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In vivo spectroscopic study of photoreceptor pigments of *Blepharisma japonicum* red and blue cells

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Abstract

In the coloured ciliate *Blepharisma japonicum*, step-up photophobic responses are triggered by the endogenous pigment blepharismin. Blepharismin, red in dark-grown cells, is intracellularly photooxidized into a blue form (oxyblepharismin), still acting as photosensing pigment. With the aim of correlating the spectroscopic properties of blepharismin and oxyblepharismin in vivo with their photophysiological features, optical absorption, steady-state and time-resolved fluorescence spectra have been measured on cell suspensions. Both in blepharismin and oxyblepharismin in their physiological molecular environment, three fluorescent species have been observed, with virtually the same lifetimes (~ 0.2 ns, ~ 1.0 ns, ~ 3.5 ns), but significantly different relative amplitudes. In red cells the long-living component has a very low relative amplitude ($\sim 4\%$) and the short-living one is largely predominant ($> 78\%$), whereas in blue cells the slowly decaying species has a slightly higher relative amplitude ($\sim 40\%$) than the intermediately ($\sim 31\%$) and the fast decaying species ($\sim 29\%$). Together with the spectral width of time-gated spectra, these data are discussed in connection with current hypotheses on the structures of the chromophores. No meaningful difference in the above-mentioned spectroscopic parameters was observed after 30 min of UV-B irradiation, showing that no significant difference exists between red and blue blepharismin as far as UV-B lability is concerned.

Keywords: Blepharismin-binding protein; Photomovement; Photoreception; Time-gated fluorescence; Ultraviolet B; (*B. japonicum*)

1. Introduction

Following a sudden increase in light intensity, the heterotrichous ciliate *Blepharisma japonicum* shows a reversal of the ciliary beating that causes the cell to swim backward and to change direction, before resuming forward swimming [1–3]. This photomotile reaction (step-up photophobic response) allows the cells to avoid brightly illuminated regions, where they can be killed by photodynamic reactions sensitized by the endogenous red pigment, blepharismin [2–4].

When *B. japonicum* is irradiated with dim visible light (fluence rates lower than about 30 W m^{-2}, at which no cell photokilling takes place), blepharismin undergoes an intracellular photooxidation process to oxyblepharismin [2,5]. When this process attains the saturation, the cells appear blue-grey but still maintain their photosensing capability [6,7]. The absorption spectrum of the extracted pigment in ethanol solution shows structural modifications [2,3,5], that make it very similar to the absorption spectrum of hypericin [3,8].

Action spectroscopy indicated blepharismin as the photoreceptor molecule which transduces the light stimulus into an intracellular signal ultimately yielding the photomotile response [6,7,9]. The pigment granules, which are regularly spread all over the cell just beneath the plasmatic membrane [1,2], were proposed to be the cellular structures where the photosensory process initiates [9,10]. In blue cells the action spectrum appeared quite similar to the absorption spectrum of oxyblepharismin in ethanol, thus suggesting that photooxidation does not modify blepharismin reactivity as far as its photodetecting and photosignalling function is concerned [6,7].

Spectroscopic studies of isolated chromophores, aimed to clarify the nature of the primary molecular reactions occurring in the photosensing unit [11,12], provided basic

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information on the excited states of blepharismin and their lifetimes, but did not adequately clarify the question of the photophysical processes triggering the sensory transduction chain. Substantial progress can be achieved by focusing on the photochemical reactivity of molecular systems closer to the physiological ones, the blepharismin-binding proteins, which are involved in the primary steps of light-signal perception and transduction. Two chromoproteins, differing in apparent molecular mass (200 kDa and 38 kDa), were isolated from *B. japonicum* red cells by Matsuoka et al. [13] and Gioffré et al. [14], and two spectrally distinct forms of blepharismin-binding proteins were isolated from red cells by Yamazaki et al. [15].

Matsuoka et al. [6] and Murakami et al. [8] observed that in vitro light irradiation of the cell homogenate, and the consequent photooxidation of blepharismin to oxyblepharismin, apparently increases the binding of the chromophore to the 200 kDa apoprotein and suggested that the blue chromophore-protein complex functions as the photoreceptor mediating the light response of blue cells.

Yamazaki et al. [15] reported a detailed time-resolved study of the spectroscopic properties of the different blepharismin binding-proteins they isolated, but could not identify which of the blepharismin species corresponds to the biologically functional photoreceptor.

