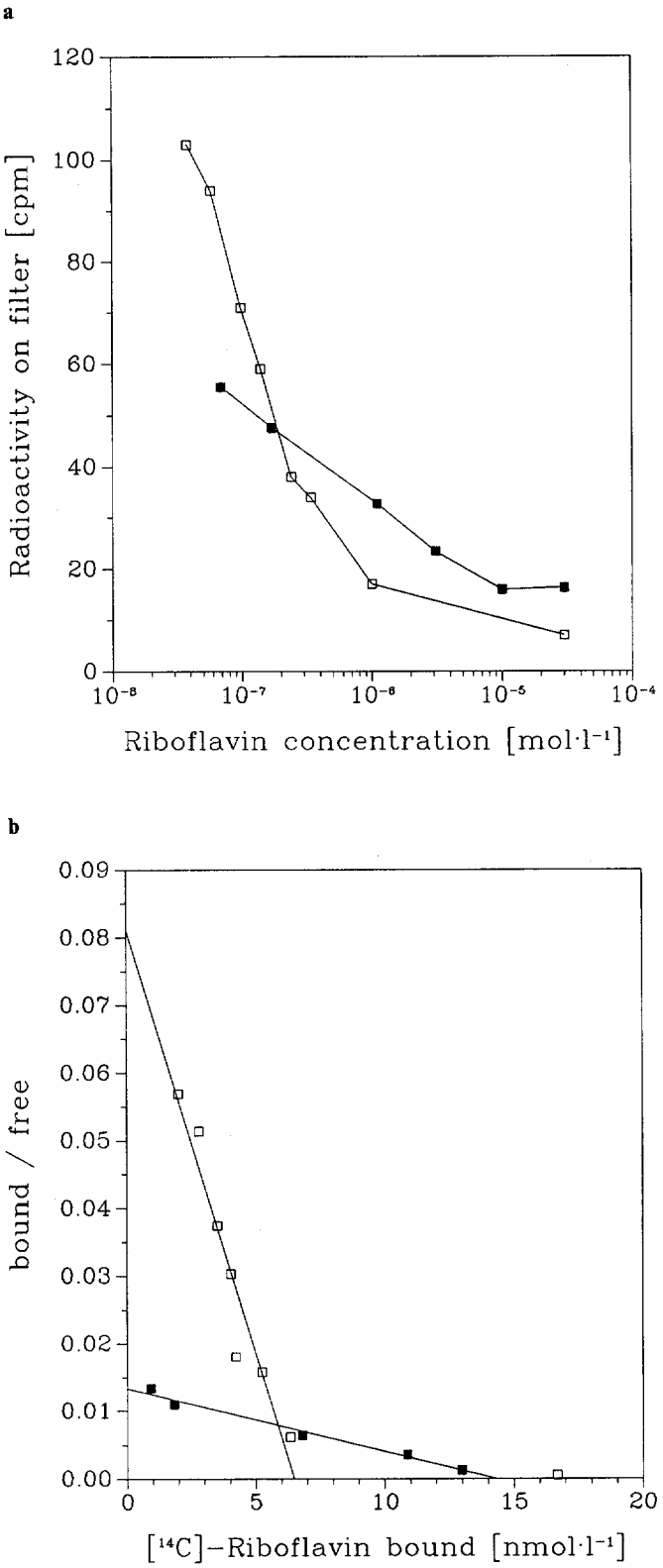

Fig. 1. a Saturation curves at increasing RF concentrations under oxidizing conditions (*open symbols*), using 60 µg of protein (approx. 6 · 10^7 flagella) in each test sample, and under reducing conditions (5 mM dithionite added; *closed symbols*), using 6.9 µg protein (4.2 · 10^6 flagella) **b** Scatchard plots calculated from data in **a** under oxidizing conditions (*open symbols*), and under reducing conditions (*closed symbols*)

