Biochimica et Biophysica Acta, 1056 (1991) 27–39
Elsevier

BBABIO 43312

An electroluminescence study of stabilization reactions in the oxygen-evolving complex of Photosystem II

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(Received 27 December 1989)
(Revised manuscript received 27 September 1990)

Key words: Electron transport; Oxygen-evolving complex; Photosystem II; Luminescence; Electric field effect; (Spinach chloroplast)

The stabilization of the photosynthetic charge separation in Photosystem II by secondary reactions was studied using chlorophyll luminescence induced by electric field pulses in a suspension of preilluminated osmotically swollen chloroplasts. This 'electroluminescence' was measured as a function of the delay time between illumination and field pulse, and as a function of the number of preilluminating flashes. The result is a survey of, in principle, all stabilization and deactivation processes beyond the state $Z^+Q_A^-$, which is formed within the approx. 20 μs time resolution of the method. Most of these could be identified with known secondary electron transfer reactions. A 20-fold stabilization with a half-time of 330 μs is attributed to Q_A^- reoxidation. No further stabilization at the acceptor side seemed to occur and no flash number dependence was detected, although a normal Q_B/Q_B^- oscillation was found in ultraviolet absorbance. With regard to the donor side, the data are consistent with the known S-state-dependent Z^+ reduction times and indicate values of 9, 5 and 65 for the equilibrium constants associated with this reaction on the transitions $S_1 \rightarrow S_2$, $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_0(O_2)$ respectively. Z^+ reduction was found to be electrogenic and exposed to about 5% of the membrane potential. An 0.1 s phase in S_0 is attributed to oxygen release. S_2 and S_3 are further stabilized in two phases of unknown origin with half-times of 15 ms and 0.4 s, followed by a final 20 s phase attributed to deactivation. In S_1, Z^+ reduction was probably hidden in an unresolved fast phase present on all transitions, but in addition a 350 μs phase was found, which might be related to proton release. In nearly 20% of the Photosystem II reaction centers electron transfer beyond Q_A^- was inhibited. In these centers Z^+ reduction appeared to take about 1 ms and charge recombination followed in phases of about 8, 80 and 800 ms half-time.

Introduction

Photosynthesis is reversible. No matter how many secondary electron transfer reactions, (de)protonations and other stabilizing processes have occurred, the product will be in Boltzmann equilibrium with the singlet excited state of chlorophyll, which will sometimes be lost by fluorescence emission before it is retrapped. Especially in Photosystem II this delayed fluorescence is easily detected. Its intensity is expected to decrease with every step during a series of stabilization reactions which follow the photochemical charge separation and should provide very detailed information on the kinetics and thermodynamics of the stabilization process [1,2]. It has proved difficult, however, to correlate the decay of delayed fluorescence with known electron transfer reactions, presumably because the signals are often dominated by a small fraction of PS II in which stabilization fails and the luminescence yield is consequently much higher [3,4].

This problem can be minimized by inducing charge recombination in all centers by applying a strong external field and measuring the resulting burst of 'electroluminescence' emission [5,6]. Here the external field

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Abbreviations: D, secondary electron donor; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; I, primary electron acceptor; M..M^{3+}, redox states of the oxygen-evolving complex; Mops, morpholinepropanesulphonic acid; P-680, primary electron donor; PS, Photosystem; Q_A, Q_B, secondary electron acceptors; S_0..S_3, redox states of the donor side; Z, secondary electron donor.

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changes the reaction rates of electrogenic redox reactions by changing the relative potentials and/or positions of the reactants. Sufficiently large membrane potentials can be generated by using the local amplification of the electric field strength in the membrane of large vesicles, e.g., the 'blebs' formed by unfolding of the thylakoid system upon hypotonic swelling of chloroplasts [7]. The time resolution of the method then is limited by the risetime of the local electric field [8], which is typically a few tens of microseconds.

At this time after a photosynthetic charge separation in PS II, the state $Z^+Q_A^-$ has already been formed. By applying the field at progressively later times after the primary charge separation one should obtain decreasing electroluminescence intensities as $Z^+Q_A^-$ is transformed into more stable states (e.g., by manganese oxidation at the donor side and Q_B or Q_B^- reduction at the acceptor side [9–11]). Under conditions where the stabilization at the acceptor side was blocked beyond Q_A^- formation such a decrease in electroluminescence was indeed observed in the μs time range and ascribed to reduction of Z^+ by the $M^+ \rightarrow M^{2+}$ transition [12]. We will use $M..M^{3+}$ as in Ref. 13 to denote the four redox states of the oxygen-evolving complex; the term S_i will be used in its original general sense [14], e.g., S_2 denotes (P-680$^+$ZM$^+ \rightleftarrows$ P-680 Z$^+$M$^+ \rightleftarrows$ P-680 ZM^{2+}), except that S_4 will not be used and S_0 will refer to whatever state is present after the turnover in S_3.

The dependence of the reaction rates of the stabilization reactions on the redox state of the reaction center [15] implies that, when dark adapted samples are illuminated with a series of saturating flashes, signals oscillating with flash number are to be expected. Indeed, the appearance of oscillating electroluminescence signals with delay times between flash and pulse in the (sub-)ms range have been reported [8,16,17]. We have extended this time range up to 20 s and decomposed the obtained data set into electroluminescence kinetics and decay phases associated with the various S-states and with non-oscillating centers.

Materials and Methods

Chloroplasts from laboratory-grown spinach were prepared and stored as described before [12]. Blebs were formed by diluting a suspension of chloroplasts with a chlorophyll concentration of 1 mg/ml 200-fold in a 1 mM Mops buffer (pH 6.6). The blebs were formed at least 10 min before the start of the measurements. To suppress contributions of PS I electroluminescence 50 μM ferricyanide was added [12]. An important difference with previous experiments on PS II electroluminescence in blebs [8,12] is that we added 1 mM $CaCl_2$ to the buffer. This raises the conductivity of the sample and decreases the maximal applicable field strength, but the use of a cuvette with an electrode spacing of 2 mm placed in the focus of an ellipsoidal mirror enabled us to impose a higher field-strength (1500 V/cm) than before [12] and to collect enough emitted light to obtain a good signal to noise ratio without much signal averaging. The emitted light was detected with a gated photomultiplier through a 686 nm interference filter and a Schott RG 665 cut-off filter. Saturating laser flashes (15 ns, 532 nm) were provided at 10 Hz, unless stated otherwise. For measurements at less than 200 μs after the flash the signal observed without electric pulse was subtracted after digitalization. At longer delay times a linear baseline correction was applied. This correction was insignificant at delay times longer than 1 ms. Absorbance difference measurements at 325 nm were performed with the appararatus described before in Ref. 18. The optical pathlength of the measuring light was 1 cm and actinic flashes were given with a xenon flash lamp (10 μs half-width) and filtered with a Corning CS 4–96 broad blue filter. All measurements were performed at 22 °C. For each measurement a fresh sample was used, which was dark-adapted for at least 10 min. The signals were decomposed into contributions by each of the different S-states and by non-oscillating centers, as will be described in the Results section. The decrease of each contribution

