MICROSECOND DELAYED FLUORESCENCE OF PHOTOSYSTEM II OF PHOTOSYNTHESIS IN VARIOUS ALGAE: EMISSION SPECTRA AND UPHILL ENERGY TRANSFER

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1. Introduction

In 1951 Strehler and Arnold [1] discovered delayed fluorescence (for sake of simplicity we use in the following the term luminescence) of chlorophyll in the green alga *Chlorella pyrenoidosa*. The emission spectrum of the luminescence was found to be similar to that of (prompt) chlorophyll fluorescence of this alga [2]. The hypothesis given in [2] that luminescence is caused by the reversal of photosynthetic reactions is generally accepted and for a recent review of this so-called 'recombination'-hypothesis we refer to [3].

So far relatively few emission spectra of luminescence have been published. As far as we know spectra of luminescence components with decay times shorter than 100 μs have not been published. We will present spectra with decay kinetics down to 0.2 μs.

Very recently Van Best and Duysens [4] reported a luminescence component in *Chlorella vulgaris* with a lifetime of ~0.8 μs. This luminescence seems to originate from reaction centers illuminated in the 'closed' state $P680Q^-$, where P680 is the reduced primary donor and Q^- the reduced acceptor of Photosystem II (PS II). The emission spectrum of this luminescence component has been measured in addition to the luminescence spectra of components with lifetimes of 20 and 30 μs in the state P680Q in the absence and presence of hydroxylamine. In the Photosystem I (PS I) lacking mutant *Fl* 5 of *Chlamydomonas reinhardtii*, μs luminescence was present too and we have measured the spectrum.

The accessory pigments of blue-green and red algae, phycocyanins and allophycocyanins are contained in particles (phycobilisomes) outside the membrane. Chlorophyll *a* and the reaction center are located in the membrane. Many species also contain phycoerythrins which we do not consider here. Excitation energy is transferred 'down-hill' from phycocyanin via allophycocyanin to chlorophyll *a*. Murata [5] concluded from the DCMU*-induced difference spectrum of fluorescence and the ms luminescence emission spectrum of the blue-green algae *Anabaena variabilis* and *Anacystis nidulans* that excitons are transferred energetically uphill from chlorophyll *a* to (allo-)phycocyanin too. The μs luminescence spectra of *Cyanidium caldarium* and *Porphyridium cruentum* did not show a marked (allo-)phycocyanin contribution to the emission. However, in the case of *Anacystis nidulans* a clear μs emission in the wavelength region 620–665 nm was found. This indicates that uphill transfer from the reaction center chlorophyll via the antenna chlorophyll to the phycobilins depends on the detailed structure of the pigment system.

* DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea

2. Materials and methods

Chlorella vulgaris was grown as described in [6]. Phycocyanin-containing algae, *Cyanidium caldarium, Anacystis nidulans* and *Porphyridium cruentum* were cultured as described in [7–9], respectively. *Chlamydomonas reinhardtii Fl* 5 was grown according to [10]. For all algae the concentration was brought to an absorbance of 0.2/5 mm at 680 nm, which was corrected for scattering by subtracting the absorbance at 750 nm. The measurements were carried out at room temperature (~22°C) in a 30 × 30 × 5 mm^3 perspex cuvette.

DCMU (20 μM) or hydroxylamine (1 mM) were added in the dark about 15 min prior to the measurements. The samples were renewed repeatedly in order to prevent anaerobiosis in the closed cuvette.