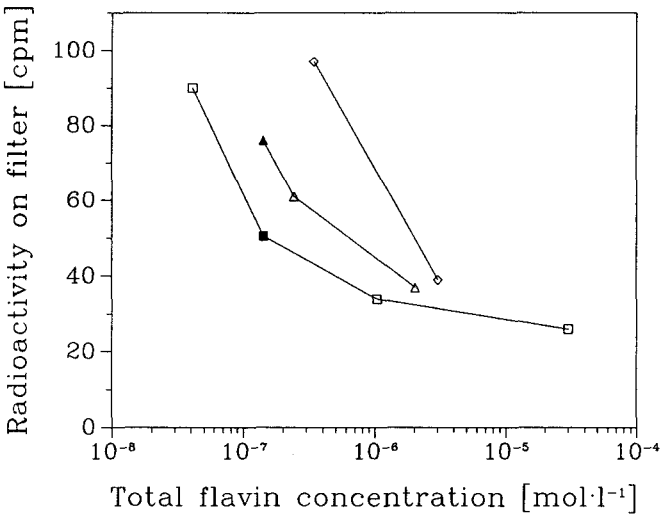

Fig. 2. Saturation curves with increasing concentrations of RF (*squares*), FMN (*triangles*), and FAD (*diamonds*). Each sample contained 40 µg protein, approx. $4 \cdot 10^6$ flagella. To this was added $4.1 \cdot 10^{-8}$ M (i.e. 2000 cpm) [^{14}C]RF and varying concentrations of unlabelled flavins, but no dithionite. The values at 10^{-7} M (*filled symbols*) represent the means of four replicates each; here, the standard error were about the size of the symbols (±2 cpm)

per assay (corresponding to 1.0 nmol · mg^{-1} protein), and their RF affinity was characterized by a dissociation constant K_D of 0.7 µM.

Centrifuge binding assays (according to Hertel et al. 1980) produced essentially the same results as did the filter tests (data not shown).

The binding tests were usually performed in laboratory daylight. Control experiments under red safelight did not reveal any difference from those under the standard conditions (data not shown), i.e. there was no effect of light on RF binding.

To determine whether RF was a specific ligand for these sites, increasing concentrations of some analogs were tested for their capacity to compete with [^{14}C]RF (Fig. 2). When tested in the absence of dithionite, FAD showed a much lower affinity for the binding sites, whereas FMN was more effective in replacing [^{14}C]RF. However, it still had significantly less affinity than RF itself, by a factor of approx. 2. The same pattern was obtained in the reduced state; substances like xanthopterin and indoleacetic acid did not compete at all (data not shown).

Estimate of number of binding sites per flagellum. The molarity of RF-binding sites in the assays can be converted to binding sites per protein or – more relevantly – the number of binding sites per flagellar piece counted. In experiments without dithionite approx. $3 \cdot 10^4$ sites per flagellum are estimated. In the case of the reduced ligands (see Fig. 1b, closed symbols), however, the number of binding sites seems to be much higher, approx. $5 \cdot 10^5$ per flagellum. (It should be noted that in six separate and independent experiments, the estimated number of binding sites per flagellum varied by a factor of 2.8.) The estimates of binding sites represent minimal values only because the number of "entire" flagella may be overestimated. On the other hand, the number of sites could be underestimated because the flavins present in vivo might still be occupying some of the sites.

Localization of binding sites. The main experimental aim was to compare flagella and cell bodies of *Euglena* with respect to RF binding. The isolation procedure according to Gualtieri et al. (1986), separates flagella with intact PFBs from the cells; thus, the presumed photoreceptor and – according to our hypothesis – the RF-binding sites should be highly enriched in flagella compared to whole cells.

In numerous experiments, using independent preparations, and under various conditions, the RF-binding capacity of isolated flagella as well as that of the cells, was assayed. The most striking and important qualitative feature in all independent tests was the high enrichment of in vitro RF binding per unit of protein in the flagella when compared to cells.