Significant contributions to relating the photophysical and photochemical properties of isolated chromophores and chromoproteins with their photophysiological functions can come from data on pigment systems in their physiological molecular environment, achievable by time-resolved fluorescence spectroscopy and imaging studies on intact cells ([16,17], and references therein). In this perspective, absorption and steady-state as well as time-resolved fluorescence techniques were used to investigate in

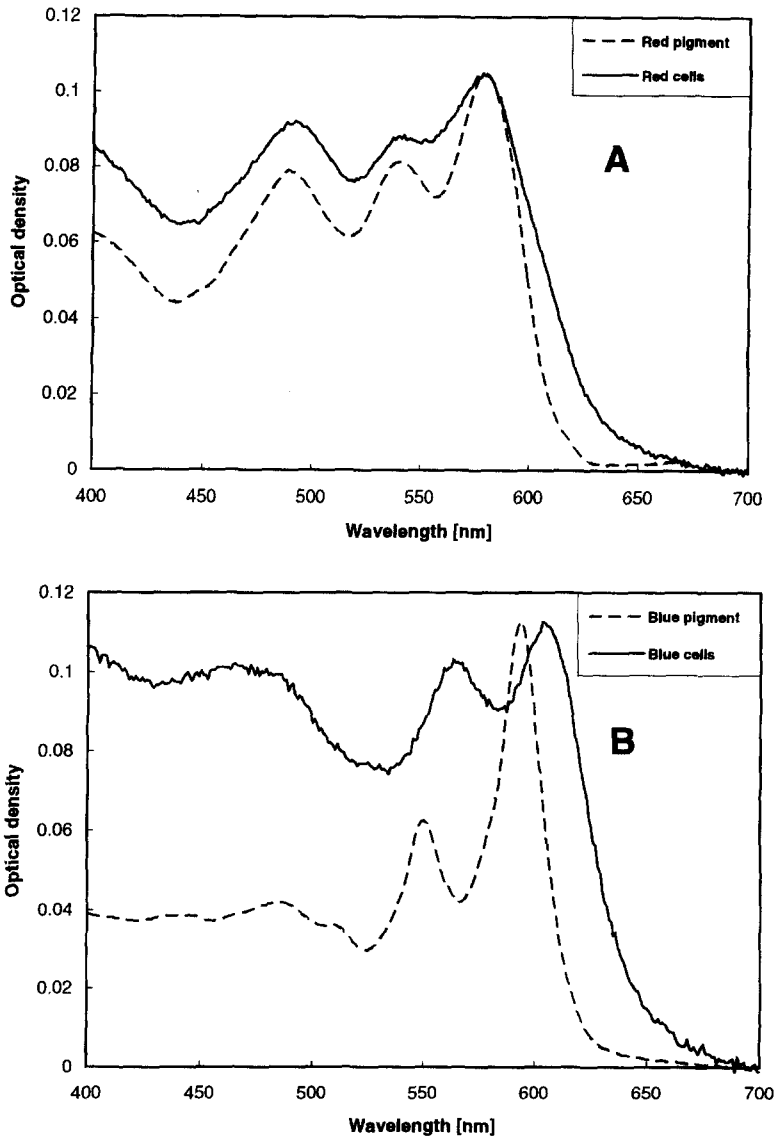

Fig. 1. (A) Absorption spectra of an agar suspension of *Blepharisma japonicum* red cells and blepharismin (Red pigment) in ethanol solution. (B) Absorption spectra of an agar suspension of *B. japonicum* blue cells and oxyblepharismin (Blue pigment) in ethanol solution.

vivo the spectroscopic properties of the different photoreceptor molecular species of red and blue *Blepharisma japonicum* in cell suspensions.

It was recently shown that, following artificial UV-B irradiation, the percentage of *B. japonicum* cells exhibiting step-up photophobic responses significantly decreased [18]. To investigate diversities in the photolability of the two photosensing pigments, the in vivo study of red and blue pigments spectroscopic properties was extended also on UV-B irradiated cells.

2. Materials and methods

Blepharisma japonicum was grown as previously described [14]. The cells were collected by gentle centrifugation at about $100 \times g$, washed and resuspended in a saline resting medium (SMB, Saline Medium for *Blepharisma*, [19]). To photo-oxidize blepharismin to oxyblepharismin in vivo, cells were kept under white cold light (about 8 W m^{-2}) for 96 h; in these conditions the native red form of blepharismin fully converts into the blue form, without any detectable impairment of cell viability [7].