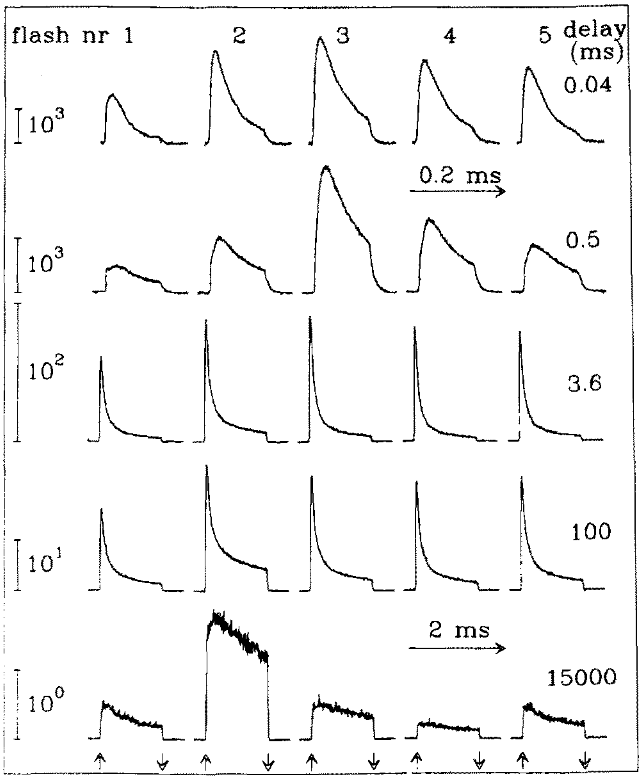

Fig. 1. Flash number dependence of electroluminescence with the onset of the pulse at various delay times. Average of 1 (0.04, 0.5 and 3.6 ms), 3 (100 ms) and 5 (15000 ms) traces. The bars at the left indicate the scaling in relative units; the arrows mark the onset and end of the pulses. Each trace is 0.2 ms (0.04 and 0.5 ms) or 2 ms (others).

as a function of the delay time between flash and electric pulse was fitted by a sum of exponentials without a priori assumptions on half-times, amplitudes, or number of exponentials needed, unless otherwise indicated. A logarithmic fit criterion was used to account for the orders of magnitude differences in the data.

Results

Electroluminescence signals were recorded as a function of flash number (1 to 11) and delay time between the last flash and pulse (40 μs to 20 s). In Fig. 1 electroluminescence kinetics at different times after the flashes one to five are displayed. Over the range of delay times studied (6 orders of magnitude) the signal amplitude decays by more than 3 orders of magnitude. The following qualitative observations can be made:

PS I electroluminescence was effectively suppressed by the use of ferricyanide. The kinetics of PS I electroluminescence during the pulse differ considerably from those of PS II; most PS I electroluminescence is emitted within 50 μs after the pulse onset at the applied field-strength [12].

The most pronounced oscillations with flash number are observed around 0.5 ms after the flash and in the seconds time range. At intermediate times a non-oscillating signal dominates the electroluminescence kinetics. This signal is lower after the first flash than after the later flashes and its initial kinetic shape resembles that of the oscillating electroluminescence at shorter times.

The oscillations at delay times around 0.5 ms display a maximum at flash 3 (and 7,11, etc.) as has been reported before [8,16,17], in agreement with the notion that Z^+ reduction is slowest in S_0 [15,19]. At very long delay times (15 s) strong oscillations are observed with a maximum on flash 2 (and 6, 10 etc). This indicates that the signal arises mainly from S_3 and possibly also from S_2 (flash 1, 5, 9 etc.). These oscillations are also present at much shorter delay times than 15 s, just after Z^+ reduction, as can be seen from the end of the electroluminescence traces in the millisecond time range (Fig. 1, 3.6 ms trace). The kinetics of the electroluminescence are much slower than at shorter times (notably around 0.5 ms) after the flash; i.e., the electroluminescence kinetics are much faster when the pulse is given in the state $Z^+Q_A^-$ than when a pulse is given after stabilization reactions have taken place at the donor- and acceptor side. As will be explained in the Discussion, this implies that the stabilization reactions at the donor side and/or the acceptor side are electrogenic.

Fig. 2 shows the electroluminescence, integrated over the pulse duration, as a function of flash number and for a series of delay times. Our data differ from electroluminescence oscillation patterns that have been reported before [8,17] in two respects: (a) the overall increase of the signal amplitude with increasing flash number is much less than in Ref. 8 and (b) the relative amplitude of the signal after the flash number 2, 6, etc. is less, suggesting a faster $Z^+M^{2+} \rightarrow ZM^{3+}$ transition. Both phenomena were found to be related to the presence of $CaCl_2$; at 1 mM (the concentration used) these effects were saturated (not shown).

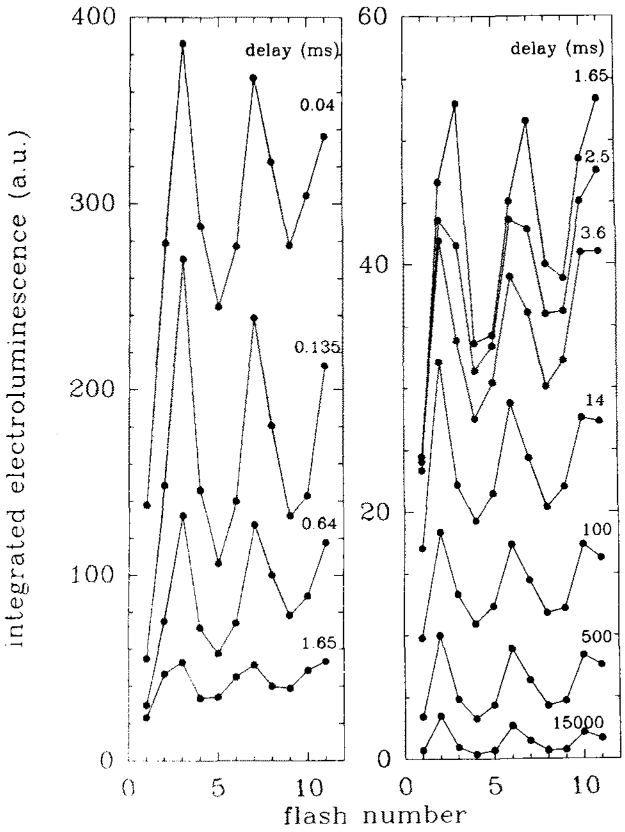

Fig. 2. Flash number dependence of the integrated electroluminescence recorded during pulses of 1.2 ms at various delay times.

Q_B/Q_B^- oscillations

As stated above, $Z^+Q_A^-$ is thought to be the electroluminescence precursor in the (sub-)ms domain. At times longer than the Q_A^- reoxidation time (about 0.5 ms [20,21]) presumably some Q_A^- is always present in equilibrium with Q_B^-. The Q_A^- concentration is expected to oscillate with flash number with a periodicity of 2. Also the rate of electron transport from Q_A to Q_B may depend on the redox state of Q_B and therefore oscillate binarily [21]. To investigate the influence of the Q_B/Q_B^- oscillation an experiment analogous to that of Lavergne in ultraviolet absorbance [22] was performed. Electroluminescence oscillations were monitored in the presence of FCCP, an agent which accelerates the deactivation of the S-states [23]. We monitored the kinetics of the deactivation by FCCP by observing the phase shift of the oscillation pattern as a function of dark time between preflashes and a measuring flash series. When 50 nM FCCP was added the half-time of deactivation was about 0.5 s (not shown). Fig. 3A and B shows