Saturation curves of RF binding to flagella and to deflagellated cells were compared under oxidizing conditions (Fig. 3). The absence of binding capacity in the cell homogenate might be attributed to the action of substances present in this crude preparation and inhibiting RF binding. However, the possibility of such an inhibitor was ruled out since a mixture of the isolated flagella and the cell homogenate showed exactly the same binding capacities as did the flagella alone (Fig. 3). For reducing conditions, the same difference between flagella and cells was found (Table 2: one experiment in detail; Table 3: summary of three tests). In these experiments (Tables 2, 3), isolated flagella were compared to a homogenate of still-flagellated cells. These cells did show RF-binding capacity which was small on a protein basis, but the numbers per cell are similar to those per flagellum (Table 2, last line).

Again, the strong enrichment of binding per protein in flagella cannot be attributed to inhibitor(s) in the cell

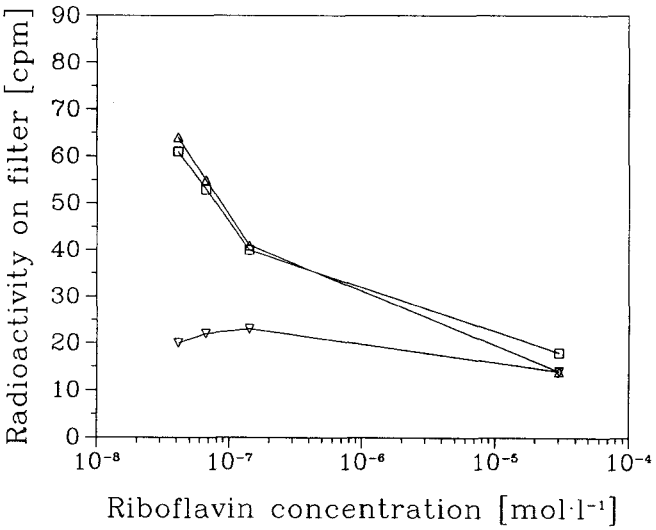

Fig. 3. Riboflavin binding to flagella and cell homogenates of *Euglena*, and mixtures thereof. The flagella and cell samples contained 30 µg and 40 µg protein, respectively. The mixtures were simple additions of 30 µg flagellar and of 40 µg cell protein. Other conditions were as in Fig. 2. (□–□ flagella, ▽–▽ cell homogenate, △–△ flagella + cell homogenate)

Table 2. Binding of RF to isolated flagella and cell homogenates of *Euglena* and to a mixture thereof. $6.5 \cdot 10^{-8}$ M (i.e. 3048 cpm) [^{14}C]RF + $3 \cdot 10^{-5}$ M unlabelled RF was added. All samples contained 5 mM Na-dithionite to achieve reducing conditions. 'Pieces' are either cells, or flagella, or fragments of flagella counted in the microscope. Numbers in parentheses are extrapolated for the mixture

	Flagella	Homogenate from cells	Flagella + homogenate from cells
Protein (μg)	6.0	67.4	(73.40)
Number of pieces	$4.9 \cdot 10^6$	$2.6 \cdot 10^6$	$(7.5 \cdot 10^6)$
cpm spec. bound	29.0	26.9	51.2
% bound/free RF	0.95	0.88	1.68
% bound/free · mg^{-1} protein	158.6	13.1	22.9
Binding sites/piece	$4.3 \cdot 10^5$	$7.4 \cdot 10^5$	$(4.9 \cdot 10^5)$

Table 3. Binding of RF to flagella and cell homogenates of *Euglena* and to mixtures thereof: three independent preparations. The cells used still carried their flagella. The protein concentration of the cell homogenate was usually about ten times that of the flagellar sample. All samples contained 5 mM Na-dithionite to obtain reducing conditions. The values are given as '% bound/free · mg^{-1} protein'. Other conditions as in Table 2

Expt. No.	RF binding in		
	flagella	homogenates from cells	flagella + homogenates from cells
1	159	13	23
2	171	8	18
3	154	11	13

homogenate because the absolute amount of saturably bound radioactivity in the mixture of flagella and cells is the sum of the values for flagella, and for cells alone (Table 2). It should be noted that the binding values per unit of protein in Tables 2 and 3 are low for the mixtures because binding sites are "diluted" by the large amount of cell protein added.

