Biochimica et Biophysica Acta, 593 (1980) 136—148

BBA 47943

PROMPT AND DELAYED FLUORESCENCE OF CHLOROPLASTS UPON MIXING WITH DICHLOROPHENYLDIMETHYLUREA

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(Received February 5th, 1980)
(Revised manuscript received June 16th, 1980)

Key words: Photosystem II; Fluorescence yield; Dichlorophenyldimethylurea; Electron transfer; Luminescence

Summary

1. The kinetics of prompt and delayed fluorescence of isolated chloroplasts or algae have been monitored after flash preillumination (in the time-range extending from 0.4 s after the flash). A rapid mixing with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) may take place after the last flash.

2. 1 s after the mixing with DCMU, the prompt fluorescence displays binary oscillations with the number of preilluminating flashes, similarly to the observation of Velthuys, B.R. and Amesz, J. ((1974) Biochim. Biophys. Acta 333, 85—94), with chloroplasts to which an artificial Photosystem II donor was added. These oscillations are due to a back-transfer of electrons from the secondary acceptor, B, to the primary acceptor, Q, caused by DCMU. At longer times after the mixing, charge recombination takes place to a variable extent according to the charge storage state S_i on the donor side, yielding the oscillatory pattern observed by Wollman, F.A. ((1978) Biochim. Biophys. Acta 503, 263—273).

3. Shifting the pH from 6 to 8 causes an acceleration of the DCMU-induced back-transfer to Q and an about 2-fold increase in the amplitude of the fluorescence oscillations. The rate of the DCMU-induced rise of fluorescence is sensitive to the pH during the mixing, whereas the amplitude of the oscillations depends on the pH during the preillumination. Even under optimal conditions, the oscillations account only for a fraction of the total variable fluorescence.

4. The delayed light emitted by isolated chloroplasts in the 100 ms—seconds range oscillates weakly (periodicity 4) with the number of preilluminating

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS II, Photosystem II; Φ, Fluorescence yield; L, luminescence (= delayed fluorescence/light); Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

flashes. Mixing with DCMU after the preillumination causes a delayed light stimulation which varies with the flash number. The enhancement factor oscillates with periodicities of both 2 and 4. The amplitude of the period-2 contribution varies with the amount of B oxidized in the dark, while that of the period-4 contribution depends on the extent of this type of oscillation in the control experiment.

5. The period-2 oscillations of the DCMU-stimulation of delayed light behave similarly to the fluorescence oscillations. It is shown that they are not due to a modulation of luminescence by the fluorescence yield, but rather to the variations of the amount of Q^- as a substrate.

6. It is concluded that in the absence of DCMU, the reduced secondary acceptor B^- is not the main source of electrons involved in radiative recombination of functional centers in the time-range we have studied. Possible models are discussed.

Introduction

The existence of a secondary acceptor B of chloroplast Photosystem II (R in the terminology of Velthuys and Amesz [1]) was revealed through experiments showing that electrons are transferred in pairs to the plastoquinone pool, whereas the primary acceptor Q is a one-electron carrier. Bouges-Bocquet [2] studied the pairwise arrival of reductants to Photosystem I during flash illumination. Velthuys and Amesz [1] showed binary oscillations of a dark DCMU-induced fluorescence rise following flash preillumination in chloroplasts to which an artificial PS II electron donor (hydroxylamine) had been added.

The model invoked by these authors may be summarized as follows: starting from Q and B oxidized, the first flash results in a one electron retention on B:

$$QB \xrightarrow{h\nu} Q^-B \rightarrow QB^-$$

After the second flash, the pair of reduced equivalents are transferred to the plastoquinone (PQ) pool, restoring the QB state:

$$QB^- \xrightarrow{h\nu} Q^-B^- \rightarrow QB^{2-},$$

and:

$$QB^{2-} + PQ \rightarrow QB + PQ^{2-}$$

(where the protonation steps of reduced quinones have been omitted for the sake of simplicity).