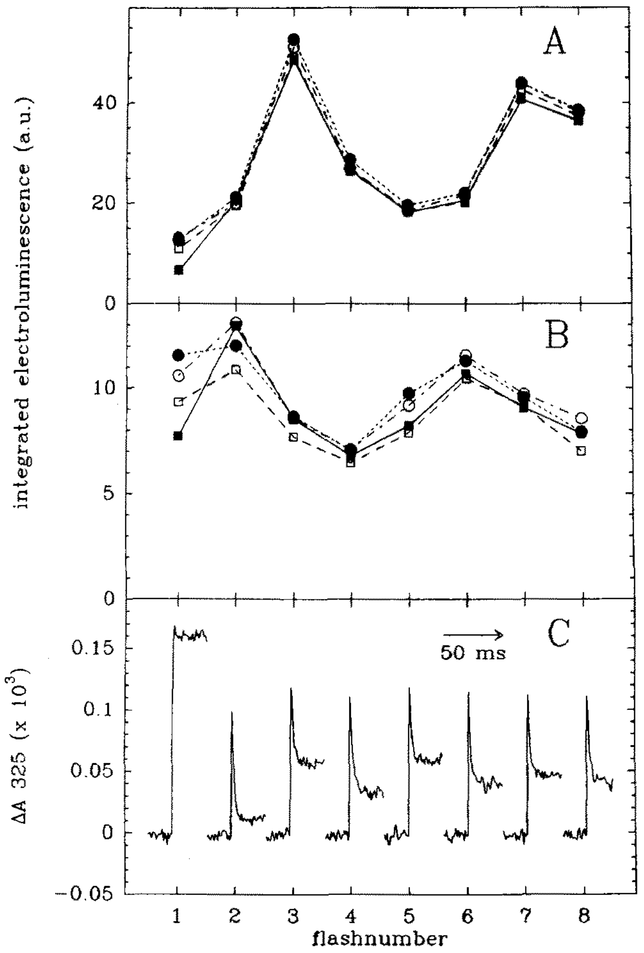

Fig. 3. The influence of the acceptor side on electroluminescence oscillations. In all experiments 50 nM FCCP was present. (A,B) 0 (solid squares), 1 (open squares), 2 (solid circles) or 3 (open circles) preflashes, spaced at 3 s, were given; 5 s after the last preflash a series of flashes (40 Hz) was given. The electroluminescence was measured with delay times of 0.4 ms (A, 0.12 ms pulse) and 50 ms (B, 1.2 ms pulse, integrated over the second half of the pulse only). (C) Flash induced absorbance difference at 325 nm. Average of 100 traces. Flash frequency 0.5 Hz.

electroluminescence oscillations measured 5 s after 0, 1, 2, or 3 preflashes, spaced at 3 s. The Q_B^- concentration before the measuring flash series should then oscillate with the number of preflashes, whereas the centers should be in the S_1 state irrespective of the number of preflashes (except for 0 preflashes if some centers are in S_0 in the dark). The oscillation patterns with delay times of 0.4 ms (Fig. 3A) and 50 ms (Fig. 3B, integrated over the second half of the 1 ms pulse time to select the oscillating part, see Fig. 1) did not change when the preflashes were given. Only the first flash signal increased upon preillumination; this is presumably due to the non-oscillating centers (see above).

Fig. 3A and B reveals no oscillation with the number of preflashes. To further substantiate this observation the electroluminescence as a function of flash number was monitored after 0.4 ms in the presence of FCCP and with a flash interval of 5 s. Under these conditions the S-state distribution should be the same after each flash (except possibly the first), whereas the redox state of the acceptor side is expected to oscillate. The electroluminescence revealed no such oscillation (not shown).

To verify whether the Q_B^- concentration did oscillate as expected, the flash induced absorbance changes at 325 nm were monitored (Fig. 3C) at a flash interval of 2 s, which was long enough to deactivate the S-states between flashes. It is clear that the Q_B^- concentration oscillated with a period 2. It might be expected that Q_B^- is reoxidized on a seconds time-scale by ferricyanide. However, using longer flash intervals (6 s) a period 2 was also observed (not shown), indicating that the used concentration of ferricyanide (50 μM) was too low to rapidly reoxidize Q_B^- between flashes. We have to conclude that the electroluminescence is not sensitive to the oxidation state of Q_B.

The dark distribution of S-states

Figs. 3A and B also show that the phase of the period 4 oscillation did not shift upon preflashing and deactivation of the donor side. This implies that the dark distribution of the S-states did not change and hence a 100% S_1 concentration in the dark must be assumed. To substantiate this assumption a similar experiment was done shortly after the S-states were randomized by strong continuous illumination and deactivated (Fig. 4). In this case the phase is retarded compared to the dark adapted case (cf. Fig. 3A). The dark adapted result is restored after 1 or 2 preflashes and deactivation. These observations confirm the presence of S_0 (presumably 25%) after the strong continous preillumination and its absence after dark adaptation.

A different way to estimate the initial S-state distribution is to analyse the electroluminescence oscillations at very long delay times by fitting to the Kok model

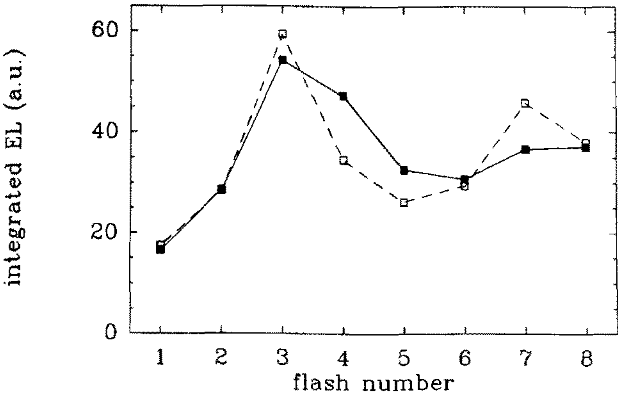

Fig. 4. Integrated electroluminescence, recorded with a 0.12 ms pulse given 0.5 ms after the last flash, in blebs which were dark adapted for 5 s after preillumination with strong light for 3 s. Oscillation patterns (flash frequency 40 Hz) were recorded without (solid squares) and with (open squares) two preflashes as in Fig. 3. 50 nM FCCP was present.

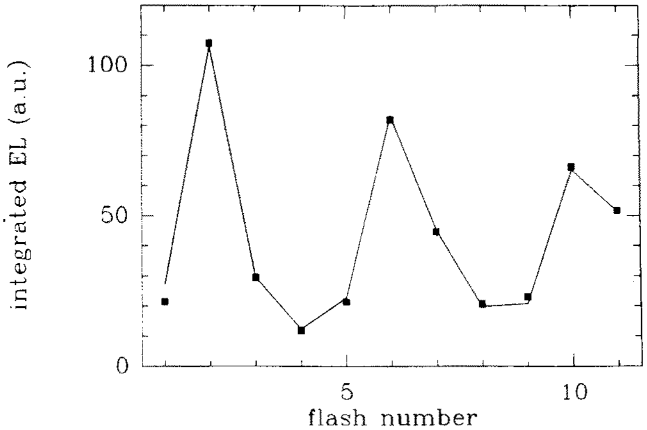

Fig. 5. Oscillations of the integrated electroluminescence recorded with a 1.2 ms pulse given 15 s after the last flash. The line is a fit assuming 7% misses, 100% S_1 in the dark, relative contributions of centers in S_2 and S_3 and non-oscillating centers of 2:11:1 and an overall decrease of the signal of 0.5% per flash.

[14]. The stable states S_0 and S_1 may then be expected not to contribute any electroluminescence. The first flash was not included in the fit (see above) and it was assumed that centers are either in S_0 or in S_1 in the dark. The fit was obtained by iteratively minimizing the sum of squares of the deviation of a calculated and the measured oscillation pattern (obtained by varying the number of preflashes in the series) when the following parameters were varied: contributions by centers which are in S_2 and in S_3 after the last flash of the series and from non-oscillating centers, the dark concentration of S_1, misses and the relative change in the total amount of active centers per flash. Thus deactivation after the last flash is reflected by a decrease in the contribution of the centers which are in S_2 and S_3 at long delay times. If any deactivation occurs in the flash interval this is reflected in the miss parameter. In Fig. 5 the optimal fit is shown to the oscillation pattern of the integrated electroluminescence measured at 15 s after the flash. The corresponding fit parameters are: contributions of S_2, S_3 and non-oscillating centers 2:11:1, dark concentration of S_1 100%, misses 7% and decrease of the number of active centers per flash −0.5%. So this analysis also indicates that S_0 is absent after dark adaptation.

Conversely, taking the experiments of Figs. 3A,B and 4 as evidence for the dark distribution of 0% S_0 and 100% S_1, the fit of Fig. 5 implies that if S_0 and S_1 contribute significantly to the electroluminescence at a delay time of 15 s their contributions have to be equal. This was true also at other delay times in the seconds time range (see below), indicating that also these contributions were equal. It is most likely, therefore, that they are due non-oscillating centers and that S_0 and S_1 indeed do not contribute to electroluminescence in the seconds time range.