It was also found that homogenates from intact *Euglena* cells display better RF binding than those from cells where flagella had been shaken off (Table 4). As another control, a "flagellar" preparation was included which was derived from cells that were previously deprived of their flagella; its binding capacity was very low (Table 4, third column).

In some experiments the flagellar content of the preparations was checked by immunodetection of tubulin in the different samples. For this we employed sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and Western blots with subsequent incubation with anti-tubulin antibodies. *Euglena* tubulin antigen appeared at the expected molecular weight of 55 kDa, and co-migrated with known bovine brain tubulin (data not shown). Figure 4 demonstrates the relative abundance of tubulin in the flagellar preparation compared to the cells. The RF binding (oxidized state) in these two fractions was determined to be 16.2% · mg^{-1} protein for flagella, and 2.0% · mg^{-1} protein for cells.

The result confirms the microscopic estimates and again shows that RF binding is enriched in the flagella. In this experiment, the cells were homogenized with an additional vigorous treatment in a "micro-dismembrator". The resulting extract was taken up in nine volumes of test buffer, and large pieces and cells were removed by a centrifugation at $1300 \cdot g$ for 10 min. This preparation showed the same binding properties as did the standard cell homogenate used in the other experiments.

Riboflavin binding to flagellar preparations and their supernatant. During this study, it was found that the supernatant obtained from a flagellar preparation contained an appreciable number of RF-binding sites. Flagella were sedimented at the intermediate centrifugal force of $22\,000 \cdot g$ (characterization and RF binding shown in Table 5, left column); the resulting supernatant was subjected to a further, high-speed centrifugation at $200\,000 \cdot g$ for 40 min. This final pellet showed RF binding (Table 5, right column), and it contained flagella, but in numbers too small to account for the large amount of binding observed. If, during preparation of flagella, some PFBs – much smaller than flagella – were broken off they might be left in the supernatant of the centrifugate at $22\,000 \cdot g$, sedimenting, however, at the higher force.

Table 4. Binding of RF in various flagellar and cellular preparations. Deflagellated *Euglena* cells were obtained as described in *Materials and methods*. The 'flagellar' preparation in the third column was derived from previously deflagellated cells by a second Ca^{2+} shock, corresponding to fraction III in Galland et al. (1990), (Fig. 1.). All samples contained 40 μg protein, except for that in the third column with only 18 μg, and a very low number of flagella. No dithionite was added. Each sample contained $3 \cdot 10^{-8}$ M [^{14}C]RF; $3 \cdot 10^{-5}$ M unlabelled RF was added to half of the samples

	Starting from			
	whole cells:		deflagellated cells:	
	flagella	homogenate	'flagella'	homogenate
–	94 cpm	58 cpm	34 cpm	32 cpm
+ Unlabelled RF	22 cpm	26 cpm	23 cpm	20 cpm
Saturable binding	72 cpm	32 cpm	11 cpm	12 cpm
% bound/free RF	3.9	1.7	0.6	0.6
% bound/free · mg^{-1} protein	96.3	42.8	32.7	16.0

