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NON-ELECTROGENIC CHARGE RECOMBINATION IN PHOTOSYSTEM II AS A SOURCE OF SUB-MILLISECOND LUMINESCENCE

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The luminescence emitted by Photosystem II at times between 1 μs and 1 ms after flash illumination was studied. Membrane potentials of up to 1 V, generated in osmotically swollen chloroplasts by externally applied electrical field pulses, had no effect on the decay components of this luminescence. Instead the field induced an additional, much slower luminescence due to a stimulated recombination of the primary charge separation in Photosystem II centers. The field-insensitive luminescence, in contrast to the field-induced luminescence, was independent of the redox state of the primary acceptor Q, and appeared not to originate from the same Photosystem II centers. The decay kinetics consisted mainly of two phases with 10 and 60 μs halftimes. The initial amplitude could be restored at all times by a second flash, indicating that both phases are due to a reversal of the field-insensitive charge separation. In the presence of DCMU and hydroxylamine, no luminescence was observed between 10 μs and 1 ms after the flash. Presumably the reoxidation of the electron acceptor was significantly slowed down, but it was still much faster than that of Q^-.

Introduction

Photosynthetic electron transport in chloroplasts is arranged vectorially in the thylakoid membrane, in such a way that upon illumination both Photosystem I and Photosystem II transfer electrons across the membrane in the same direction. The photoreactions create an electrical potential difference between the inside and outside of the thylakoids, positive on the inside, and hence an electrical field in the membrane, which can be detected by the concomitant electrochromic absorbance changes of antenna pigments (for a review, see Ref. 1). The membrane potential thus formed exerts a 'back pressure' on the light-induced electron transfer. This can be demonstrated very clearly for Photosystem II, where reversed electron transport is accompanied by a significant reexcitation and luminescence of chlorophyll. Arnold and Azzi [2] observed that the luminescence intensity increases dramatically when a suspension of osmotically swollen chloroplasts is subjected to an electrical field of several hundred volts per cm. This luminescence burst is quantitatively correlated with a reoxidation of Q^-, the first stable reductant generated in System II, by reversed electron flow [3]. By the same method it was recently found that a sufficiently large membrane potential can even prevent the photoreduction of Q [4].

Contrary to these findings, several lines of evidence seem to indicate that Photosystem II can also produce a charge separation that does not cross the membrane. Such a 'non-electrogenic' charge separation appeared to occur when Q was

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; P-680 and Q, the primary donor and acceptor in the reaction center of Photosystem II, respectively; X, alternative primary acceptor (equivalent to X_a [23] and Q_2 [7]); Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

in the reduced state and has therefore been postulated to involve some other electron acceptor [5–7], which we shall denote X in this paper. On the other hand, the photoreduction of Q in about one third of the System II reaction centers, the so-called β-centers [8], has also been proposed to be non-electrogenic [9]. In these studies a lack of correlation with the electrochromic absorbance changes was noted. A different indication of a non-electrogenic charge separation was found by Jursinic et al. [10] who noted that the luminescence emitted within a millisecond after a flash illumination was not stimulated by a membrane potential.

We have studied the sub-millisecond luminescence, using the sensitivity to an externally applied electrical field as a tool to discriminate between electrogenic and non-electrogenic System II activity. The results indicate that the charge pair, involving X is the source of virtually all of the sub-millisecond luminescence, is indeed non-electrogenic and is formed in Photosystem IIβ only.

Materials and Methods

Spinach leaves obtained from local shops were ground in a cooled blender in 40 mM Tricine buffer, pH 7.8, containing 0.4 M sucrose/10 mM KCl/2 mM $MgCl_2$. After filtration through a 25 μm mesh nylon cloth, the chloroplasts were sedimented by 5 min centrifugation at 8000 $\times$ g resuspended and stored on ice. Just before measurement the suspension was diluted 200-fold in distilled water, to obtain a suspension of thylakoid membrane vesicles ('blebs') with an absorbance at 680 nm of 0.1–0.2 per cm.