Kinetic decomposition

The kinetic shapes of the different contributions to the electroluminescence kinetics during the pulse were obtained by performing a fit as above for each point of the kinetics rather than for the integrated electroluminescence. We assume that, after the reduction of Z^+, centers which are in S_1 do not contribute to the electroluminescence (see above) and hence the contributions of S_0, S_2, S_3 and the non-oscillating component are taken as fit parameters for each point of the kinetics. The values for the misses (7 %) and dark-distribution of S-states (100 % S_1) were kept fixed in those fits. The result of this procedure is a set of curves representing the contribution of centers in each separate S-state S_0, S_2 and S_3, and of the non-oscillating centers to the observed signals. The results for the delay time of 15 s are shown in Fig. 6B, right column of traces. These traces clearly illustrate the absence of S_0 electroluminescence at this delay time. The decay of S_2 electrolumines-

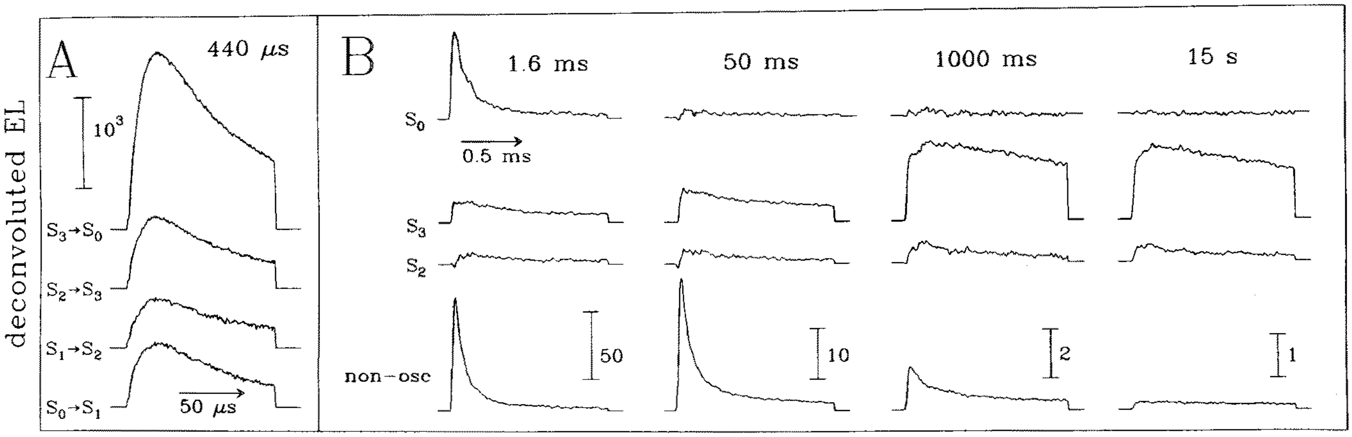

Fig. 6. Decomposition of electroluminescence contributions at various delay times as described in the text. The bars indicate the scaling (a.u.). (A) 0.12 ms pulse; only contributions by oscillating centers were assumed. (B) 1.2 ms pulse; contributions by centers in S_0, S_2 and S_3 and by non-oscillating centers were assumed.

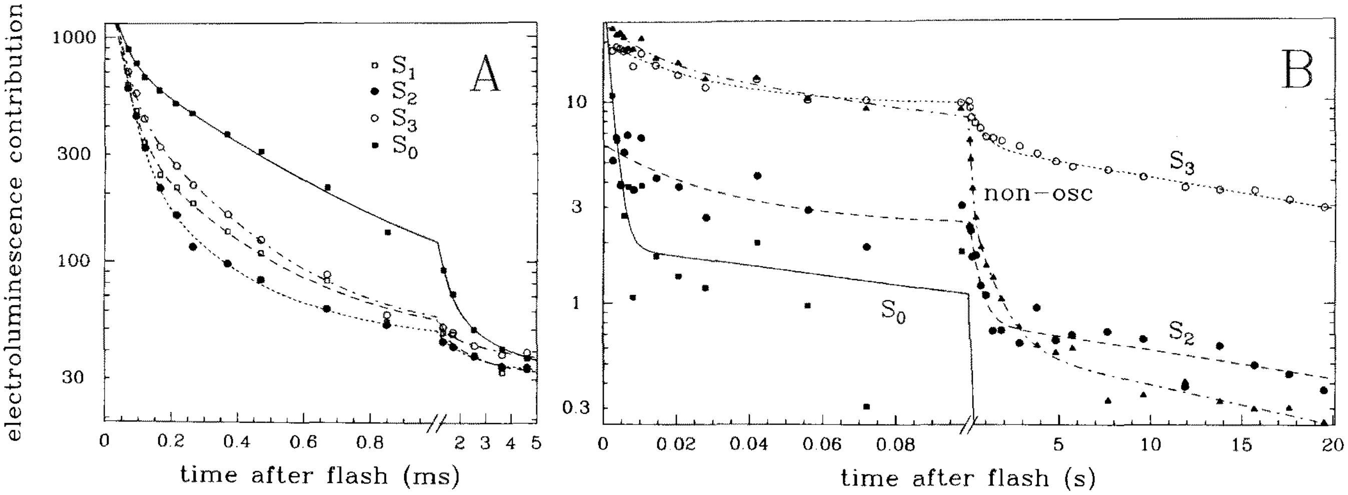

Fig. 7. Decay of integrated electroluminescence contributions as a function of time after the last flash. The curves are based on the fit parameters listed in Table I (A) and Table II (B) .Note the different time-scales. (A) Contributions of centers reaching S_1 (open squares), S_2 (solid circles), S_3 (open circles) and S_0 (solid squares) on the last flash as in Fig. 6A. (B) Contributions of centers which are in S_0 (solid squares), S_2 (solid circles) and S_3 (open circles) after the last flash and from non-oscillating centers (solid triangles) as in Fig. 6B.

cence during the pulse is found to be somewhat faster than that of S_3.

This procedure allows us to decompose the electroluminescence oscillations into contributions of four different precursors at all delay times. Consequently, the kinetic shape of the electroluminescence during the pulse and the kinetics of the precursors can be obtained for all S-states or, alternatively, for three S-states and a non-oscillating component. Thus, at longer delay times we can decompose the observed signals into contributions from centers in S_0, S_2 and S_3 and from non-oscillating centers as above. At shorter delay times, however, all four S-states may be associated with Z^+ and contribute to the electroluminescence. This implies that, at shorter delay times, we have essentially one free parameter too many. For this reason the analysis was performed in two domains of delay times: one from 2.5 ms to 20 s and one up to 5 ms.

At all delay times a small change of the amount of active centers with increasing flash number had to be taken into account. At long delay times this parameter is slightly negative (see above). At delay times of up to about 1 s this parameter was found to have a small positive value. It did not exceed 2% per flash. This effect is presumably due to the filling of the plastoquinone pool. In the delay time range of about 1 ms to 1 s a much stronger increase was found when the flash sequence was extended beyond 11 flashes (not shown).

Delay times of 40 μs to 5 ms

As explained above, one cannot simultaneously extract contributions by all four S-states and a non-oscillating component from the data. At short delay times all those are expected to be present. To cope with this problem only S-state-dependent contributions were assumed; decay phases which are equal for all S-states may therefore be regarded as arising from a non-oscillating precursor. Another difference with the analysis at longer delay times (Figs. 5,6B) is that only the centers which were hit by the last flash and thus performed an S-state transition were considered to contribute to the electroluminescence.