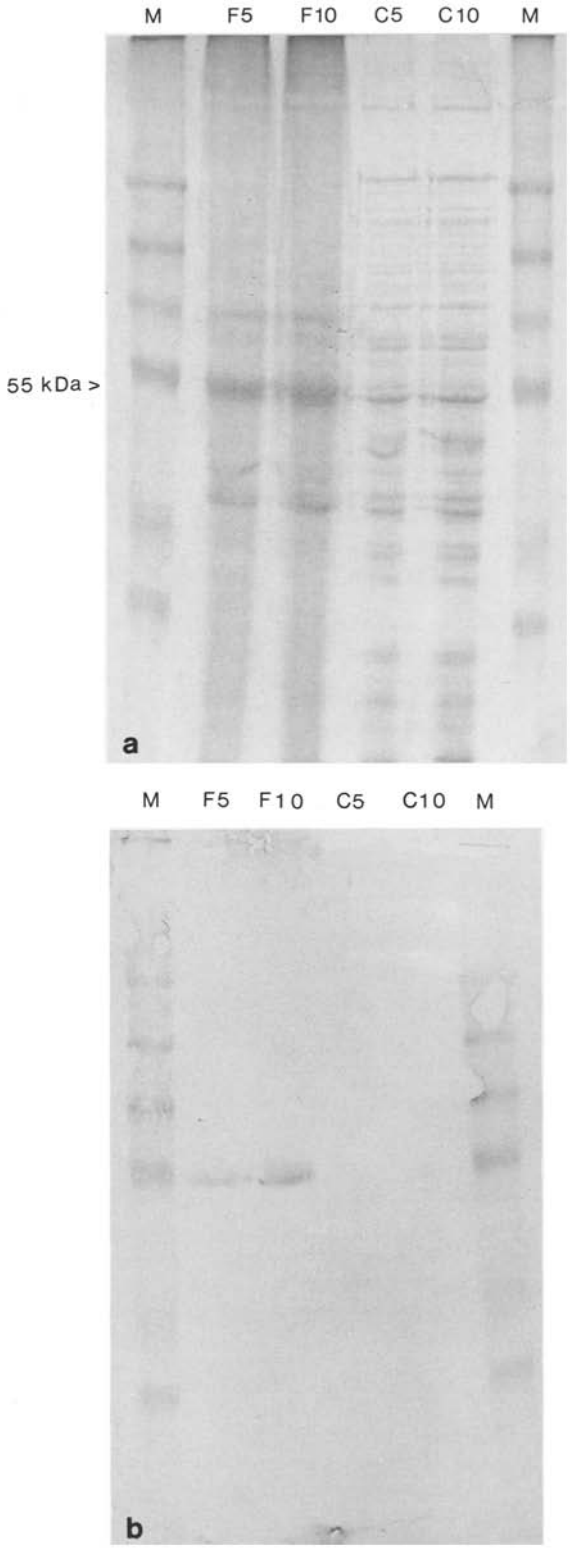

Fig. 4. a Analysis of flagella and cell preparations by SDS–PAGE, and **b** subsequent Western blot with anti-α-tubulin antibodies. The proteins in **a** and **b** were separated on one common 10% gel which subsequently was cut into two halves for protein staining and blotting, respectively. The lanes were loaded with 5 μg and 10 μg protein, respectively, as indicated above each lane. *F*, flagellar preparation; *C*, cell extract; *M*, molecular-weight markers (pre-stained markers from Sigma, FRG)

Table 5. Binding of RF to isolated flagella of *Euglena* and to the high-speed pellet from a 'flagellar' supernatant (centrifugation at 200000 · *g* for 40 min). The [^{14}C]RF concentration was $7 \cdot 10^{-8}$ M. All samples contained 5 mM Na-dithionite. The number of binding sites was calculated assuming a K_D of 0.7 μM

	Flagella	Pelleted supernatant
Protein (μg)	6.9	6.9
Number of flagella	$4.2 \cdot 10^6$	$1.6 \cdot 10^6$
Saturable binding (cpm)	39.2	39.3
Bound/free · mg^{-1} protein (%)	170.8	171.0
Binding sites in assay	$2.6 \cdot 10^{12}$	$2.6 \cdot 10^{12}$
Binding sites/flagellum	$6.2 \cdot 10^5$	$1.6 \cdot 10^6$

Furthermore, preliminary tests indicate that flagella without a PFB bind much less RF. The isolated flagella had been subjected to an additional Ca^{2+} shock. After a subsequent centrifugation, according to Brodhun and Häder (1990), the pelleted flagella should contain no PFBs. In parallel assays, both regularly prepared flagella and flagella with their PFBs removed were exposed to [^{14}C]RF: in the oxidized state, the latter showed a saturable RF binding (as bound/free · mg^{-1} protein) of only 0.21 as compared to 0.96 for control flagella.