An automatic stopped-flow system brought the sample into a cubical plexiglass vessel of 1 cm^3 with the in- and outlet on one side. Two opposite walls were covered with platinum electrodes connected to a power supply, which generated pulses of up to 1200 V with a 90% rise and decay time of about 1 μs. The remaining three sides of the vessel allowed for excitation by two flash lamps and for detection of luminescence.

Luminescence was measured either in a phosphorescope with xenon flash excitation and a minimum delay of 70 μs before measurement, or in the apparatus described in Ref. 11 with Nd-Yag laser excitation (15 ns halfwidth) and electronic gating of the photomultiplier which allowed measurements after 1 μs. In the phosphorescope the flashes were filtered by a Corning CS 4-96 and the photomultiplier shielded by a Schott AL 685 filter. In the laser apparatus the photomultiplier was protected by Schott KV 550 and AL 688 filters, and for double flash experiments a dye laser pulse (Phase R, orange-2) of 150 ns halfwidth was fired through a Schott BG 23, 2 mm filter, at a variable time before the Nd-Yag flash. Via a transient recorder, the signal was fed into a minicomputer for averaging and digital filtering.

Results

An electrical field of ca. 1 kV/cm applied to a suspension of osmotically swollen chloroplasts, 'blebs', will generate electrical fields in the thylakoid membrane of up to about $2 \cdot 10^6$ V/cm [12]. We have shown that the photoreduction of Q can be prevented by the presence of a strong electrical field [4]. Hence the field should not only affect the flash-induced increase of the fluorescence yield, but also the intensity of luminescence

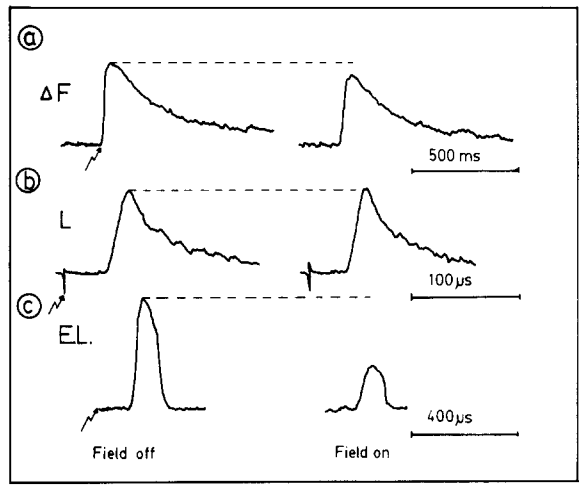

Fig. 1. Measurements performed in the presence and the absence of an electrical field during a saturating xenon flash (right- and left-hand side, respectively). The external field (1 kV/cm) was switched of at the onset of the flash. (a) Flash-induced fluorescence change; (b) luminescence; (c) stimulated emission induced by a pulse of 100 μs duration at 100 μs after the flash. The normal luminescence was subtracted. 10 μM DCMU was present in all cases. The rise kinetics in (a) and (b) were instrument-limited.

originating from a back reaction of Q^- with an oxidized donor.

Fig. 1a shows the fluorescence changes induced in a suspension of blebs with 10 μM DCMU by a 10 μs xenon flash in the presence and in the absence of an electrical field. The pulse was switched off at the onset of the flash. The field in the membrane disappeared in about 50 μs [11]. Expectedly, the presence of an artificial membrane potential during the flash led to a smaller fluorescence change, indicating that less Q had been reduced. Due to the geometry and structure of the swollen chloroplast [12] only a small fraction of System II is exposed to a large field of the desired polarity, which explains why only 15% of the fluorescence change was abolished. No corresponding decrease was observed in the luminescence: the presence of an electrical field during the flash had no detectable effect on the luminescence intensity measured 70 μs after the flash (Fig. 1b). Jursinic et al. [10] noted that the luminescence emitted on a sub-millisecond timescale after a flash appeared to be insensitive to the membrane potential. Our experiments show that the flash-induced state which generates this luminescence is still formed in the presence of a membrane potential large enough to prevent the photoreduction of Q. We conclude that Q^- is not involved in this state.