The interpretation of the experiments of Velthuys is that mixing with DCMU causes a back-transfer of electrons from B^- to Q, presumably because of a negative shift of the B/B^- couple midpoint potential due to DCMU binding:

$$QB^- \xrightarrow{DCMU} Q^-B$$

Consequently the yield of fluorescence, which is mainly controlled by the redox state of Q, rises after DCMU addition in a way which reflects the amount of QB^- centers present before the addition. Velthuys could only observe this

phenomenon in chloroplasts in which the oxygen-evolving system was damaged and to which electrons were provided by an exogenous donor such as *p*-phenylenediamine or hydroxylamine. The likely reason for this limitation was that Q^- undergoes a reoxidation by charge recombination in the intact system. Evidence for this process was brought by Wollman [3] who studied the period-4 oscillations of the prompt fluorescence yield at long times after mixing with DCMU and showed that they could be explained by assuming a recombination of Q^- with states S_2 and S_3 [4] of the oxygen evolving apparatus. Wollman further showed that the results obtained with *Chlorella* cells were consistent, too, with these assumptions and that more than half of the PS II centers in algae are in the QB^- state when dark-adapted. Dark oxidation of B^- in *Chlorella* cells was shown to take place with benzoquinone addition.

In this paper we confirm these points by continuously monitoring the fluorescence yield after a rapid mixing with DCMU following flash preillumination. At about 1 s after the mixing binary oscillations of the Velthuys type are observed, followed by recombination kinetics which lead to oscillations of the Wollman type.

Another aim of this study was to investigate the role of B^- as an electron source to radiative recombination. In the time range extending from a few milliseconds to several seconds after a flash the reoxidation of Q^- by B has been completed, so that the likely reduced substrate for delayed light is B^- (whereas the charge storing device of the oxygen evolving apparatus provides for the likely oxidized substrate). We shall show that a binary contribution to delayed light oscillations is observed after mixing with DCMU but not in its absence, which suggests that, contrary to our expectation, B^- is not the main source of reduced equivalents in the delayed light reaction.

Material and Methods

Broken spinach chloroplasts were prepared by grinding the leaves in a 0.3 M NaCl/5 mM $MgCl_2$/50 mM Tricine (pH 7.5) medium containing ascorbate and bovine serum albumin. After filtration and slow speed centrifugation the chloroplasts were pelleted and washed in a Sorbitol (0.4 M), NaCl (10 mM), $MgCl_2$ (5 mM), Tricine (50 mM, pH 7.5) medium. Final resuspension was made in the same medium which was further used (except for different buffers when mentioned) for diluting the chloroplasts to a concentration of 20 μg/ml total chlorophyll for the prompt and delayed fluorescence experiments. *Chlorella* cells, grown and harvested daily as previously described [5], were diluted with distilled water to the same chlorophyll concentration as chloroplasts.

The experiments were made in an apparatus already described [5]. A sample of suspension flows to a preillumination chamber were it is submitted to the desired number of short saturating flashes (General Radio 'Strobotac', 2 μs duration at half peak, energy 0.1 J) spaced 0.5 s apart. During another flow step (0.4 s duration) the sample is mixed with an equal volume of medium, containing DCMU, if present, at a concentration of $2 \cdot 10^{-4}$ M (such a high concentration is required to obtain a rapid binding of DCMU [5]). It stops again in an observation chamber from which delayed light is observed with a EMI 9558 B photomultiplier. The anode current is fed into a nanoamperometer

(Lemouzy) and displayed as an oscilloscope trace.

The same attachment is used for prompt fluorescence monitoring, with a weak modulated light for excitation (two green light emitting diodes, Hewlett Packard 5082/4958 modulation rate 1 kHz) filtered through a 4-96 Corning filter (a complementary red 2-64 Corning filter and Wratten filter are mounted on the photomultiplier window). The modulated fluorescence signal is processed by a PAR lock-in amplifier and the kinetics recorded with the oscilloscope. The actinic effect of the fluorescence excitation light was such as to elicit a fluorescence induction rise from a DCMU + hydroxylamine-treated sample with $t_{1/2} = 140$ s.

Results

Fluorescence

The kinetics of the DCMU-induced fluorescence rise of chloroplasts after preillumination with 0—4 flashes are shown in Fig. 1. A binary oscillatory pattern is observed about 1 s after the mixing. Maxima occur on uneven flashes, which shows that a majority of centers have B oxidized in the dark-adapted state. Adding 50 μM ferricyanide decreases or cancels the DCMU-induced rise on the dark adapted sample, showing that ferricyanide oxidizes B^-. The same result is obtained with $2 \cdot 10^{-4}$ M benzoquinone, although some quenching of fluorescence may then be observed. B remains oxidized in the dark when the incubation with ferricyanide or benzoquinone is followed by a washing of the chloroplasts, similarly to Wollman's observation [3] in the case of algae treated with benzoquinone.

