Biochimica et Biophysica Acta 806 (1985) 305–310
Elsevier

BBA 41707

External electric-field effects on photosynthetic vesicles. The relationship of the rapid and slow phases of electrophotoluminescence in hypotonically swollen chloroplasts to PS I and PS II activity

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(Received July 24th, 1984)

Key words: Electric field; Electrophotoluminescence; Photosystem I; Photosystem II; (Chloroplast)

The external electric-field induced luminescence in hypotonically swollen thylakoid vesicles consists of two kinetically different phases, rapid R and slow S, which have been characterized previously (Symons, M., Malkin, S. and Korenstein, R. (1984) Biochim. Biophys. Acta, 767, 223–230). We show that the R phase is elicited by precursors originating in Photosystem I and that S is created through Photosystem II activity. This is substantiated by the difference of the two luminescence phases regarding their action spectrum, sensitivity to electron acceptors, electron-transport inhibitors and heat treatment.

Introduction

Delayed luminescence from photosynthetic membranes has been frequently used to probe electron transport reactions in chloroplasts and photosynthetic bacteria (Refs. 1–5; for recent reviews, see Refs. 6 and 7). Delayed luminescence from green plants has been found to originate predominantly from PS II [1–4]. However, luminescence originating from PS I has been reported in DCMU-treated chloroplasts [8] and has been characterized in PS-I-enriched D-144 subchloroplasts particles (Refs. 8–10, see also Ref. 41). Furthermore, some of the glow peaks in thermoluminescence experiments have been attributed to PS I [11,12,42,43].

The sensitivity of the delayed luminescence to the physical conditions of the membrane has been well characterized [1–4]. Luminescence is strongly stimulated by diffusion potentials (positive inside) [13] and by application of an external electric field [14]. The latter phenomenon has been called electrophotoluminescence [15]. In blebs formed by swelling of chloroplasts in a hypotonic medium, externally applied electric fields can stimulate the luminescence by up to three orders of magnitude [15–17]. This electric-field-induced luminescence in blebs shows two subsequent phases [15], rapid (R) and slow (S). In some conditions (i.e., between pH 4.5 and 6) these two phases can be studied separately [18]. The analysis showed that the two components are created by different precursors which we suggested to be located in different parts of the swollen membrane system: the R precursors in the bleb's wall and the S precursors in the patches attached to the bleb [18]. In this paper we further investigate the dependency of the R and S phases on photosynthetic parameters and inhibition of the electron transport. The results are consistent with the S phase being elicited by PS II and the R phase by PS I.

Abbreviations: ADRY, acceleration of the deactivation reactions of the water-splitting enzyme system Y; ANT 2p, 2-(3-chloro-4-trifluoromethyl) anilino-3,5-dinitrothiopene; DCMU, 3-(3′,4′-dichlorophenyl)-1,1′-dimethylurea; DCIP, 2,6-dichlorophenol-indophenol; PS I, Photosystem I; PS II, Photosystem II.

Materials and Methods

Class C chloroplasts from lettuce and spinach were prepared as in Ref. 18. The chloroplasts were washed and stored in a medium containing 0.4 M sucrose and 10 mM Tris (pH 7.5). Storage of chloroplasts, supplemented by 30% ethylene glycol, was at liquid nitrogen temperature [19]. The experimental set-up was described previously [18]. A typical experiment started by preillumination with either a 10 μs flash or with continuous illumination of variable duration usually filtered by a Corning 4–96 filter, thus limiting the wavelength to a band of about 400–600 nm. For action spectrum measurements the excitation light was selected by appropriate interference filters (Ditric Optics 3 cavity type). After a variable dark time, t_d, an external electric field pulse was applied and the resulting luminescence was monitored. The minimum t_d value was limited by the shutter system used to protect the photo multiplier: 8 ms after a flash and 21 ms after continuous illumination. Unless otherwise stated the luminescence emission was selected by a RG 665 cut-off filter (Schott). Blebs [14,16,18] were formed by dilution of the broken chloroplasts in various hypotonic media (typically by a factor 500). The blebs had a distribution of diameters ranging up to 20 μm in distilled water, while the average diameter varied between 3 and 11 μm, depending on the preparation. Experiments were performed at room temperature. Chlorophyll concentration was about 10 μg/ml.

Results and Discussion

Fig. 1 shows the kinetics of the external-field-induced luminescence with flash preillumination selected for two different wavelengths in the red (640 nm) and the far-red (720 nm) region. The difference in kinetics in the two cases is caused by the difference in extent of the two phases R (rapid) and S (slow), which, under the present conditions, have a rise time of 5 and 60 μs, respectively. The contamination of the S phase by R, at 60 μs after the onset of the field, is minimal shown by the fact that the extent of S at this point is only inhibited by a few percent after addition of 50 μM methylviologen (cf. later on the sensitivity of the R phase to methylviologen).