All absorption and fluorescence measurements were performed diluting 1:1 (v/v) the cell suspensions in a 0.2% agar suspension in SMB. In such a viscous medium cells do not undergo sedimentation and, at the same time, keep their shape and remain alive for many hours.

Absorption and steady-state fluorescence spectra were recorded with a JASCO 7850 spectrophotometer, equipped with a JASCO TIS-417 integrating sphere to minimize loss of spectral resolution due to scattering, and with a JASCO FP 770 spectrofluorimeter, respectively.

Time-resolved fluorescence measurements were performed using a computer-controlled apparatus [20]. An Argon-ion laser, operating in the mode-locking regime, supplied the excitation pulse (at 514 nm). The experimental data were obtained by the time-correlated single-photon-counting technique. The instrumentation provided the simultaneous acquisition of the spectrum-integrated decay waveform, the time-integrated emission spectrum ("cw", continuous wave emission spectrum) and two time-gated emission spectra. The first time gate was undelayed and 0.5 ns wide (0.0–0.5), whereas the other one was delayed by 2.0 ns and 2.0 ns wide (2.0–4.0). The overall time resolution of the system was 120 ps and its spectral resolution was 2 nm. The fluorescence decay waveforms were analysed by a non-linear least-square fitting procedure [21] and the quality of the fitting was evaluated on the basis of the reduced χ^2 value, the weighted residuals and of their autocorrelation function [22].

In UV-B experiments, cell suspensions in SMB, at a concentration of about 1500 cells/mL, were irradiated by means of two TL40W/12 Philips lamps, filtering the light by means of cellulose acetate films, in order to remove the UV-C wavelengths. Samples were exposed to an UV-B irradiance of about 5 W m^{-2}; each sample was irradiated only once and examined immediately after irradiation.

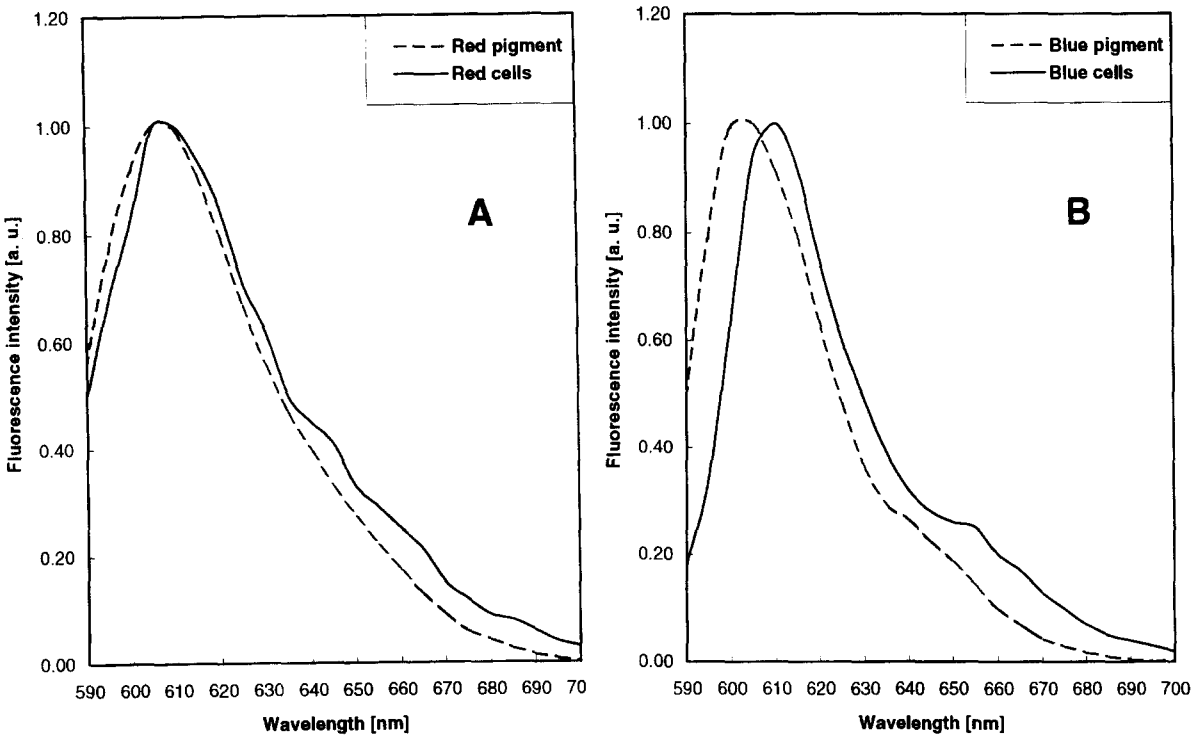

Fig. 2. (A) Fluorescence emission spectra of an agar suspension of *Blepharisma japonicum* red cells and blepharismin (Red pigment) in ethanol solution. (B) Fluorescence emission spectra of an agar suspension of *B. japonicum* blue cells and oxyblepharismin (Blue pigment) in ethanol solution.