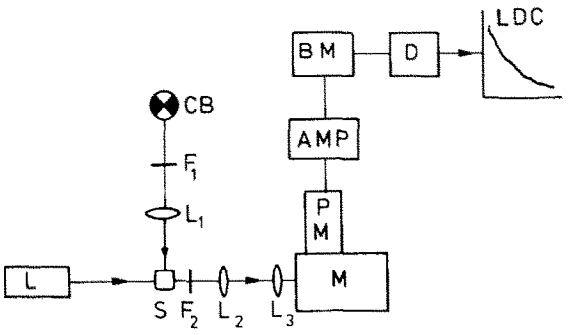

Fig.1. Scheme of apparatus used for luminescence and fluorescence emission measurements. L, YAG-laser JK ($t_{1/2}$ = 15 ns, λ = 530 nm); CB, lamp for continuous blue light illumination; S, sample; L_1, L_2, L_3, lenses; F_1, filter Balzers K1; F_2, filter Schott KV 550/3; M, Bausch and Lomb monochromator (slitwidth = 5 nm); PM, Philips XP 1002 photomultiplier (S-20 cathode) which could be gated during the laser flash; AMP, Tektronix 7A13 oscilloscope preamplifier; BM, Biomation 8100 transient digitizer (maximal speed 10 ns/channel with 2048 8-bit channels); D, DEC 11/45 computer; LDC, luminescence decay curve.

A schematic diagram of the apparatus is shown in fig.1. The algae were illuminated by repetitive flashes (1 Hz) of a frequency doubled Nd^+-YAG-laser ($t_{1/2}$ = 15 ns and λ = 530 nm). The laser-induced emission passed through a filter Schott KV550/3, which cuts off the laser beam without producing luminescence itself, and a Bausch and Lomb monochromator (slitwidth 5 nm) to an XP 1002 Philips photomultiplier (S20 type cathode). The PM was gated to about 200 ns after the flash in order to protect the cathode against the high intensity fluorescence of the algae during the laser flash (see [11] for details). The anode of the PM was connected to a 7A13 Tektronix oscilloscope preamplifier by a cable terminated with 50 Ohm. After amplification the signal was stored in a Biomation 8100 transient recorder and further processed by a DEC 11/45 computer. Continuous blue background illumination up to 5 ms before the laser flash was given by a tungsten iodine lamp (24 V, 250 W).

The luminescence signals of the algae were corrected for 'false' luminescence from optical components, which was less than 10% of the total signal, and for an electronic perturbation by a gate voltage present during the first 2 μs after the laser flash, by taking the difference of the average of $N \leq 32$ signals from a cuvette filled with algae and that of N signals from the same cuvette with only culture medium. The luminescence decay kinetics could be represented by one or two exponential components. For each particular component the luminescence emission spectrum was determined.

For reasons of comparison fluorescence spectra were recorded on the same apparatus by measuring the integrated fluorescence emission of the algae during the laser flash of very low intensity ($\leq$0.1 μJ/cm^2). The emission spectra presented below are corrected for the spectral response of the apparatus by means of a calibrated tungsten ribbon filament lamp of which the relative spectral emission was known as a function of band temperature. The spectra are plotted in arbitrary energy/s units per wavelength interval.

3. Results

Fig.2 shows typical luminescence decay curves of *Chlorella* without additions (decay times τ_1 = 0.8 μs and $\tau_2 \simeq$ 20 μs), in the presence of DCMU and with