The results from this procedure, using the initial dark distribution (0% S_0, 100% S_1) and misses (7%) obtained earlier, are shown for a delay time of 0.44 ms in Fig. 6A. It can be seen that the electroluminescence kinetics during the pulse are similar for all S-states. In the submillisecond delay time range the decomposed electroluminescence kinetics do not change; only at delay times of tens of μs the kinetics are somewhat faster (not shown, cf. Fig. 1). The decays of the integrated contributions were fitted by a multi-exponential decay and are shown in Fig. 7A. In all cases a mimimum of four

TABLE I

Fit parameters from Fig. 7A

Fit parameters of the decay of integrated electroluminescence contributions from pulses of 0.12 ms given at delay times up to 5 ms corresponding to the fits in Fig. 7A (only centers making a turnover on the last flash were assumed to contribute to EL). Half-times in milliseconds; amplitudes (between parentheses) in relative units. The half-time of the 0.03 ms phase was kept fixed. The > 5 ms phases and most of the approx. 1 ms phases (except for S_0) are ascribed to the non-oscillating electroluminescence (see text).

S_1	S_2	S_3	S_0
0.03 (1800)	0.03 (2300)	0.03 (1700)	0.03 (1000)
0.17 (330)	0.14 (270	0.17 (500)	0.26 (750)
0.9 (33)	0.8 (30)	1.5 (19)	1.2 (67)
> 5 (33)	> 5 (35)	> 5 (38)	> 5 (35)

exponentials was required. The fit parameters are listed in Table I. It is clear that on all contributions a phase is present with a half-time of approx. 30 μs. This phase is too rapid for a reliable determination of its half-time and amplitude. The slowest decay phases all have a similar amplitude. This suggests that they originate mainly from the non-oscillating centers. The amplitude of the slowest phase associated with the $S_2 \rightarrow S_3$ transition is slightly, but significantly, higher. This is consistent with the observation that electroluminescence from S_3 dominates the oscillations at longer time-scales (Figs. 5, 6B). The two intermediate phases have half-times of around 0.2 and 1 ms. The time constants of the approx. 1 ms phases, which have relatively low amplitudes, could not be determined precisely due to the scatter in the data; satisfactory fits for all half-times between 0.8 and 4 ms could be obtained with similar amplitudes. The reoxidation of Q_A^- is expected to contribute to the approx. 0.2 ms phase on all S-state transitions; a 1.2 ms phase is expected to occur on the $Z^+M^{3+} \rightarrow ZM(O_2)$ transition only [15,19]. The approx. 1 ms phase on the other transitions is in fact much smaller and may indicate a contribution by the non-oscillating centers. Note also that the amplitude of the approx. 0.2 ms phase is smaller for the $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$ transitions than for the $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_0$ transitions.

Delay times of 1.6 ms to 20 s

The electroluminescence contributions in the delay time range of 1.6 ms to 20 s were obtained the same way as described before for the contributions at 15 s. For a few delay times the results are shown in Fig. 6B. The integrated contributions were fitted to exponential decays. The results are displayed in Fig. 7B and the fit parameters are listed in Table II.

A minimum of three exponentials was required to fit the decrease of electroluminescence contributions by S_2 and by S_3 as a function of the delay time between flash and pulse. Both for S_2 and for S_3 the half-times were about 15 ms, 400 ms, and 20 s. The half-time of the slowest phase is in the time range of deactivation of S_2 and S_3 [24,25]. The ratio of the amplitudes of the phases for S_2 and S_3 contributions is significantly higher for the 15 ms (0.35) and 400 ms (0.45) phases than for the 20 s phase (0.13). Also note that the kinetic shape of the S_2 and S_3 electroluminescence contributions remains similar throughout the time range (Fig. 6B).

In the decay of the electroluminescence precursor associated with centers which are in S_0 after the flash, in addition to the 1.2 ms phase (Fig. 7A, Table I), ascribed to centers in which Z^+M^{3+} is still present, a minor but definitely significant phase with a half-time of about 0.1 s is found. The kinetics during the pulse of the electroluminescence associated with this phase resemble those of S_2 and S_3. The 0.1 s phase may reflect a stabilization of S_0 by oxygen release, which has about the same time constant ([26]; oxygen evolution in blebs was measured as in Ref. 26 and was found to occur with a similar time constant (not shown)).

The precursor of the non-oscillating electroluminescence decays multi-exponentially. At least four phases of 8, 80 and 800 ms and 15 s were required to obtain a reasonable fit. The time constant of the 8 ms phase could not be determined precisely; the scatter in the data allows fits with half-times of 2 to 10 ms. Hence this phase might be the same as the approx. 1 ms phase mentioned above. The kinetics of the non-oscillating electroluminescence during the pulse are similar for all phases, except for the minor 15 s phase. The electroluminescence associated with this phase decayed much more slowly during the pulse (Fig. 6B).

Non-oscillating electroluminescence and the effect of DCMU

To investigate the origin of the non-oscillating electroluminescence we monitored the flash saturation characteristics of electroluminescence with delay times of 0.5 ms (when the S-state dependent signal dominates) and 100 ms (when the non-oscillating signal dominates). No differences were observed (not shown), suggesting that the α-β heterogeneity [27] is not involved here.

The precursor of the non-oscillating electroluminescence decays in a multiphasic and perhaps non-exponential manner. Such kinetics have been reported before for $S_2Q_A^-$ recombination in the presence of DCMU [28,29]. Therefore, this emission may be ascribed to centers in which the reoxidation of Q_A^- by Q_B is inhibited. The existence of such centers has been proposed before to explain an anomalous ultraviolet absorbance change on the first flash of a series [22,30]. Also the electroluminescence kinetics in the presence of DCMU (not shown) are similar to those of the electroluminescence from non-oscillating centers at intermediate delay times. At longer delay times (the 15 s phase) the kinetics of the non-oscillating electroluminescence are different; the decay during the pulse was

TABLE II

Fit parameters from Fig. 7B

Fit parameters of the decay of integrated electroluminescence contributions from pulses of 1.2 ms given in the time range of 2.5 ms to 20 s after the flash series corresponding to fits in Fig. 7B (all centers were assumed to contribute to EL). Half-times in milliseconds; amplitudes (between parentheses) in relative units and corresponding to those in Table I as described in the text.

S_2	S_3	S_0	Non-osc.
16 (3.3)	15 (9.6)	1.2 (37)	8 (9.1)
350 (2.0)	460 (4.4)	100 (2)	80 (12.6)
			800 (2.5)
22000 (0.8)	19000 (6.0)		15000 (0.6)

slower. This suggests that the signal originates from a different precursor at this time scale.

A remarkable feature of the non-oscillating electroluminescence is that its amplitude after the first flash is lower than after subsequent flashes (Fig. 1). The amplitude of the electroluminescence upon the later flashes depended on the flash interval and the presence of ferricyanide. In the presence of ferricyanide this amplitude was about 20% higher when the flash interval was several seconds than when it was 0.1 s. The half-time of this phenomenon was 0.9 s (not shown). In the absence of ferricyanide this effect was not observed. We propose that the increase in amplitude is related to reoxidation of Q_A^- by ferricyanide; upon a second flash possibly $S_3Q_A^-$ could then be formed.

Finally, it should be noted that the electroluminescence of non-oscillating centers, which presumably originates from $S_2Q_A^-$, displays much faster kinetics during the pulse than electroluminescence from $S_2Q_B^-$ (Fig. 6B).

Discussion

As pointed out in the Introduction, the time resolution of the technique used here is limited to a few tens of microseconds by the rise of the local electric field and therefore only reactions beyond the state $Z^+Q_A^-$ can be studied. The field-induced luminescence increase in PS II presumably arises from an increase in the concentration of P-680$^+$I$^-$ and, if electron transport from P-680 to I is electrogenic, an increased recombination rate from this state. If the reaction centers are in the state $Z^+Q_A^-$ at the onset of the pulse, the new (pseudo-)equilibrium is brought about by reversed electron flow from Q_A^- to I and from P-680 to Z^+. The former reaction is strongly electrogenic [31,32]; the latter presumably is not [33]. Therefore the oxidation of P-680 is presumably the rate-limiting step in the kinetics of electroluminescence in this case. Indeed, comparison of electroluminescence measured under conditions in which P-680$^+Q_A^-$ [34] and $Z^+Q_A^-$ [29] are precursors reveals that in the former case the electroluminescence kinetics are faster. Also the PS I electroluminescence [12], which arises from a charge pair that is not stabilized at the donor side, is much faster than PS II electroluminescence from $Z^+Q_A^-$.