The DCMU-induced rise is followed by decay kinetics (except with the non-preilluminated sample), with $t_{1/2} \simeq 3$—5 s. The extent of this decay varies with the flash number: it is large after 1 flash, small after 3 flashes. This is consistent with the idea that states S_2 and S_3 of PS II donor side are able to recombine with Q^-, contrary to states S_1 and S_0. In the dark-adapted state the centers are in states S_1 (about 75%) and S_0 (25%). No recombination will occur in this case, whereas after 1 and 2 flashes the centers are mostly in the recombining

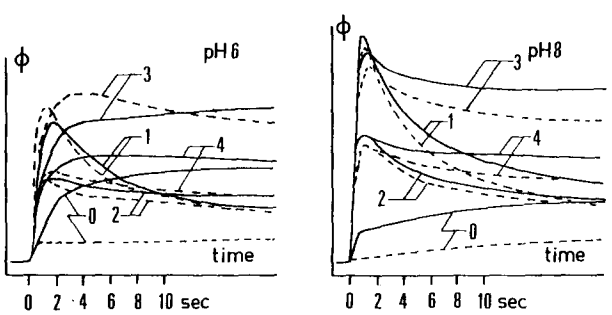

Fig. 1. DCMU-induced fluorescence rise of chloroplasts, at pH 6 (50 mM Mes), and 8 (50 mM Tricine). Preillumination by 0 to 4 flashes as indicated. A 400 ms flow period takes place after the last flash in order to mix the sample with an equal volume of DCMU solution ($2 \cdot 10^{-4}$ M) and to bring it into the observation chamber. The kinetics start at about 0.4 s after the last flash. The amplitude of the 1-flash peak at pH 8 accounts for about one third of the total variable fluorescence. Dashed curves: chloroplasts were incubated for 5 min with 50 μM ferricyanide.

states S_2 and S_3. After 3 flashes a majority of centers have evolved oxygen and returned to the S_0 state: a small extent of recombination is thus observed. The oscillatory pattern a few tens of seconds after the mixing is similar to that published by Wollman [3], with a periodicity of 4 and a maximum after 3 flashes.

Basically, the same observations remain true with algae. One difference is that slightly more than half the centers have B reduced in the dark-adapted state. These centers may be oxidized by benzoquinone.

The pH of the medium controls several features of the phenomena that have been described. Fig. 1 shows typical result at pH 6 and pH 8. It can be seen that:

(i) The amplitudes of the fluorescence transients are larger at pH 8 (by a factor which is generally about 2). This is not due to a gross change of the fluorescence yield determined by the pH: the Φ_0 level (base level) is almost the same at both pH values, and the maximum variable fluorescence $\Delta\Phi$ is slightly smaller at pH 8. Moreover, the enhancement of fluorescence oscillations at pH 8 still takes place in the presence of an oxidant ensuring total dark oxidation of B at both pH values so that the phenomenon is not simply a consequence of a change in the dark-adapted redox state of B (see below).

(ii) The dark-adapted state of B is more reduced at pH 6 as can be seen by comparing the zero flash kinetics with and without ferricyanide at both pH values.

(iii) The rate of the DCMU-induced rise of Φ is about 3-times faster at pH 8 (0.2—0.3 s) compared to pH 6 (0.7 s).

(iv) The rate of the reoxidation kinetics of Q^- varies with the pH. A maximum is observed at about pH 6.5 and the rate decreases at higher or lower pH.

The titration of the pH dependence described in (i) revealed a midpoint close to pH 6.8. A question one may ask is whether the phenomenon is due to a pH dependence of the DCMU action in transferring electrons from B^- to Q, or is more deeply related to the functioning of PS II. An experiment which gives support to the latter hypothesis is the following: the chloroplasts are incubated and preilluminated at pH 6 (with a low buffer concentration) and mixed with a DCMU solution at pH 8 (high buffer concentration, so that the resulting pH is 8). The amplitude of the fluorescence kinetics is then the same than at pH 6, whereas the rate of the DCMU-induced rise is fast, typical of pH 8 (see point (iii) above). It thus seems that the effect on the amplitude is controlled by the pH during the preillumination and not by the pH during the DCMU action.