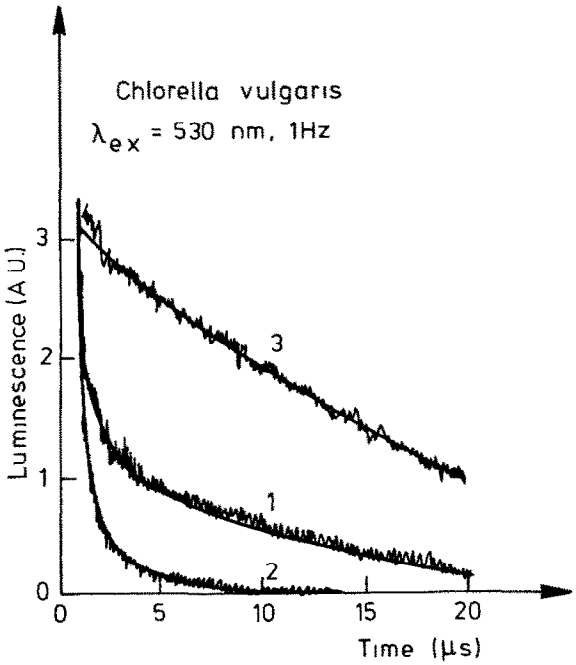

Fig.2. Typical luminescence decay kinetics of aerobic *Chlorella vulgaris* in a series (1 Hz) of oversaturating laser flashes ($t_{1/2}$ = 15 ns and λ = 530 nm); τ is the time for a luminescence component to decay to 1/e; (1), no additions, $\tau_1 \simeq$ 0.8 μs and $\tau_2 \simeq$ 20 μs; (2), +20 μM DCMU, $\tau \simeq$ 0.8 μs and (3), +1 mM hydroxylamine, $\tau \simeq$ 30 μs.

continuous blue background illumination so that all reaction centers were in the state $P680Q^-$ ($\tau \simeq 0.8$ μs and a small contribution of a slow luminescence component), and in the presence of hydroxylamine ($\tau \simeq 30$ μs). The smooth lines are the exponential fits of the noisy experimental curves.

The results obtained with the mutant *Fl* 5 of *Chlamydomonas*, which lacks PS I, were very similar to those for *Chlorella*. The decay kinetics of luminescence were similar to those of curve 1 in fig.2 and showed 0.8 and 20 μs components. Upon repetitive laser flashes the kinetics rapidly changed into those of curve 2 in fig.2. We attribute this effect to the accumulation of Q^-. This accumulation is probably due to the lack of PS I so that Q^- is not reoxidized efficiently during a series of flashes.

We carefully checked that the emission was indeed luminescence and not in some way caused by fluorescence. To show this we boiled *Chlorella* for 5 minutes which abolished almost all luminescence in the microsecond range but not the fluorescence. Moreover the luminescence of a cuvette with chlorophyll *a* in ethanol of about the same concentration as in vivo was negligible. Further the influence of DCMU and hydroxylamine in fact shows that we did not measure some effect of fluorescence because these additions did not change fluorescence emission essentially but altered drastically the luminescence kinetics and amplitude.

The emission spectra of fluorescence and microsecond luminescence components of *Chlorella* obtained under various conditions are shown in fig.3. The wavelengths of the peaks shown in this figure are close to 685 nm and all spectra show a more or less pronounced secondary maximum in the wavelength region 720–750 nm. The microsecond luminescence spectrum of the mutant *Chlamydomonas Fl* 5 was found within the rather wide limits of precision to be the same as for *Chlorella* in this figure.

In fig.4 the emission spectra of luminescence in the μs range of several phycocyanin containing algae are presented. The fluorescence spectra shown in this figure are composed of the emission of (allo-)phycocyanin with bands between 645 and 665 nm and of chlorophyll *a* between 680 and 683 nm. It is possible