Discussion

Proteins, in the plasma membrane or in another "fixed" position, that bind RF have been proposed as blue-light photoreceptors in fungi and plants (Hertel 1980). The results here document the occurrence of a strong and saturable binding of RF to flagellar preparations from *Euglena*. Both oxidized and reduced RF can bind specifically, i.e. better than FMN or FAD. Affinity for reduced as well as for oxidized RF was reflected by K_D values in the range of 0.1 – 1 μM. The number of RF-binding sites seems to be higher in the presence of dithionite.

The difference between the number of sites in the reduced and the oxidized state suggests that one is dealing with two different types of binding proteins. The decision as to whether there are two or more different binding sites involved, or just different affinity patterns at one site only, must await isolation and characterization of the respective proteins. Such a study is presently under way.

From the amount of RF binding and from the number of flagella (or flagellar pieces) in the assay, approx. $5 \cdot 10^5$ RF-binding sites per flagellum were estimated under reducing conditions. The amount per PFB might actually be higher by a factor of two, or more, since probably only half of the "flagella" counted carried their PFB.

The number of 10^6 binding sites per PFB should be compared to: (i) the amount of binding sites from one intact cell (Table 2); (ii) the number of photoreceptor molecules in the PFB that can be expected from microspectrophotometric observations (Colombetti and Lenci 1980); (iii) the number of flavin molecules in/at the PFB determined by Colombetti and Lenci (1980) by microspectrofluorimetry; and (iv) the number of unit cells of the PFB's protein crystal (Piccini and Mammi 1978).

Since all these numbers are in the range of approx. 10^6, it seems reasonable to propose that the RF-binding sites are localized in the PFB or at the surrounding membrane, and that these proteins are involved in photoreception for euglenoid phototaxis.

It has been observed that the flavin-type fluorescence in the PFB region of *Euglena* described by Benedetti and Checcucci (1975) and by Schmidt et al. (1990) is lost when preparing and washing flagella (Galland et al. 1990, fraction II in their Fig. 1). These findings agree very well with a "loose" flavin binding to the flagellar structures. The finding by Brodhun and Häder (1990) of some covalently (or tightly) bound flavin in flagellar preparations is not incompatible with the large amount of reversible RF binding shown here.

Gualtieri et al. (1989) measured absorption spectra from a single "dried" PFB in situ. The amount of pigment must be very high, in the range of 10^7 molecules per PFB. The interpretation of the spectrum as being that of a rhodopsin is not in line with the reported action spectra (see *Introduction*). We rather suggest that PFBs contain a protein-stabilized "red" flavin semiquinone radical (see Massey and Palmer 1966, where e.g. D-amino acid oxidase shows a spectrum like the one measured by Gualtieri et al. 1989).

The pterins – probably present at the PFB – may act as a second photoreceptor, or as a cofactor for the photoreactions (Brodhun and Häder 1990; Galland et al. 1990; Schmidt et al. 1990). Interference of pterins with RF binding, however, was not seen when xanthopterin was added.

The determination of the mechanism of photoperception in *Euglena* is of general interest because this protozoon/phytoflagellate may be at the base of eukaryotic evolution. *Euglena*'s – and most protists – precise phylogenetic relationships are far from clear, but it might actually represent a protozoon that carries a eukaryotic alga as an endosymbiont. For example, its pellicle lacking cellulose is atypical of cell walls of green algae, and the "chloroplasts" have one additional membrane which has been interpreted as the remnant of an endocytotic event in which a eukaryotic green alga was incorporated into the pre-*Euglena* cell (Gibbs 1978).

In view of these relationships it is most interesting that other non-green algae also show a strong flavin-like fluorescence in one of their flagella (Kawai 1988; Coleman 1988). Riboflavin-binding sites in these flagella would be another strong argument for a photophysiological role of such flavoproteins. It should be noted, however, that in *Euglena,* flavin fluorescence of the flagellum – in contrast to the PFB – is very weak (Kawai 1988).

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