Luminescence

1. Oscillating pattern. Barbieri et al. [6] were the first to study delayed light emission after a series of preilluminating flashes. They showed that in *Chlorella*, the delayed light amplitude oscillates strongly according to the number of flashes, with a periodicity of 4 revealing the involvement of the charge accumulation process on PS II donor side. The maxima occur on the 2nd, 6th . . . flashes, and minima on the 4th, 8th . . . flashes. An analysis of these results led the authors to conclude that delayed light originates from centers in states S_2 and S_3. Similar experiments were later undertaken by Zankel [7] who monitored delayed light at shorter times after the last preilluminating flash. Period-4

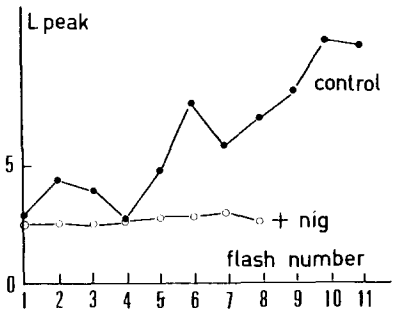

Fig. 2. Delayed light oscillations after flashes preillumination in chloroplasts (50 mM Tricine, pH 7.5). L peak (arbitrary units) is the peak of the delayed light kinetics as shown in Fig. 3 (solid lines). Upper curve: chloroplasts without addition, mixing with the same buffer; lower curve: $1 \cdot 10^{-6}$ M nigericin was added to the chloroplasts 5 min before running the experiment.

oscillations were again observed, with maxima on the 3rd, 7th ... flashes. Zankel observed these oscillations in chloroplasts and algae, and further confirmed that the first type of luminescence oscillation (maxima after 2, 6 ... flashes) occurs as soon as about 10 ms after the flash in *Chlorella*. The likely explanation for this shift in flash number dependency is that state S_4 is a better luminescence substrate than states S_3 or S_2, and controls the emission as long as it has not completely decayed through the oxygen-evolving reaction.

In isolated chloroplasts, the oscillatory behaviour of delayed light in the 10 ms—seconds time-range is much less pronounced than in algae, as was noted in [6]. The overall signal is weaker and the oscillations are generally little contrasted or even absent. We observed (Fig. 2) that they can be completely suppressed (in the 100 ms—seconds time-range) by the addition of the uncoupler nigericin which allows exchange of H^+ and K^+ across the thylakoid membrane. Inversely, imposing a proton gradient (ΔpH) across the membrane by an injection of alkaline buffer enhances the delayed light and restores contrasted oscillations similar to those of algae (see Ref. 8 for a similar observation in the case of sodium benzoate-stimulated luminescence). The rising pattern of the control experiment shown in Fig. 2 is probably due to the build-up of a flash-induced ΔpH, for it is cancelled by nigericin. No similar rise of the fluorescence yield is observed under the same conditions.

These observations are close to those reported recently by Bowes and Crofts [9] that a photo-induced ΔpH (obtained through a cyclic electron flow around Photosystem I in a phosphorscope experiment with DCMU present) not only stimulates the millisecond-delayed emission but increases the contrast of the oscillations (of the Zankel type in these experiments).

The different behavior of algae and isolated chloroplasts with respect to the oscillations of the slow phase of luminescence may thus be satisfactorily explained by the recent finding of Joliot and Joliot [10] that algae mantain a ΔpH in the dark. We observed that treating algae with $2 \cdot 10^{-4}$ M benzoquinone causes a significant decrease in delayed light intensity and damping of the oscillations, very similar to that which is obtained with isolated chloroplasts: this suggests that the benzoquinone treatment, which is known to allow permeation of species which otherwise would not penetrate the chloroplast of intact

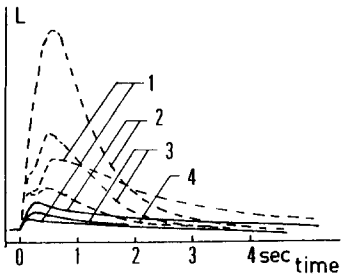

Fig. 3. Kinetics of delayed light after flashes preillumination in chloroplasts (50 mM Mes, pH 6). Solid lines: control (1—3 flashes). Dashed lines: mixing with DCMU (final concentration, $1 \cdot 10^{-4}$ M).

algae, somehow results in a collapse of the dark ΔpH.

As with the ΔpH, we observed that a transmembrane electric field (KCl injection in the presence of valinomycin) causes an enhanced and oscillating luminescence emission in chloroplasts.

2. Effect of mixing with DCMU. Mixing with DCMU after the last preilluminating flash causes an enhancement of delayed light, roughly contemporary of the fluorescence transient under the same conditions (Fig. 3).