3. Results and discussion

The in vivo absorption spectra of red and blue blepharismin, measured on cell suspensions by means of an integrating sphere, are reported in Fig. 1A and 1B, respectively. For comparison, the absorption spectra of both pigments dissolved in ethanol, after extraction with acetone, are also reported.

From Fig. 1 it appears that the absorption bands of red blepharismin in vivo overlap those in ethanol solution, with only a slight broadening of the highest peak in cell suspension spectra. In the case of blue blepharismin, on the contrary, all the absorption bands in cell suspension spectra show a marked bathochromic shift (about 15 nm) with respect to ethanol solution spectra.

Coherently, the fluorescence emission spectrum of blue blepharismin in vivo is shifted toward longer wavelengths (about 8 nm) with respect to that of the pigment in ethanol solution, whereas no significant difference is observed in the spectral distribution of emission spectra of red blepharismin in ethanol and in vivo (Fig. 2).

Both in red and blue blepharismin in vivo the presence of three fluorescent molecular species, with different fluorescence lifetimes, was revealed. The decay time values with their relative amplitudes are reported in the first two columns of Table 1. The reduced χ^2 values were always in the range of 0.94–1.24. Different measurements resulted in fluctuations smaller than 5% in the lifetimes and their relative amplitudes.

Table 1
Time-resolved fluorescence of agar suspensions of *Blepharisma japonicum* red and blue cells

	τ (ns)	A (%)	cw (%)	Gate 0.0–0.5 (%)	Gate 2.0–4.0 (%)
Red cells	3.22	4.46	31.07	8.56	75.12
	0.82	18.08	32.07	28.06	24.84
	0.22	77.46	36.86	63.37	0.04
Blue cells	3.54	39.77	74.07	49.62	81.86
	1.37	31.33	22.58	35.12	18.14
	0.22	28.90	3.35	15.26	0.00

First two columns: fluorescence decay times (τ) and relative amplitudes (A) of the three fluorescent species detected in blepharismin and oxyblepharismin. The reported numerical values are the direct outcome of the non-linear fitting of experimental decay curves. Different measurements resulted in fluctuations smaller than 5% in the lifetimes and their relative amplitudes. The reduced χ^2 values were always in the range of 0.94–1.24. Last three columns: relative contributions of the three fluorescent species to the time-integrated (cw), the undelayed (0.0–0.5) and the delayed (2.0–4.0) time-gated spectra (calculated on the basis of the mathematical approach reported by Cubeddu et al. [19])

Typical examples of full spectrum fluorescence decay curves and best-fit curves for red and blue cells are reported in Fig. 3A and 3B, respectively, together with the weighted residuals and autocorrelation function.

In the case of red cells the decay times were: 3.22 ns, 0.82 ns and 0.22 ns. The fast-decaying molecular species was the predominant one (77%, in comparison with 18%