As could be expected from ultraviolet absorbance studies [22,30] a substantial contribution was found to come from 'inactive centers' that do not exhibit the characteristic period 4 oscillation with flash number. The analysis of the kinetics was seriously complicated by the highly polyphasic decay of this contribution. On the other hand, analysis of the oscillating components was simplified by two findings. Firstly, all centers were found to be in the S_1 state after dark adaptation (perhaps due to the presence of ferricyanide [35]). Secondly, the Q_B/Q_B^- oscillation did not influence the electroluminescence, at least up to 50 ms after the flash; this was quite unexpected, but clearly demonstrated by the data shown in Fig. 3.

Some of the decay phases listed in Tables I and II are easily identified. The deactivation of S_2 and S_3 probably accounts for the approx. 20 s phases associated with these states. The pronounced 1.2 ms component which is responsible for the phase shift of the period 4 oscillation (from maxima on flash 3, 7, 11 to maxima on 2, 6, 10) is undoubtedly due to the $Z^+M^{3+} \rightarrow ZM(O_2)$ transition. The small but significant 0.1 s phase remaining after this transition is in the time range of oxygen release [26]. This indicates that bound oxygen is in fact detectable via the equilibrium $ZM(O_2) \rightleftarrows Z^+M^{3+}$, which illustrates the sensitivity of the method. The 0.26 ms phase preceding the $Z^+M^{3+} \rightarrow ZM$ transition (Table I) may safely be attributed to reoxidation of Q_A^- by Q_B. This reaction should have a half-time of 0.33 ms in order to account for the observed 0.26 ms half-time ($1/0.33 + 1/1.2 = 1/0.26$), which is in the range expected [20,21,25]. Moreover, in view of its flash number independence (Fig. 3, in contrast to Ref. 21), the same Q_A^- reoxidation time (0.33 ms) would contribute to the 0.17 ms phase observed in S_3, which indicates that the reaction $Z^+M^{2+} \rightarrow ZM^{3+}$ should then have a half-time of 0.35 ms. This value coincides with data from ultraviolet absorbance and EPR spectroscopy [15,19].

By the same reasoning the 0.17 ms and the 0.14 ms phases in electroluminescence observed in S_1 and S_2 (Table I) would reflect the reactions $Z^+M \rightarrow ZM^+$ and $Z^+M^+ \rightarrow ZM^{2+}$ with half-times of 0.35 ms and 0.24 ms, respectively, together with the 0.33 ms Q_A^- reoxidation. The $Z^+M^+ \rightarrow ZM^{2+}$ transition is expected to take 0.11 ms [15,19] and should have resulted in an 0.09 ms phase. Imposing this time in the analysis instead of the observed 0.14 ms led to an acceptable fit. However, the 0.35 ms reaction in S_1 cannot be ascribed to the $Z^+M \rightarrow ZM^+$ stabilization as this reaction is known to be about 10 times faster [15]. This indicates that it must reflect some other process which stabilizes the S_1 state, perhaps deprotonation, which was reported to take 0.25 ms [36], and that the $Z^+M \rightarrow ZM^+$ transition is hidden in the 0.03 ms phase (Table I). It should be noted that the above interpretation of the various approx. 0.2 ms phases implies that no such phase is attributed to electroluminescence from non-oscillating centers.

The 15 ms and approx. 400 ms phases seen after the $Z^+M^+ \rightarrow ZM^{2+}$ and the $Z^+M^{2+} \rightarrow ZM^{3+}$ transitions (Table II) may in principle be due to stabilizations at the donor side and/or the acceptor side. The stabilization is more prominent in S_2 than in S_3, whereas no period 2 effect on the acceptor side was monitored. This indicates that at least one of the stabilization phases is due to the donor side. The nature of the stabilization remains unknown. No charge transport processes in this time domain are known at the donor side. However, the electroluminescence presumably is very sensitive to any structural changes or charge displacements. The absence of a 15 ms phase in the decay of the signal associated with centers which are in S_0 indicates that the 15 ms phases in S_2 and S_3 are not due to to a stabilization at the acceptor side. On the other hand, it cannot be excluded that the 400 ms phase is due to oxidation of the acceptor side by ferricyanide. It was observed that without ferricyanide the electroluminescence after flash 1, 5, 9 etc. was higher relative to the other flashes than in the presence of ferricyanide (not shown). This might be interpreted as an oxidation of the acceptor side which is accelerated in centers connected with S_2, which state is correlated with a high concentration of Q_B^- (Fig. 3C). The absence of a period 2 effect in the electroluminescence measured after 50 ms (Fig. 3B) does not support such an explanation for the 15 ms phase, but we could not obtain information on the period 2 effect on a longer time-scale due to the FCCP-induced deactivation of the S-states.

Electrogenicity

The charge recombination kinetics during the field pulse depend on the stage of the stabilization process at which the field pulse is applied. Its change as a function of the delay time between flash and pulse should not only reflect the stabilization process, but also reveal any electrogenicity of the reactions involved. This point is clarified by calculations based on the reaction scheme depicted in Fig. 8. Here one stabilization reaction at the donor side and one at the acceptor side are taken into account and before the charge separation all reaction centers are assumed to be in the state $M^{i+}ZQ_AQ_B$. In the absence of an electric field efficient photosynthesis requires that the rate constant of recombination from $Z^+Q_A^-$, k_1, is small compared to the sum of the rate constants of the stabilization reactions, k_2 and k_4. After equilibration at the donor and the acceptor side the equilibrium concentrations Z^+M^+ and $Q_A^-Q_B$ are determined by the equilibrium constants k_2/k_3 and k_4/k_5 respectively and four different redox states are present. In the presence of an electric field k_1 is strongly enhanced. We have calculated the kinetics of $Z^+Q_A^-$ (and therewith of the electroluminescence) according to this scheme assuming that the external field would also increase k_3 with a factor α, but not change k_2, k_4 and k_5. In this case the transition $Z^+M^{i+} \rightarrow ZM^{(i+1)+}$ is electrogenic and the corresponding equilibrium constant $K = k_2/k_3$ is decreased by a factor α. The result for the onset of the field (assumed to be *immediate*) before equilibration is shown in Fig. 8A. The amplitude decreases mainly with $k_1 + k_2 + k_4$. When a field pulse is applied after equilibration (Fig. 8B, solid curves) the initial amount of $Z^+Q_A^-$ is smaller by a factor $\{k_3/(k_2 + k_3)\}.\{k_5/(k_4 + k_5)\}$ (k_3 without field) and this amount will decay with the same kinetics as in Fig. 8A, but now the slow phases due to regeneration of this state via k_3 and k_5 are not negligible anymore. It can be shown that, if k_3 is enhanced by the field by more than a factor $(1 + k_1/k_2)$, the $Z^+Q_A^-$ concentration will actually increase initially. Thus the kinetics of the electroluminescence during the pulse are very sensitive to electrogenicity of the stabilizing reactions, whereas its initial amplitude is simply proportional to the amount of $Z^+Q_A^-$ present just before the pulse.