The amplitude of the delayed light transient after DCMU mixing varies with the number of preilluminating flashes. The oscillating pattern obtained may be described as follows: whenever the dark state of B is not too far from the equipartition B/B^-, (e.g. algae, or chloroplasts at pH 6), large period-4 oscillations are

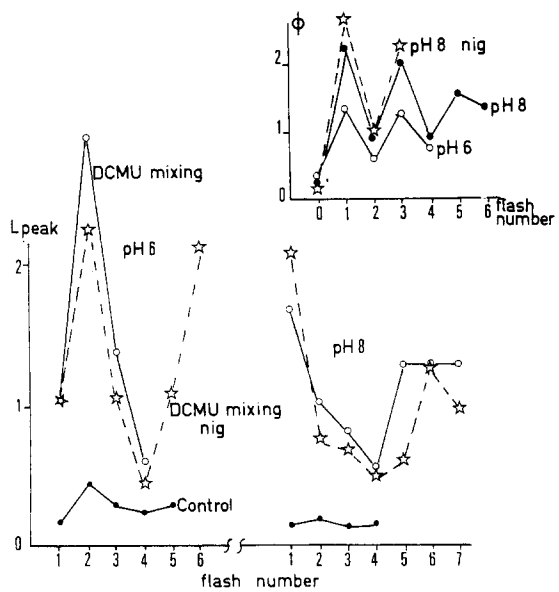

Fig. 4. Delayed light oscillations after flash preillumination in chloroplasts at pH 6 and 8. The buffers are: 50 mM Mes at pH 6, and 50 mM Tricine at pH 8. ●———●, control; ○———○, mixing with DCMU (final concentration, $1 \cdot 10^{-4}$ M); ☆- - - - -☆, mixing with DCMU after 5 min incubation of the chloroplasts with $1 \cdot 10^{-6}$ M nigericin. Insert: fluorescence oscillations with the same chloroplasts, after mixing with DCMU. The fluorescence levels are measured 1 s after the mixing, on traces similar to those shown in Fig. 1.

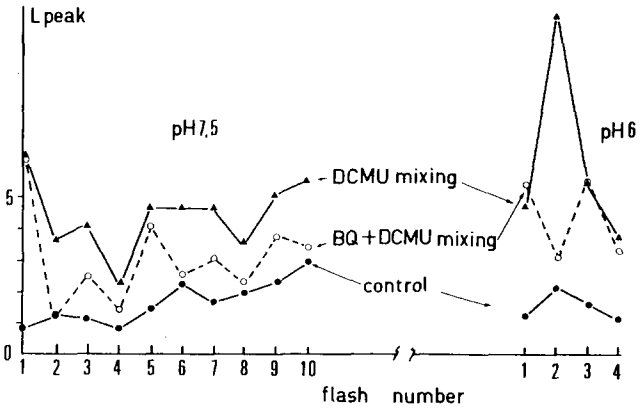

Fig. 5. Delayed light oscillations after flash preillumination in chloroplasts at pH 7.5 and 6 (different batches of chloroplasts were used). The buffers are: 50 mM Tricine at pH 7.5 and 50 mM Mes at pH 6. ●——●, control; ▲——▲, mixing with DCMU (final concentration 10^{-4} M); ○- - - - -○, mixing with DCMU after 5 min incubation of the chloroplasts with $2 \cdot 10^{-4}$ M benzoquinone.

observed (maxima after 2—6 flashes) as shown in Figs. 4 and 5 for chloroplasts at pH 6. These oscillations are, as a first approximation, similar to those obtained with ΔpH stimulation. However, when B is more oxidized in the dark (e.g. chloroplasts at pH above 7, algae or chloroplasts treated with benzoquinone), a distortion corresponding to a period 2 contribution appears, which increases delayed light after flashes 1, 3, 5 ... and decreases it after flashes 2, 4, 6 .... This can be seen in Figs. 4 and 5 for chloroplasts at pH 8 or 7.5. If chloroplasts are incubated with benzoquinone (or ferricyanide) which completely oxidizes B in the dark, at pH 8 (or 7.5) the period-2 contribution becomes more contrasted (Fig. 5), and appears markedly for chloroplasts at pH 6 (Fig. 5). Binary oscillations of the DCMU-stimulated luminescence are observed in algae as well, after preincubation with benzoquinone. Another effect of benzoquinone in algae is to slow down markedly the decay of the DCMU-stimulated luminescence, together with a decrease of its amplitude.