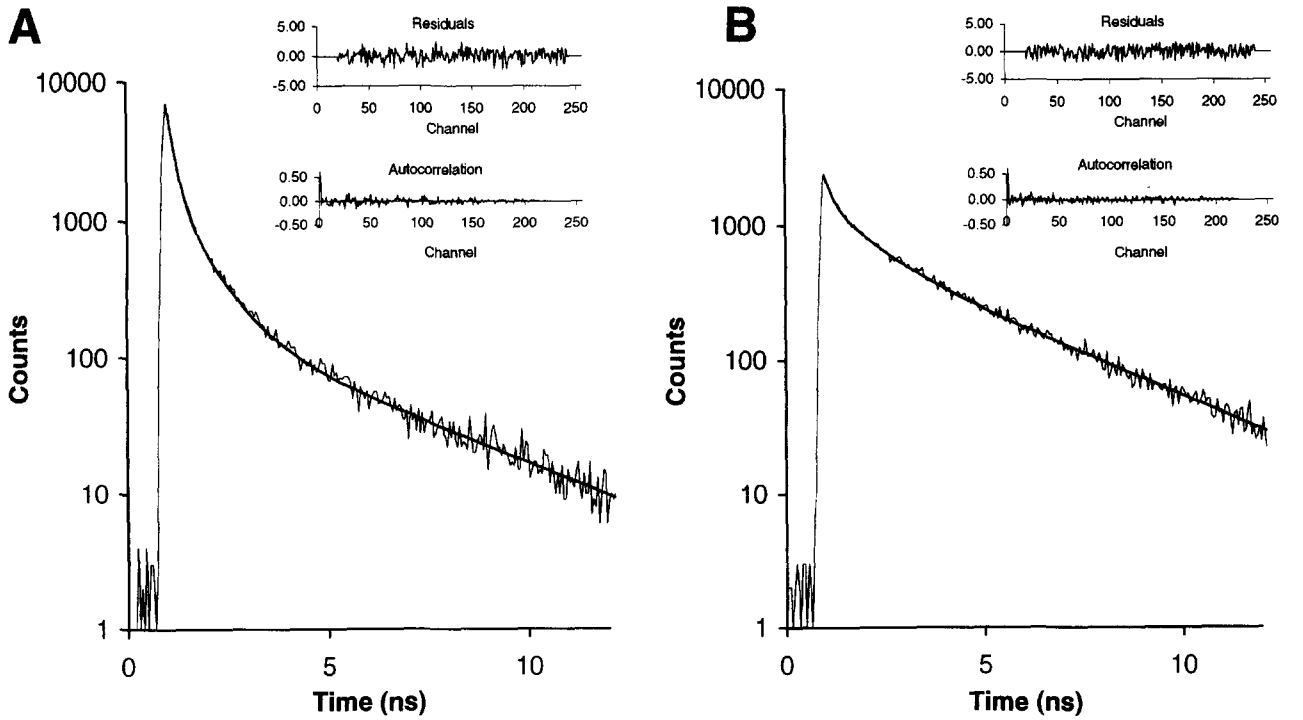

Fig. 3. (A) Full spectrum fluorescence decay data and best-fit curve (smooth line), weighted residuals and autocorrelation function of an agar suspension of *Blepharisma japonicum* red cells. (B) Full spectrum fluorescence decay data and best-fit curve (smooth line), weighted residuals and autocorrelation function of an agar suspension of *Blepharisma japonicum* blue cells.

and 5% of the intermediately and the slowly decaying species, respectively). These data are in good agreement with the results of previously reported microspectrofluorometric measurements on intact single cells [16] and, as far as the fluorescence lifetimes values are concerned, with those of Yamazaki et al. on Blepharismin A2 [15].

In blue cells similar fluorescence decay times values were measured: 3.54 ns, 1.37 ns and 0.22 ns. The relative amplitude of the fast component, however, markedly decreased (29%) to the advantage of the intermediate (31%) and the slow ones (40%).

Interestingly enough, fluorescence lifetimes longer than 5 ns are observed in Blepharismin C protein isolated from red cells [15], but not in vivo, either in red or blue cells. This might suggest that Blepharismin A2 [15], or a similar protein-pigment complex (it should be noted that Yamazaki et al. [15] point out the heterogeneous character of their preparations), is the photobiologically functional photoreceptor in red cells.

Time-gated spectra collect only the fluorescence photons which decay within the selected time-window and, assuming that all the molecular species fluoresce independently of each other, their relative contribution to the gated spectrum depends on the time width of the gate and on its delay time with respect to the excitation pulse.

The contribution of the different emitting species to an undelayed gated spectrum is proportional to their peak amplitudes, provided that the time window is narrow enough (i.e., shorter than the shortest lifetime). This was only approximately verified with the 0.5 ns gate, since, as mentioned above, a decay time of 0.22 ns was present for both red and blue cells (Table 1).

A delay longer than the longest lifetime allows isolation of the contribution of the slowest component. However, such a long delay usually implies a prohibitive attenuation of the detected signal. Therefore, in the present study the delay was limited to 2.0 ns and the corresponding spectrum, even though enhancing the slow emission, was partly due also to the intermediate-living species.

The relative contributions of the three emitting species to the time-integrated and the two time-gated spectra, calculated on the basis of the mathematical approach described by Cubeddu et al. [20], are reported in Table 1.