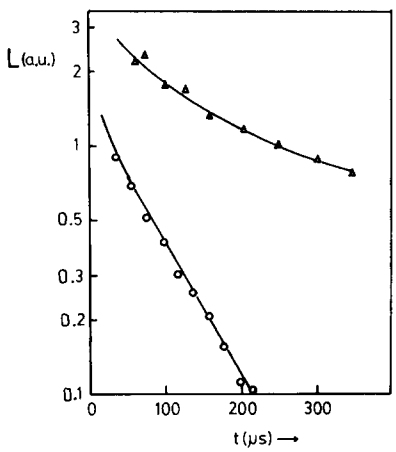

Fig. 2. Decay of the ordinary luminescence (circles) and the decay of the precursor of the field-induced luminescence (triangles) after a xenon flash. The latter decay was measured as the amplitude of the stimulated emission during a 100 μs pulse in a series of experiments with a varying delay between the flash and the pulse. A fresh sample was taken for each experiment. a.u., arbitrary units. 10 μM DCMU present.

An electrical field pulse, applied after photoreduction of Q has taken place, causes a strong luminescence emission which is quantitatively correlated with a reoxidation of Q^- [3]. Fig. 1c shows that this field-induced luminescence was largely prevented when a field of the same polarity had been present during the flash. The failure to observe this effect in earlier experiments [3] was due to a synchronisation error in the apparatus. Because the stimulated emission originates from the same centers that are affected by a field pulse during the flash, it is diminished more strongly than the variable fluorescence. The remaining luminescence probably arises from membrane regions where the field has not been strong enough to prevent the flash-induced charge separation. These data show that also on a sub-millisecond time-scale a considerable field-induced luminescence can be measured and suggest that Jursinic et al. [10], using much smaller field strengths, missed it only because in this time-domain it is superimposed on a large field-insensitive emission of different origin.

The decay of the normal luminescence and that of the amount of field-inducible luminescence support this interpretation. Fig. 2 shows that the amplitude of the luminescence burst induced by a field pulse decreases at longer times between flash and pulse (triangels), but the intensity of the normal luminescence after the flash decreases much faster (circles). The decrease of the field-inducible luminescence on this time-scale may be due to a stabilization of the charge pair by secondary electron transfer reactions which, because DCMU was present, must be ascribed to the oxidizing side (e.g., the formation of the S_2 state [13]). Since these reactions are not electrogenic [14], they cannot explain the apparent increase of the luminescence enhancement by the field at longer times after the flash.

The solid circles in Fig. 3 show the amplitude of the field-induced emission for much longer times between the flash and the field pulse. The open circles indicate the amount of field-induced luminescence observed when a second flash was fired at 200 μs before the pulse, expressed in per

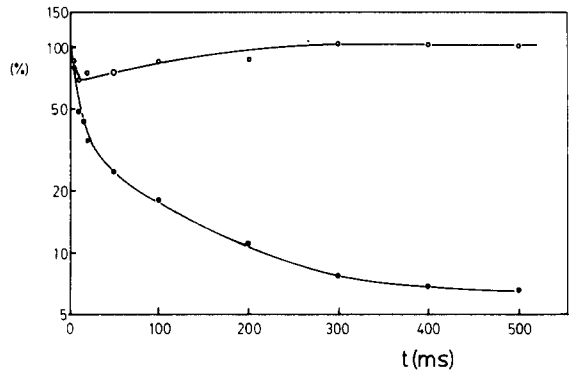

Fig. 3. Solid circles: amplitude of the stimulated emission induced by a field pulse of 100 μs duration as a function of time after a xenon flash. Open circles: identical experiment, except that at 200 μs before the pulse a second xenon flash was fired. The stimulated emission due to a pulse 200 μs after one flash was taken as 100%. A fresh sample was taken for each experiment. 10 μM DCMU present.

cent of the amount induced by a pulse at 200 μs after a single flash. The data confirm that the decay is largely due to stabilization during the first few milliseconds, because at this time the second flash did not restore the amplitude. After about 10 ms the decay appears to be due only to recombination, as indicated by the restoration by the second flash and by the concomitant decrease of the fluorescence yield (cf. Fig. 1a). The remarkably non-exponential kinetics of this recombination are known [15].