The addition of nigericin modifies only slightly the oscillating pattern observed in chloroplasts upon mixing with DCMU (Fig. 4) whereas the oscillations of the control are completely cancelled (Fig. 2). One can define a DCMU enhancement factor of delayed light as the ratio of the peak of DCMU-stimulated delayed light to that of the control without DCMU mixing. When nigericin is present the delayed light sequence of the control is flat and the DCMU enhancement factor oscillates similarly to the DCMU-stimulated sequence, with usually a dominant period-4 contribution combined with a period-2 contribution more-or-less pronounced depending on the initial redox state of B. Without nigericin the control sequence generally already displays (weak) period-4 oscillations so that the corresponding contribution in the DCMU enhancement factor pattern is attenuated. It is possible to cancel completely this period-4 component in the enhancement factor oscillations by running the experiment with chloroplasts submitted to a ΔpH. In this case the control is already fully oscillating with a periodicity of 4, and the DCMU stimulation is now purely binary. Fig. 6 shows the result of such an experiment where chloroplasts

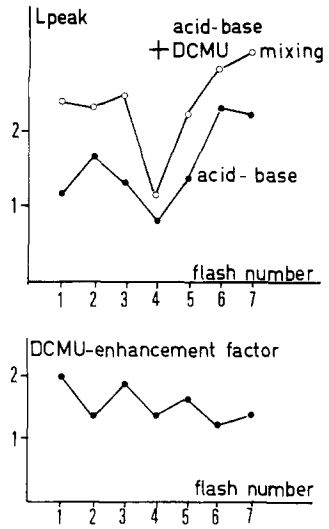

Fig. 6. Delayed light oscillations of chloroplasts submitted to a ΔpH. Top: same representation as in Figs. 4 and 5. Lower curve: the chloroplasts are incubated at pH 6 (5 mM Mes) and mixed with Tricine (100 mM, pH 8) after flash preillumination. The L peak levels are about 10-times higher than in a control experiment with a mixing medium at pH 6. Upper curve: the mixing medium contains Tricine (100 mM, pH 8) and DCMU $2 \cdot 10^{-4}$ M. Bottom: the DCMU enhancement factor is the ratio of the values plotted above for L peak with and without DCMU mixing.

(which have undergone a benzoquinone treatment in order to oxidize completely B) were incubated and preilluminated at pH 6, then mixed with concentrated buffer at pH 8 with or without DCMU. The lower part of the figure is a plot of the enhancement factor which oscillates period-2 without period-4 contribution.

Comparison of the delayed light results in chloroplasts at pH 6 and 8 (see Fig. 4) may be summarized as follows:

(i) The luminescence intensity (particularly that of the control) is higher at pH 6, and period-4 oscillations are more pronounced.

(ii) The binary contribution to the oscillations of the DCMU-stimulated luminescence is more readily visible at pH 8 (when no oxidant is added). As suggested above, this is probably due to the difference already noticed in the fluorescence section (see Fig. 4, insert): at pH 8, B is more oxidized in the dark, and besides, the amount of quencher involved in the DCMU-induced fluorescence rise is greater.

Discussion

1. The amplitude of the DCMU-induced fluorescence oscillations

It has been noticed by Wollman [3] that only a fraction of the variable fluorescence is involved in the DCMU-induced fluorescence oscillations. For instance, even when B is completely oxidized in the dark, the fluorescence rise after one flash and mixing with DCMU is not greater than about half the maximum variable fluorescence. It is well known that the fluorescence yield levels are not linearly correlated to the amounts of quencher (mainly because of exci-

tation transfer between PS II units), so that a more relevant way of estimating this quantity is to take the relative area bounded by the fluorescence induction curve under continuous illumination and its Φ_{max} asymptote. Using this type of procedure, Wollman estimates about 70% of the total amount of quencher to be destroyed by DCMU addition after one flash (100% B oxidized in the dark).

In our experiments, the amount of quencher involved under the same conditions is of the same order of magnitude at pH 8, about 70% of the total photochemical quenching in the presence of hydroxylamine, and about 50% without hydroxylamine. We have shown that this quantity depends on the pH, being about twice smaller at pH 6 than at pH 8. A likely explanation for this dependence would have been that DCMU reveals only a part of the QB^- centers, to an extent which is controlled by pH. However we showed that it is the pH during the preillumination but not during the DCMU action which controls the amount of back-transfer. It thus seems that we are dealing with a phenomenon which does not simply involve the inability of DCMU to reveal all the B^- present.

Discussing this problem, Wollman put forward two hypotheses: (i) There are two types of PS II center, one in which Q is linked to B and the other in which electron transfer after Q by-passes B; (ii) Q does not account for all the quenching due to PS II centers, and an auxiliary quencher such as that hypothesized by Joliot and Joliot [11] may be involved.