In all our sets of measurements, in the case of both red and blue cells, the undelayed gated spectrum is broader than the delayed spectrum, as shown in Fig. 4. This indicates a greater spectral width of the fast emission with respect to the slow one. In the two gated spectra of red cells, for example, where this broadening is particularly evident (see Fig. 4A), the relative weights of the slow and fast component vary from 9% and 63%, respectively, in the undelayed gate to 75% and 0%, respectively, in the delayed gate. The contribution of the intermediate component, on the contrary, is about the same (28% and 25%) and therefore does not affect the above-described broadening of the undelayed spectrum respect to the delayed one.

By comparing the two gated spectra of blue cells, it is more difficult to investigate the difference in the spectral features of the three components, since the weight of all of them changes with the delay of the acquisition window.

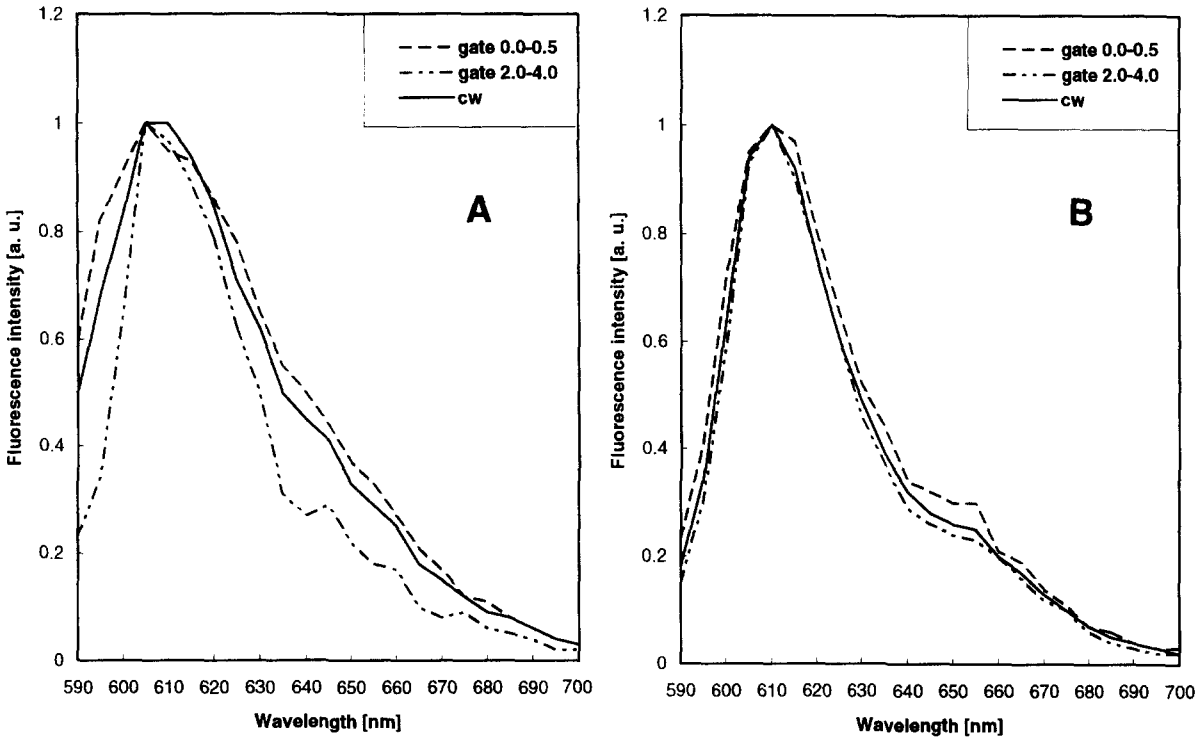

Fig. 4. Time-integrated (cw) and time-gated spectra of agar suspensions of red (A) and blue (B) *Blepharisma japonicum* cells.

On the other hand, the fluorescence profile of the different emitting species is the same in red and blue cells. This is clearly shown for the slow and the intermediate component by the similarity in shape of the delayed spectra of both cell types. In fact, these fluorescent species contribute with about the same percentages (75% and 82% for the long-living species and 25% and 18% for the intermediate-living one) to the two delayed spectra.

Finally, also when the two undelayed spectra are compared, the relevant broadening always observed in the case of red cells can be attributed to the higher contribution of the fast component (63% instead of 15% as for blue cells).

On the basis of these observations, therefore, considering both the time-integrated (cw) and the time-gated spectra, it can be concluded that the higher the contribution of the short-living species, the larger is the spectral width at half height, as shown in Fig. 5.