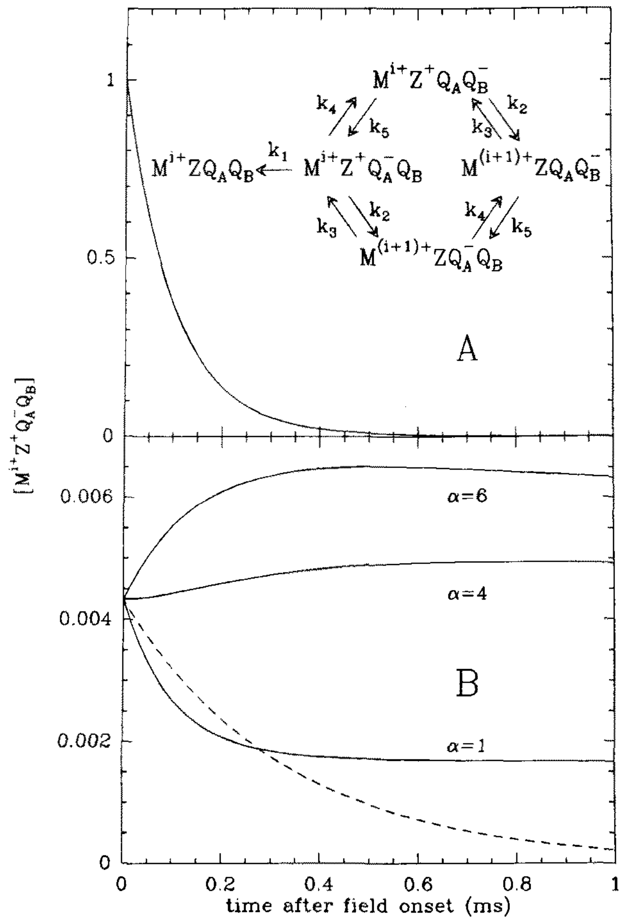

Fig. 8. Calculation of the time-course of the concentration of the state $Z^+Q_A^-$ after the onset of an immediately rising electric field according to the depicted scheme when all centers are in the state $Z^+Q_A^-$ (A) and when equilibrium between the four states has been reached before the pulse, assuming $k_1 = 0$ (B). The solid curves are calculated with $k_1 = 6$ ms^{-1} (in the presence of the field), $k_2 = 2$ ms^{-1} and $k_3 = 0.2$ ms^{-1}(in the absence of the field), $k_4 = 2$ ms^{-1} and $k_5 = 0.1$ ms^{-1}; k_3 is enhanced by a factor α by the pulse. The dashed curve is calculated for the case of no Q_A^- reoxidation by Q_B, i.e., $k_4 = 0$, and modified rate constants at the donor side: $k_2 = k_3 = 0.6$ ms^{-1} (in the absence of a pulse) and $\alpha = 6$. The dashed curve is normalized on the initial value of the solid curves. Its significance will be discussed later.

Analogous results are obtained for the case of an electrogenic acceptor side stabilization reaction and a non-electrogenic donor side stabilization reaction. However, the fact that the kinetics during the pulse of the electroluminescence associated with centers performing the $Z^+M^{3+} \rightarrow ZM$ transition do not significantly change upon reoxidation of Q_A^- implies that the stabilization reaction at the acceptor side is not electrogenic. Electron transfer from Q_A^- to Q_B has also been found to be non-electrogenic in the purple bacterium *Rhodopseudomonas viridis* [37].

The electroluminescence due to the centers in the state Z^+M^{2+} decays during the pulse with a half-time of roughly 0.1 ms (Fig. 6A). As discussed above, the $Z^+M^{2+} \rightarrow ZM^{3+}$ transition itself has a main half-time of about 0.35 ms. At times longer than this transition time an equilibrium concentration Z^+M^{2+} must still be present. If the equilibrium between Z^+M^{2+} and ZM^{3+} were not altered by an external field, i.e., if the $Z^+M^{2+} \rightarrow ZM^{3+}$ would not be electrogenic, then upon the onset of the field the centers which are in Z^+M^{2+} should give rise to an electroluminescence which decays initially in 0.1 ms and be followed by a much slower phase due to the newly installed (pseudo-)equilibrium between Z^+M^{2+} and ZM^{3+} (Fig. 8B, solid curve, $\alpha = 1$). Such kinetics are not observed (Fig. 6B). Instead, after the initial rise in about 10 μs, presumably reflecting the rise of the field, a further rise with a half-time of about 0.1 ms is observed. The signal then decays with a half-time much longer than 1 ms. We therefore conclude that the $Z^+M^{2+} \rightarrow ZM^{3+}$ transition is electrogenic.

The values for the rate constants in the calculations of the solid curves of Fig. 8 were chosen such that they fit with our data for the $Z^+M^{2+} \rightarrow ZM^{3+}$ transition. Comparison of Fig. 6B with Fig. 8B (solid curves) would indicate that, if the electrogenic effect is only reflected in an increase of k_3, this reaction is accelerated about 6-fold. Our data exclude the possibility that the electrogenicity only affects the stabilizing rate constant k_2 (by decreasing it), because in this case the electroluminescence kinetics would always display an initial decay (not shown). If the electrogenicity both decreases k_2 and increases k_3, as would be expected on the basis of reaction rate theory [38], the decrease of K would have to be larger than a factor 6 to describe the observed kinetics. It is reasonable to estimate that in our conditions K is enhanced with a (Boltzmann) factor which is in the order of 10, which corresponds to an electric field-induced potential difference of about 50 mV between Z and the component which is oxidized at the $M^{2+} \rightarrow M^{3+}$ transition.

In our samples a distribution of local field strengths (and hence free energy differences between the redox components) is present during the pulse [7], but the higher field strengths dominate the electroluminescence contribution. At the external field used here, 1500 V/cm, this corresponds to a membrane potential of about 1 V, the maximum membrane potential, above which dielectric breakdown occurs [7]. It is concluded that M^{2+} oxidation by Z^+ spans about 5% of the hydrophobic core of the membrane.

The kinetics of the electroluminescence during the pulse are very similar for the various S-states both before (Fig. 6A) and after (Fig. 6B) Z^+ reduction. This indicates that the reasoning leading to the conclusion that the $Z^+M^{2+} \rightarrow ZM^{3+}$ transition is electrogenic can also be applied to the $Z^+M^+ \rightarrow ZM^{2+}$ and the $Z^+M^{3+} \rightarrow ZM$ (with bound oxygen, see below) transitions. As no evidence for electroluminescence from ZM^+ after Q_A^- reoxidation was obtained, no statement on the electrogenicity of $Z^+M \rightarrow ZM^+$ can be made. We conclude that Z^+ reduction is moderately electrogenic at least in S_2, S_3 and S_0. The predicted percentage of about 5% is within the noise of the electrochromism data of Conjeaud and co-workers [33], which did not reveal any electrogenic stabilization reactions on this time-scale.

Amplitudes of kinetic phases and stabilization potentials of the S-state transitions

One of the aims of this investigation was to determine the equilibrium constants between Z^+ and the S-states. The decrease of the $Z^+Q_A^-$ concentration with each stabilization step is reflected in the decrease of the initial electroluminescence amplitude, i.e., the amplitude reached with the risetime of the field, with increasing delay time between illumination and electric pulse. To enhance the precision, the kinetic analysis was performed on the integrated electroluminescence rather than on the initial amplitudes. When necessary a correction will therefore be made when comparing amplitudes of kinetic phases associated with different signal shapes.

The equilibrium constant of the stabilization reaction due to the Q_A^- reoxidation equals the ratio of the amplitudes of the 0.26 ms and the sum of the amplitudes of the 1.2 ms and 0.1 s phases associated with the $Z^+M^{3+} \rightarrow ZM$ transition. The amplitude of the latter phase is negligible compared to those of the former phases. The amplitude scaling of Table II was chosen such that the amplitude of the 1.2 ms phase due to $Z^+M^{3+} \rightarrow ZM(O_2)$ corresponds to the scaling of Table I; no correction is needed for a difference in kinetics during the pulse as they remain similar before and after Q_A^- reoxidation. It follows that this stabilization reaction has an equilibrium constant of 20 and hence a standard free energy difference of 75 meV. This result is in accordance with reported values for the equilibrium constant $[Q_A^-Q_B]/[Q_AQ_B^-]$ [25,39]. However, we have to mention again that the assumption that the electroluminescence precursor of the acceptor side is in equilibrium with Q_B^- is hard to reconcile with the absence of a period 2 effect on the observed oscillations. Further research is needed to solve this puzzle. For the interpretation of the equilibria at the donor side it suffices at

TABLE III

Equilibrium constants

Amplitudes of electroluminescence at 20 μs after the onset of the pulse and equilibrium constants associated with Z^+ reduction. The latter were calculated as desribed in the text.