2. The problem of the substrates of the slow components of delayed emission

Donor side. The fact that in the 100 ms—seconds time-range, the amplitude of the flash-excited luminescence in chloroplasts oscillates poorly (period 4) or does not oscillate at all (in the presence of nigericin) raises a problem. For even without any specific assumption on the respective ability of states S_2 and S_3 to serve as oxidized substrates for delayed light, the stability of states S_0 and S_1 is well established, and contrasted period-4 oscillations are therefore expected whenever a normal functioning of PS II donor side takes place. On the other hand, the PS II origin of this non-oscillating luminescence is clearly established by its normal sensitivity to hydroxylamine. This emission may originate from PS II centers which have a deficient water-splitting system (possibly because of damage occurring during the isolation, although the fact that a poorly oscillating luminescence is observed in algae, too, upon benzoquinone treatment does not support a preparation artifact hypothesis), similarly to Tris- (or hydroxylamine-) washed chloroplasts. The hypothesis that the emission from centers unable to evolve oxygen may predominate does not imply that these centers are present in large amount, but may be explained by assuming that their intrinsic luminescence is rather high, and that, in this time-range, delayed light from functional centers is very weak in the absence of enhancement factor (ΔpH, electric field, DCMU).

The occurrence of a period-4 oscillating pattern upon stimulation by ΔpH or membrane potential suggests that these stimulations preferentially enhance luminescence from normal centers, possibly in the case of ΔpH because of protonation equilibria of the S system [9].

The rationale for luminescence stimulation upon mixing with DCMU is the back-transfer of electrons from B^- to Q, assuming, for example, that the activa-

tion energy for radiative recombination is smaller from Q^- than from B^- (or from the other electron source that we shall introduce later). As previously shown, the DCMU enhancement factor oscillates with both period 4 and 2. We shall deal in the next section with the binary component. As to the period-4 component, which is most prominent in the presence of uncouplers, (when the control pattern is 'flat'), we have to assume that the DCMU stimulation somehow selects for centers with a functional oxygen-evolving system. Possibly, the centers with a deficient S system which we have postulated above undergo a comparatively fast cyclic rcombination with B^-, so that most of the Q^- formed after DCMU-mixing is contributed by normal centers.

Acceptor side. Our working hypothesis was that, as soon as Q^- has been reoxidized after a flash ($t_{1/2}$ <1 ms), the likely electron source for radiative recombination was the charge storage system B. The pathway for this process being (dashed arrows):

$$SQB \underset{L}{\overset{h\nu}{\rightleftharpoons}} S^+Q^-B \rightleftharpoons S^+QB^-$$

Should this be true, the following consequences are to be expected: (i) a noticeable contribution of period-2 oscillations to the luminescence sequence as soon as the ratio B/B^- in the dark differs appreciably from 1; (ii) no further enhancement of this binary contribution upon mixing with DCMU (i.e. the enhancement factor should have no period-2 component). To explain this point, let us denote by K the equilibrium constant $[Q^-B]/[QB^-]$ and by N the number of centers which store an electron in the QB system after a given preillumination. The amount of recombining substrate is given by $[Q^-B] = KN/(K + 1)$, so that when DCMU is added, the intensity of the emission should be enhanced by a factor

$$\left(\frac{K}{K+1}\right)_{DCMU} \Big/ \left(\frac{K}{K+1}\right)_{control},$$

but still be controlled by N in the same way, and the oscillatory pattern should not change.

Experimentally, point (i) is not verified. No important contribution of binary oscillations appears in the absence of DCMU. The oscillatory pattern of algae or of chloroplasts submitted to a ΔpH is similar, although algae have a dark B^-/B ratio greater than 1 whereas that of chloroplasts is close to zero. We have shown that point (ii) is not verified, either. Binary oscillations of the DCMU-enhancement factor are easily observed and appear neatly when the period-4 contribution is eliminated (by imposing a ΔpH).

However, this argument might be questioned on the basis of the $L = \Phi_L \cdot J$ relationship. This expression was proposed by Lavorel [12] to take into account that delayed ligh excitons injected in the antenna at a rate J have a probability Φ_L to be re-emitted as photons. If these excitons have very little probability to jump out of their unit of origin, Φ_L will be a mere constant, whereas if they may freely spread over the antenna and visit neighboring units, Φ_L will vary like the fluorescence yield, depending on the amount of quenching centers present. If this is the case, then the binary oscillations of the DCMU-enhancement factor may plainly reflect the oscillations of the fluorescence yield under the same conditions and not a substrate effect as we have assumed.