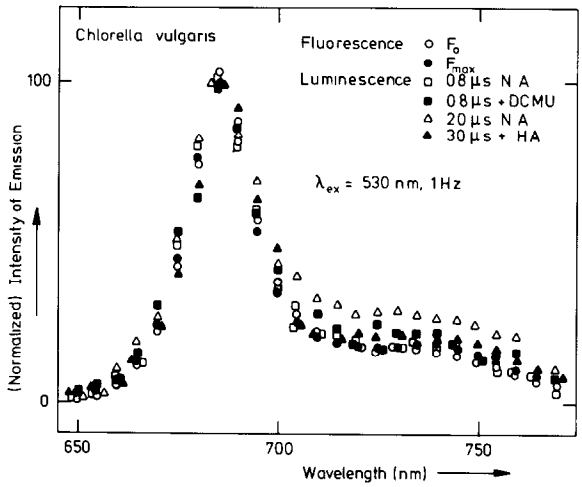

Fig.3. Emission spectra of fluorescence and luminescence of aerobic *Chlorella vulgaris* in a series (1 Hz) of undersaturating and oversaturating laser flashes ($t_{1/2}$ = 15 ns and λ = 530 nm), respectively. F_o is the fluorescence in the state P680Q (open reaction centers) and F_{max} for all centers in the state $P680Q^-$. Luminescence spectra are given for different components with lifetimes and conditions as indicated; NA stands for no additions and HA for hydroxylamine. The points, equally spaced by 5 nm, are obtained from the original points by normalizing the peak at 685 nm to (about) 100.

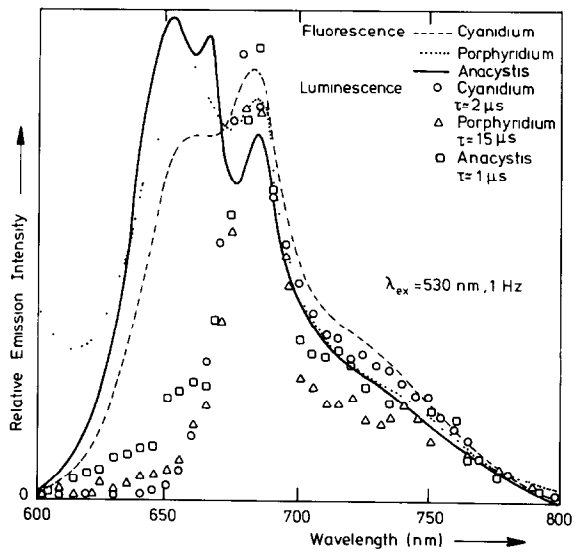

Fig.4. Emission spectra of fluorescence and luminescence of different algae in a series (1 Hz) of non- and oversaturating laser flashes (λ = 530 nm and $t_{1/2}$ = 15 ns), respectively. The fluorescence spectra have been measured in the condition for open reaction centers; the curves are obtained by drawing a smoothed line through the original points. The luminescence spectra are given for components with lifetimes τ as indicated and the peaks at 680–685 nm are normalized to about equal intensities.

that the latter band contains some contribution of allophycocyanin B, which has a band at about 680 nm. We observed, however, that in the three species investigated 3 mM *m*-dinitrobenzene quenched the hump around 680 nm almost completely, without affecting markedly the phycocyanin fluorescence. Thus even if allophycocyanin B contributes to the 680 band, it is in excitation equilibrium with chlorophyll *a*. It can be observed that of the luminescence spectra only the spectrum of *Anacystis* contains a distinct contribution of (allo-)phycocyanin in the wavelength region 620–665 nm.

4. Discussion

It has been assumed that the chlorophyll *a* of PS II is the source also for the μs luminescence. However, no spectra had been determined. The strong similarity of the fluorescence and luminescence spectra displayed in fig.3 shows that the fluorescence and μs luminescence are both emitted by chlorophyll *a*. The characteristic effect of hydroxylamine and DCMU on the luminescence (see also [4]) provides evidence that PS II is the source of luminescence. Further Van Best [11] reported a 20 μs component in the fluorescence induction kinetics of *Chlorella* after a short saturating laser flash. This component was probably due to fluorescence quenching by the oxidized primary donor $P680^+$ and may be correlated with the 20 μs component of the luminescence decay kinetics which was found under the same conditions. The results obtained with the mutant *Fl* 5 of *Chlamydomonas* also strongly suggest that PS II is the source of the μs luminescence. This PS I lacking mutant showed almost the same luminescence as found for *Chlorella*. Thus we can conclude that mainly if not only PS II is involved in the 0.8, 20 and 30 μs components of luminescence.