The normal sub-millisecond luminescence, on the contrary, could fully be restored by a second flash immediately. Fig. 4 shows its decay on a shorter time-scale than Fig. 2, revealing that it contains, in addition to the 60 μs phase seen in Fig. 2, a faster phase with a half time of about 10 μs (dashed lines). But even at 10 μs after the flash, a full restoration to the initial amplitude by a second flash was observed (circles). Thus the normal sub-millisecond luminescence appears to originate from a field-insensitive charge pair which recombined several thousand times faster than that involving Q^-. This result could be obtained with dark-adapted blebs in the presence of DCMU, but the data shown in Fig. 4 were actually measured on chloroplasts with 300 μM ferricyanide without DCMU, after a preillumination of 15 flashes at 5 Hz. These conditions allow a direct comparison to the measurements by Eckert and Renger [6], which revealed a flash-induced absorbance decrease at 690 nm with a similar rapid recovery, presumably due to oxidation of the primary electron donor P-680.

In the presence of DCMU and an artificial System II electron donor, 4 mM hydroxylamine or 10 μM tetraphenylboron, only a small luminescence was observed after one flash and none after a few flashes. This confirms that the field-insensitive luminescence is due to a back reaction in System II and suggests that the electron acceptor X may be kept in the reduced state, just like Q in these conditions. We have tried to measure its reoxidation kinetics after preillumination in the presence of DCMU and hydroxylamine, by measuring the flash-induced luminescence after rapid removal of the hydroxylamine by dilution. However, the amplitude of the flash-induced luminescence was completely restored within the 10 s needed for dilution and transfer to the cuvette. Fig. 5 shows the results of such an experiment, carried out with chloroplasts that had been incubated for 15 min with 10 mM hydroxylamine. This pretreatment, which inactivates oxygen evolution, had little influence on the result and was included only to allow a direct comparison of the

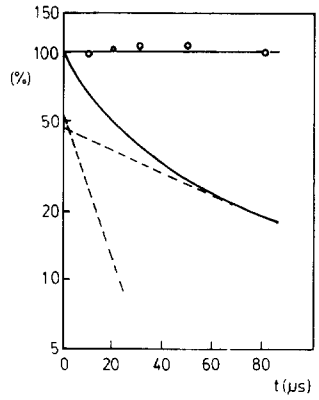

Fig. 4. Continuous line: decay of the amplitude of the normal emission after a 15 ns 530 nm laser flash in chloroplasts. Open circles: sum of this emission and the additional emission induced at 1 μs after such a flash when it was preceded by a 150 ns dye laser flash given at $t = 0$. The data are scaled to 100% on the luminescence yield 1 μs after a flash. To gain correspondence with the experiments in Ref. 6 the samples were preilluminated with 15 flashes at 5 Hz in both cases. No DCMU, but 300 μM ferricyanide present.

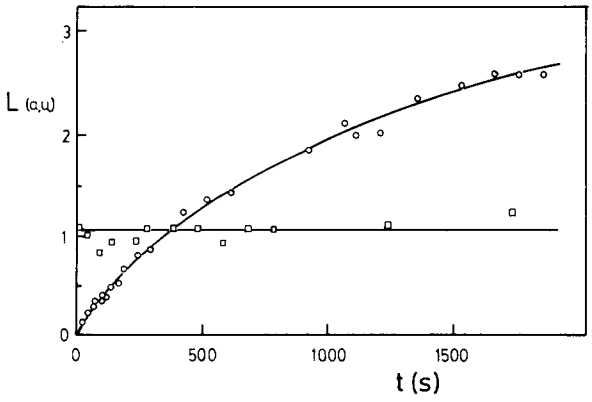

Fig. 5. Amplitude of the field-stimulated (circles) and the normal luminescence (squares) in 'hydroxylamine-treated' blebs induced by a non-saturating xenon flash as a function of time after preillumination. The 100 μs pulse was given 200 μs after the measuring flash. To obtain 'hydroxylamine-treated' blebs, chloroplasts were incubated for 15 min at pH 6.5 in the presence of 10 mM hydroxylamine. Just before preillumination with several intense flashes the suspension was diluted 2.5-fold and 10 μM DCMU added. After preillumination, the chloroplasts were diluted 200-fold in distilled water containing 10 μM DCMU to obtain blebs.

data to those reported by Joliot and Joliot [16]. The squares indicate the amplitude of the luminescence observed 200 μs after the monitoring flash, which was non-saturating. At that moment an electrical field pulse was applied and the field-induced emission is indicated by the circles. The recovery of the field-inducible luminescence corresponds approximately to the reoxidation kinetics of Q^- [16], as expected. Control experiments without preillumination showed that this slow recovery was not due to a slow bleb formation. Remarkably, the reoxidation of Q^- did not affect the amplitude of the field-insensitive luminescence. Since the measuring flash was non-saturating, the photoreduction of Q does not seem to compete for excitations with the non-electrogenic charge separation.