It is interesting to note that the larger fluorescence distribution of both the cw and the undelayed spectrum in red cells, due to the relevant contribution of the fast component, has a correspondence in the absorption spectrum. In fact, the absorption spectrum of blepharismin is broader than that of oxyblepharismin in ethanol as well as in vivo. This may indicate that the molecular species showing the fast decaying fluorescence is also responsible for the larger width of blepharismin absorption spectra.

On the basis of these observations, it is reasonable to conclude that in both red and blue blepharismin the same molecular species are present, even though in different relative amounts. The above-described spectroscopic features can be, finally, related to some structural data: in fact, it had been proposed that, at least in free chromophores, the predominance of a phenolic form is associated with a higher contribution of a short-living fluorescent species, whereas longer fluorescence lifetimes had been observed in quinonic forms [11].

As previously mentioned, a dramatic inhibition of photoresponsiveness of *Blepharisma japonicum* after 30 min of UV-B irradiation was reported [18]. Should such an effect be due to an impairment of the photosignalling

Table 2
Effect of 30 min UV-B irradiation on the fluorescence of agar suspensions of *Blepharisma japonicum* red and blue cells

	τ (ns)		A (%)	
	$t = 0$	$t = 30$	$t = 0$	$t = 30$
Red cells	3.22	3.31	4.46	6.36
	0.82	0.89	18.08	16.28
	0.22	0.20	77.46	77.36
Blue cells	3.54	3.58	39.77	33.83
	1.37	1.07	31.33	28.16
	0.22	0.23	28.90	38.01

First two columns: fluorescence decay times (τ) of blepharismins in vivo, before ($t = 0$) and after irradiation ($t = 30$). Last two columns: relative amplitudes (A) of blepharismins in vivo, before ($t = 0$) and after irradiation ($t = 30$). The numerical values reported above are the direct outcome of the non-linear fitting of experimental decay curves. Different measurements resulted in fluctuations smaller than 5% in the lifetimes and their relative amplitudes. The reduced χ^2 values were always in the range of 0.94–1.24

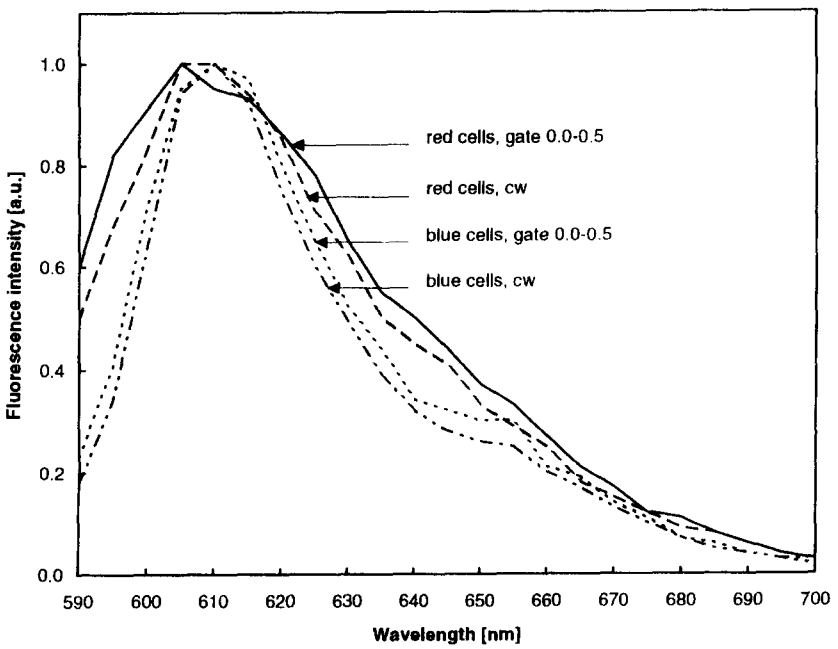

Fig. 5. Time-integrated (cw) and time-gated spectra of agar suspensions of red and blue *Blepharisma japonicum* cells. The higher the contribution of the fast-decaying species, the larger the spectral width at half height. The relative contributions of the fast component is: 63% for red cells, gate 0.0–0.5; 37% for red cells, cw; 15% for blue cells, gate 0.0–0.5; less than 4% for blue cells, cw, for red cells, gate 2.0–4.0 and for blue cells, gate 2.0–4.0 (last two spectra not reported).