We have two pieces of evidence to support the contention that we are indeed dealing with a substrate and not a yield effect.

(i) We ran the DCMU-mixing experiments with chloroplasts washed in a magnesium-free medium and with the same chloroplasts after adding 10 mM $MgCl_2$. The luminescence sequence was unchanged, whereas the Φ oscillations amplitude was doubled. The fluorescence induction of these chloroplasts was assayed and showed the usual magnesium effect: small $\Delta\Phi_{max}$ and quasi-exponential induction kinetics for the Mg-free sample, doubling of $\Delta\Phi_{max}$ and sigmoidal shape of the induction upon Mg addition. If a $L = \Phi J$ effect was involved, the addition of Mg^{2+} should have noticeably affected the luminescence results, not only because of the doubling of $\Delta\Phi$ but also because of the increase of exciton transfer between PS II units induced by Mg^{2+} (sigmoidal shape of the induction).

(ii) At pH 6, we have noticed that there is generally some B^- present in the dark (see, for example, Fig. 1). The DCMU-stimulated luminescence (Fig. 5) oscillates predominantly with a periodicity of 4. When an oxidant is added in the dark, the fluorescence kinetics are modified by a relatively small amount (Fig. 1), whereas the delayed light pattern is drastically changed (Fig. 5), displaying binary oscillations with maxima after 1, 3, 5 . . . flashes. This sensitivity of L to the amount of Q^- is more easily understood as a substrate effect.

We are thus led to conclude that, in the absence of DCMU, B^- is not the source, or at least not the main source, of electrons involved in radiative recombination in the time-range we have studied (whereas in the presence of DCMU, the electrons originate mainly from Q^-). This needs, however, some qualification: if the non-oscillating (nigericin-insensitive) delayed light originates from deficient centers which may be unfit for non-cyclic transfer, we have no evidence about the electron source for that component of luminescence. It is, rigorously, only for luminescence from functional centers identified through the period-4 oscillations that the involvement of another (main) sourve of electrons, that we shall denote Q′, is required. As mentioned previously, this type of luminescence is observed when a stimulation such as ΔpH or electric field (by mixing with K^+ in the presence of valinomycin, experiments not shown) is applied. Field stimulation yields period-4 oscillations similar (as a first approximation) to those induced through ΔpH, without significant period-2 contribution. Thus the involvement of Q′ does not rely on one particular type of stimulation. Two possibilities can be envisaged for Q′: either there are two types of center, (those of the Q-B type and those of the Q′ type), or Q′ is present in all centers in parallel with the Q-B system. In the latter kind of model we have to assume parallel independent acceptors rather than an equilibrium between Q′ and Q (for in this case the Q-Q′-B system would behave as a whole, which is not consistent with the fluorescence and luminescence data). This is close to the model proposed by Joliot and Joliot [11], where Q′ is reduced together with Q by double hitting on the first flash.

In the first kind of model with two types of center we have to face a contradictory requirement for the Q′ centers, which must be capable of non-cyclic transfer and still retain an electron, consistent with their role as luminescence substrates. One way to accomodate single electron transfer from Q′ with electron retention on Q′ would be that it acts as a single electron carrier in the

form of the Q'^{-}/Q'^{2-} couple, with the initial reduction of Q' to Q'^{-} occurring on the first flash. Q' might then be a secondary acceptor (connected to a normal primary acceptor Q), possibly a modified form of B.

To account for the data presented in this paper, we have been led to introduce inhomogeneities of the centers (or duality of acceptors within the centers) in three different instances:

(i) The existence of the non-oscillating (period 4) luminescence (nigericin insensitive) suggests that, in the absence of stimulation, the main oxidized substrate of delayed light (in the seconds-range) is not the S system. This may be due, but not necessarily, to centers with a deficient S system.

(ii) The DCMU-induced fluorescence oscillations (period 2) account only for a part of the total photochemical quenching.

(iii) The delayed light in the seconds time-range involves electrons from another source than the Q-B system (at least when a ΔpH or field stimulation is applied).

The first problem, which possibly reveals a cyclic functioning of a part of PS II centers, may contribute to the second one, whereas it is clearly unconnected to the third which involves centers able of non-cyclic transfer. On the other hand, it is possible that the second and third points have a common explanation, involving acceptor Q'.

Acknowledgements

The authors wish to thank Drs. C. Vernotte, J. Lavorel and P. Joliot for helpful discussions and critical reading of the manuscript.

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