Discussion

Luminescence of chloroplasts, i.e., the delayed emission of chlorophyll fluorescence after illumination, is generally ascribed to the reversal of a light-induced charge separation in Photosystem II. The luminescence observed at times in the order of 100 μs after illumination is probably no exception to this rule. It could be prevented completely by preillumination in the presence of DCMU and specific System II electron donors, e.g., hydroxylamine or tetraphenylboron. It was not affected when P-700 was kept in the oxidized state by preillumination in the presence of ferricyanide (data not shown), but it was abolished when also P-680 was kept in the oxidized state by preillumination in the presence of ferricyanide at pH values below 4 [17].

In other respects, however, the field-insensitive luminescence studied here seems to be independent of the redox state of known components of System II electron transport, in contrast to the field-stimulated emission. The field-insensitive charge pair was formed in the presence of a membrane potential high enough to prevent the photoreduction of Q (Fig. 1) and it was formed again as soon as the luminescence decayed (Fig. 4), whereas the lifetime of Q^- in the same conditions was several thousand times longer. Also when the recombination was prevented, by preillumination in the presence of hydroxylamine, the reoxidation of the reductant involved in this luminescence was much faster than that of Q^- (Fig. 5). The field-insensitive luminescence thus seems to originate from the recombination of a charge pair which does not involve Q. The peculiar kinetic properties correspond to those of the alternative acceptor X under the same circumstances, as described by Eckert and Renger [6] and by Joliot and Joliot [16]. Both groups reported that the photoreduction of X was not accompanied by electrochromic absorbance changes indicative of a membrane potential [5–7]. Our data show that membrane potentials of up to 1 V have no effect on the extent of photoreduction of X or the kinetics of its reoxidation by back reaction.

Our data indicate that the photoreduction of X, also in the conditions used by Eckert and Renger [6] and those used by Joliot and Joliot [16], is a single hit process which does not compete with the photoreduction of Q. The luminescence was efficiently induced by a laser flash of 15 ns halfwidth [18], irrespective of the redox state of Q. Since the reduction of $P\text{-}680^+$ takes at least 30 ns [19,20], a double turnover of P-680 during the flash is unlikely. The amount of luminescence induced by a non-saturating flash was independent of the redox

state of Q. This indicates that the photoreduction of Q and that of X are associated with different pigment systems.

Thielen found that about all of the chlorophyll in a chloroplast suspension is accounted for by the Photosystems I, IIα and IIβ [21,22]. Since X appears to be associated with a sizable fraction of P-680 [6], but not with the main part of the variable fluorescence, we propose that the luminescence studied here originated in Photosystem IIβ.

This hypothesis would explain why several characteristics of X correspond to those attributed to Q in system IIβ: the photoreduction of both acceptors is non-electrogenic and requires about 3-times higher flash energy than System IIα or System I [22,23,24] and they are not connected to the two-electron gating mechanism reducing the plastoquinone pool [9,25]. On the other hand, Q_β^- is a plastosemiquinone anion [26] and X^- is not (Ref. 16, and confirmed in this laboratory). Therefore, the efficient, single hit reduction of X by System IIβ proposed here is not consistent with the efficient and exclusive reduction of Q by System IIβ in the conditions used by Thielen. Under these conditions a non-saturating flash given 10 ms after a saturating flash induced a much smaller 60 μs luminescence than in blebs in a non-buffering medium. Various reports in the literature can be interpreted as indicating a shift from Q reduction to X reduction by a pH decrease inside the thylakoids [27,8]. Experiments to test this hypothesis are now in progress.

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