	$Z^+M^+ \rightarrow ZM^{2+}$	$Z^+M^{2+} \rightarrow ZM^{3+}$	$Z^+M^{3+} \rightarrow ZM(O_2)$
Ampl. $Z^+Q_A^-$	270	500	790
Ampl. ZQ_A	1.5	5.0	0.62
$[Z]/[Z^+]$	9	5	65
ΔG^0 (meV)	55	40	105

present to know that the reoxidation of Q_A^- is accompanied by a decrease in $Z^+Q_A^-$ concentration by a factor of 20, which does not oscillate with flash number.

We determined the amplitude of the electroluminescence in S_2, S_3 and S_0 before the stabilization reactions had occurred (Table III, row 1) and also when Q_A^- was reoxidized and Z^+ rereduced (Table III, row 2). These data were obtained from Tables I and II after correcting for the difference in kinetics during the pulse of the electroluminescence. The data in the upper row correspond to the sum of the amplitudes of the decay phases ascribed to the $Z^+M^i + \rightarrow ZM^{(i+1)+}$ transition and with Q_A^- reoxidation (the approx. 0.2 ms phases and also the 1.2 ms phase in S_0); those in the second row with the sum of the amplitudes of all decay phases slower than the 1.2 ms phase.

Special care was taken when determining the amplitude of the 0.1 s phase associated with $S_0(O_2)$. The results of Table II were based on S-state fits assuming that all centers can give rise to electroluminescence, irrespective if they made a turnover on the last flash. This may cause minor errors in the time range in the order of the flash interval. When it was assumed that only centers which made a turnover on the last flash contributed to electroluminescence an about 1.5-times larger $S_0(O_2)$ contribution was found. The decay time of 0.1 s equals the flash interval and hence for the value in Table III the average of the amplitudes found with the two procedures was used. It should be noted that the much larger amplitudes of the S_2 and S_3 electroluminescence were relatively much less sensitive to the S-state fit procedure.

With the known 20-fold stabilization at the acceptor side the equilibrium constants and standard free energy changes for electron transfer between Z and the oxygen-evolving complex could be calculated and they are also listed in Table III. It should be stressed that these values apply to the states before and immediately after Z^+ reduction. If the 15 ms and the 400 ms phase are due to stabilizations at the donor side a further stabilization by a factor of at most 6 in S_2 or 2.5 in S_3 is reached (Table II).

The stabilization in S_0 by Z^+M^{3+} reduction is about a factor 65 (Table III). Further stabilization in this state is achieved by a process with a time constant of about 100 ms. As in this state oxygen is released this stabilization is probably of a different nature than the 15 and 400 ms phases in S_2 and S_3. The half-time of oxygen release measured with a bare platinum electrode at weak polarization is, in contrast to that measured with more indirect methods [40,41], also about 100 ms [26], The electroluminescence intensity after completion of the 100 ms phase was at least 10-times lower than the amplitude of the 100 ms phase. This implies that the stabilization by $S_0(O_2) \rightarrow S_0 + O_2$ exceeds a factor 10.

Table III also shows that the electroluminescence depends not only on the amount of $Z^+Q_A^-$ present when the pulse is applied, but also on the S-state. As the electroluminescence kinetics during the pulse do not depend much on the S-state, it seems likely that the luminescence yield is S-state-dependent. A higher luminescence yield in S_3 than in S_2 can also be deduced from 'normal' delayed luminescence data [42] and has also been reported for thermoluminescence [43].

The miss parameter needed to fit oscillation patterns in Photosystem II is presumably due to the presence of equilibrium concentrations of Z^+ and of Q_A^- at longer times after a flash. The equilibrium concentration which we calculated for Z^+ is on the average less than 8% (< 1.5% in S_0, negligible in S_1, 10% in S_2 and 17% in S_3; if the 15 ms and/or the 400 ms phase is due to stabilization at the donor side this number becomes smaller at longer times after the flash). With an equilibrium concentration of 5% Q_A^-, this is roughly in agreement with the observed value of 7% for the average miss parameter.

Non-oscillating electroluminescence

A fast phase with a half-time in the order of 30 μs was present in all decays. The time resolution of our method did not allow a detailed study of this phase. As mentioned above, one would expect a contribution by Z^+ reduction in S_1 [15], but most of it may be due to the non-oscillating centers. It may also be related to the 35 μs phases observed in ordinary luminescence [3,4] and in P-680$^+$ reduction [44], which have been attributed to an apparently non-electrogenic charge separation [29,45]. A small electrogenicity is sufficient to explain the appearance of the 30 μs phase in our electroluminescence measurements, because the involvement of P-680$^+$ would otherwise predict a much larger and more rapidly decaying electroluminescence.

On a longer time-scale the non-oscillating component displays a polyphasic decay and we tentatively attributed it to centers in which Q_A^- reoxidation by Q_B is inhibited. Assuming that no kinetic phases faster than approx. 1 ms are associated with this signal, the amplitude of the signal is about 20% of that associated with

$M^+Z^+Q_A^-$. This indicates that about 17% of PS II was non-oscillating.

The approx. 1 ms phase may reflect modified Z^+ reduction kinetics in these centers. The amplitude ratio of this phase and the sum of the slower phases of the non-oscillating electroluminescence is about 1 (Table I) and hence the equilibrium constant of this stabilization reaction then is also about 1. This value is much lower than the value of 9 found for Z^+ reduction in S_2 in centers in which Q_A^- is reoxidized. With rate constants corresponding to this interpretation the simulation of the $S_2Q_A^-$ electroluminescence kinetics during the pulse as in Fig. 8 with $\alpha = 6$ (dashed curve) indeed confirms the observed kinetics. The inhibition of Q_B reduction alone cannot account for this (a rise of the signal would still occur, not shown). The fact that similar kinetics were observed for 'normal' $M^{2+}ZQ_A^-$ in the presence of DCMU suggests that the modified electron transfer between Z and the oxygen-evolving complex in non-oscillating centers is caused by the presence of Q_A^-. The similarity of the non-oscillating centers with 'normal' centers in the presence of DCMU [29] further suggests that the slower decay phases are, at least partly, due to charge recombination. The 0.8 s phase is attributed to charge recombination, simultaneously with some re-oxidation of Q_A^- by ferricyanide.

Summarizing, the approx. 1 ms decay of the non-oscillating electroluminescence can be ascribed to a $Z^+M^+ \rightarrow ZM^{2+}$ transition, modified by the presence of Q_A^-; the slower decay phases are due to recombination. Further research is necessary to clarify the influence of the redox state of Q_A on the stabilization reactions at the donor side.

Concluding remarks

The electroluminescence technique used here allowed the detection of stabilization reactions in PS II over six time decades. Decomposition procedures for the period 4 oscillations of the S-states yielded the decay of the amplitude and the relaxation of the shape of the signal for each S-state separately. These results constitute a first general survey by the electroluminescence technique of all stabilization reactions in PS II after the state $Z^+Q_A^-$. The interpretation was necessarily tentative and incomplete; many questions must await more extensive studies on specific points. Nevertheless, the results presented here have already allowed several important conclusions and revealed some apparent inconsistencies which may prove useful guides for future research.

Acknowledgements

We thank A.H.M. de Wit, M.L. van der Erf and M.C. Nieveen for technical assistance and Dr. J. Amesz for reading the manuscript. This research was supported by the Netherlands Foundation for Chemical Research (SON), financed by the Netherlands Organization for Scientific Research (NWO).

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