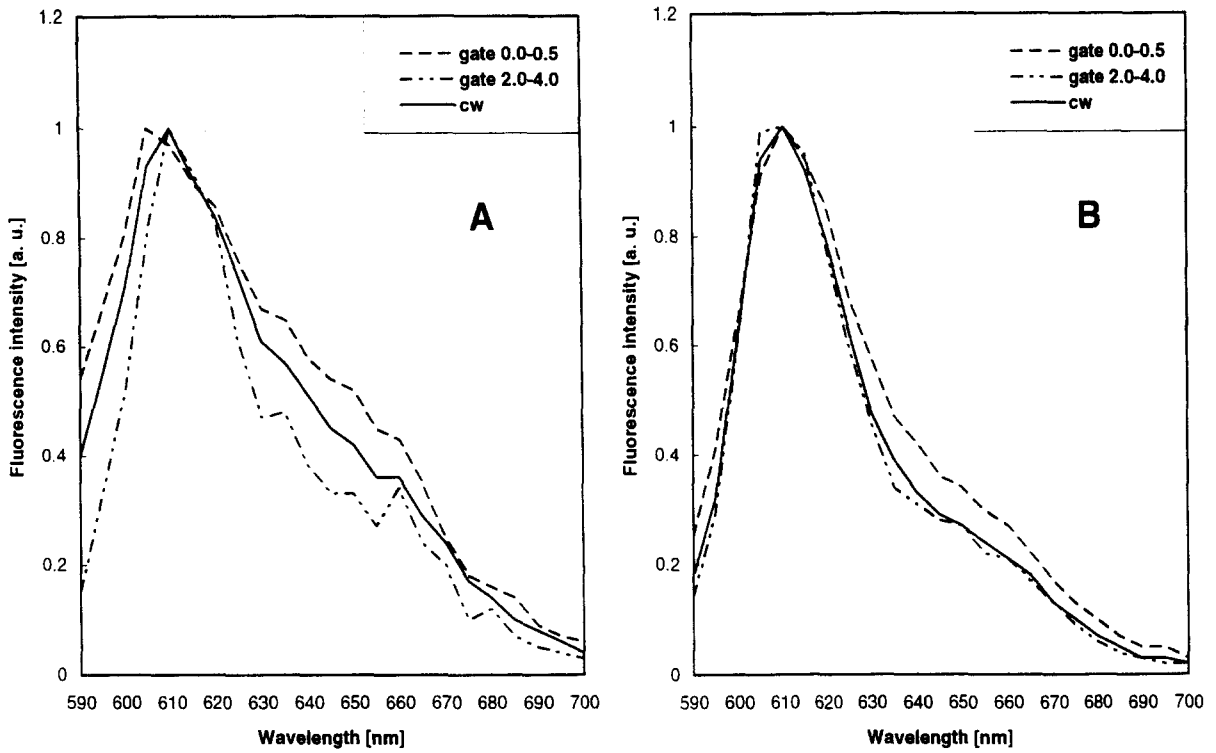

Fig. 6. Time-integrated (cw) and time-gated spectra of agar suspension of red (A) and blue (B) *Blepharisma japonicum* cells after irradiation with UV-B light for 30 min.

capability of the photoreceptor pigment, an increase in the radiative dissipation processes should be observed [17]. In this case the relative amplitude of the shortest living species, assumed to be the functional photoreceptor as it deactivates through non-radiative pathways initiating the sensory transduction chain, should decrease.

The already described set of spectroscopic measurements was, therefore, repeated on cell suspensions after 30 min of UV-B irradiation. As shown in Table 2, fluorescence lifetimes and relative amplitudes of the different emitting species, detected in red and blue cells, are almost the same before and after UV-B-treatment. Even after UV-B irradiation, both in red and blue cells, the higher is the contribution of the short-living species, the larger is the spectral width at half height, as shown in Fig. 6 (see Fig. 4).

Increasing the irradiation time over 30 min, the number of cells exhibiting photomotile responses further decreased and a global impairment of cell viability was observed [18]. The cells showed a significant increase in the fluorescence intensity, resulting from the irreversible severe damages to the plasma membrane, and the photosensing network. In these condition, it was not possible to correlate the spectroscopic properties of pigments with their functional activity.

From these findings it is possible to conclude that UV-B irradiation in the above-described experimental conditions does not damage the photoreceptor structure and does not influence the early photosensing steps, but does spoil the transduction chain. From an ecological point of view, this means that exposure to UV-B radiation for relatively short times, of the order of 30 min, can render *B. japonicum* unable to react appropriately to dangerous light conditions, leading to cell death.

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