In the past a number of authors suggested heterogeneity of PS II. Such a heterogeneity is also suggested by the differences in the spectra of fig.3 around 730 nm. In general the ratio of intensities at the main peak (685 nm) and the secondary peak (≃ 730 nm) is higher for fluorescence than for luminescence. Because of the rather high noise level for the luminescence at 730 nm the differences may not be significant for all spectra. However, it is significant for the 20 μs luminescence at 730 nm which is relatively higher than the fluorescence at 730 nm.

The occurrence of (allo-)phycocyanin emission around 650 nm in the μs luminescence spectrum of *Anacystis* (see fig.4) shows that excitation energy originating from the reaction center chlorophyll is transferred energetically uphill. This is to be expected for physical reasons. Let C* and A* be numbers of excited chlorophyll *a* and (allo-)phycocyanin molecules of PS II, k^C and k^A be the rate constants for the de-excitation (fluorescence, internal conversion, trapping, but not energy transfer) of C* and A* and k_u and k_d be the rate constants for uphill energy transfer from C* to A and downhill transfer from A* to C. Then the time dependence of A* is given by

$$dA^*/dt = -k^A\,A^* - k_d\,A^* + k_u\,C^* \qquad (1)$$

In the steady state (one may think of continuous generation of excitations at the reaction center) $dA^*/dt = 0$ and it follows that

$$A^*/C^* = k_u/(k_d + k^A) \qquad (2)$$

If the low fluorescence yield of the (allo-)phycocyanin in vivo (compared to that in isolated phycobilisomes) is mainly caused by energy transfer to chlorophyll *a*, then k^A in eqn. 2 must be small compared to k_d. Then the ratio of fotonintensities emitted in two narrow equal wavelength bands at the maxima 660 and 683 nm of allophycocyanin and chlorophyll is given by

$$A^*\,k_f^A/(C^*\,k_f^C) = k_f^A\,k_u/k_f^C\,k_d \qquad (3)$$

where the k_f's are the frequencies of emissions in the wavelength intervals under consideration. The Boltzmann distribution law implies

$$k_u/k_d = (N_A/N_C)\exp(-\Delta E/kT) \qquad (4)$$

where N_A and N_C are the numbers of A and C molecules. From eqns. 3 and 4 it follows that the ratios of luminescence contributions of A* and C* at 660 and 683 nm

$$L(660)/L(683) = (k_f^A\,N_A/k_f^C\,N_C)\exp(-\Delta E/kT) \qquad (5)$$

From the luminescence spectrum of *Anacystis* using the emission spectra of isolated phycobilisomes [12] and of chlorophyll *a* (fig.3) we estimate L(660)/L(683) ≃ 0.2 in reasonably good agreement with the value of about 0.15 for the ratio of the corresponding

fluorescence intensities from the DCMU-induced fluorescence difference spectrum in the same species [13]. The Boltzmann factor (for T = 300 K) in eqn. 5 then is ~0.07. If it is assumed that $k_f^A \simeq k_f^C$, then $N_A/N_C \simeq 3$. From action spectra a value of about 4 for the ratio N_A/N_C was found [14].

The observation from fig.4 that the 650 nm luminescence is relatively low in *Porphyridium* and *Cyanidium* indicates that the ratio of the number of (allo-)phycocyanin and the number of chlorophyll molecules between which energy transfer occurs is higher in *Anacystis* than in the latter species, and/or that in these species the luminescence emitted by chlorophyll *a* not in energy exchange with phycobilins is relatively high. For *Cyanidium* it was indeed found that half of the reaction centers of PS II were not coupled with the phycobilisomes [15].

The fact that excitation energy generated at the reaction center by backreactions is emitted by (allo-)phycocyanin in *Anacystis* shows that this excitation is not restricted to the reaction center or to chlorophyll *a* molecules in its vicinity, but in this species can reach antenna molecules of PS II including allophycocyanin. This conclusion is consistent with the conclusion [16] from different experiments that excitation energy repeatedly passes through the reaction center in the open state P680Q and through the whole pigment system before finally being trapped.

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