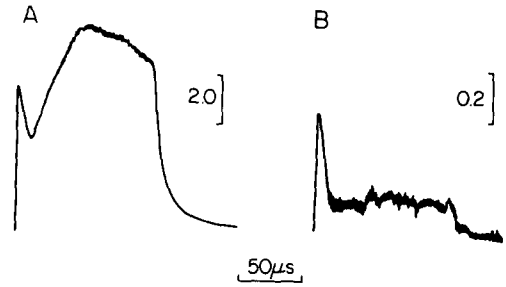

Fig. 1. Kinetics of electric-field induced luminescence elicited by flash illumination at two different wavelengths. Applied field strength was 1.6 kV·cm^{-1}. The dark time, t_d, between flash illumination and electric-field application was 10 ms. Suspension medium contains 10 mM Tris (pH 5). Bleb formation time was 30 min. Luminescence is in arbitrary units. Preillumination wavelength was 640 (A) and 720 nm (B).

In Fig. 2 we plot the R/S ratio vs. the preillumination wavelengths to obtain the ratio of action spectra of the two phases. This determination is relatively easy and devoid of errors that may occur in the determination of each action spectrum separately, as we eliminate the need for light intensity and percent absorption measurements. A criterion for meaningful ratio measurements is that the R/S ratio is intensity independent, for any given wavelength. We checked and verified indeed that the R and S phases show virtually the same dependence on the intensity of the preillumination flash, as varied by neutral density filters, so that R/S was constant. The difference in R/S ratio, varying the preillumination intensity by more than 3 orders of magnitude, is less than the standard

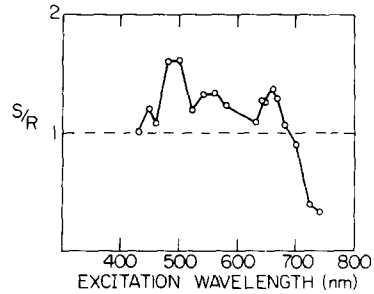

Fig. 2. Relative action spectrum of the R and S phases of field-induced luminescence. R/S ratio is presented as a function of preillumination wavelength (selected by interference filters). Conditions as in Fig. 1. Points are an average of two or three experiments.

deviation (4%) of the measurement. The three prominent maxima around 490, 550 and 660 nm, as well as the sharp drop in S/R above 700 nm are similar to the features of the action spectra ratio of PS II/PS I as determined directly [20,21]. This may be explained readily if one assumes that the R component is elicited by precursors originating in PS I, while the S component originates in PS II. It is interesting to note that when the luminescence emission was filtered through different cut-off filters, a significant enhancement of the R/S ratio (by more than 50%) was observed for cut-off filters transmitting above 700 nm (data not shown), indicating a far red shift of the R component compared to S, in favor of their assumed origins. Indeed it was noted that room-temperature PS I luminescence is predominant at 710 nm [22], while that of PS II peaks at 687 nm [23].

In order to test the attribution of the R phase to PS I and the S phase to PS II we proceeded to test the effect of various electron acceptors. As shown in a previous report [18], the experimental conditions for which the mutual kinetic interference of the R and S phases is minimal is at external-field strengths at around 1.6 $kV \cdot cm^{-1}$, where, at pH 5, the R and S phases have equal extent. Upon addition of a specific PS I acceptor such as methylviologen, the R phase was strongly inhibited while S was only slightly depressed (Fig. 3). The question arises whether the small effect of methylviologen on the apparent S phase is due to some real inhibition of S or perhaps to the tail of R luminescence, which is hidden under S. Fig. 3 demonstrates that the latter case is much more

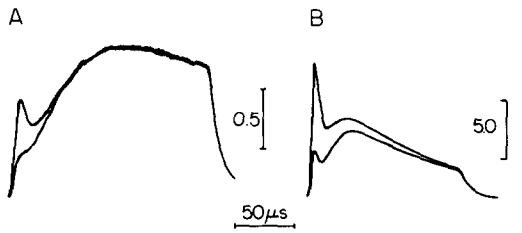

Fig. 3. Inhibition of the electric-field induced luminescence by methylviologen at different applied field strengths. t_d after flash was 10 ms. Suspension medium contained 10 mM Tris (pH 5). Bleb formation time was 30 min. Top trace, control; lower trace, kinetics in the presence of 1.25 μM methylviologen. Luminescence is in arbitrary units. Applied field strengths, 1.2 $kV \cdot cm^{-1}$ (A), 3 $kV \cdot cm^{-1}$ (B).

probable: at low external field strengths, where S is very minimally contaminated by R, S is not affected by methylviologen at all (Fig. 3A), while at higher field strengths, where S is significantly contaminated by the decaying R phase, an apparent inhibition of S is observed. We can thus conclude that the pure S luminescence is not affected by methylviologen at all, substantiating our attribution of R and S to PS I and PS II, respectively. The inhibitory effect of methylviologen on the R component is probably due to the faster decay of the delayed light precursors formed on the reducing side of PS I because of their reaction with the added electron acceptor. A faster decay of PS I delayed luminescence after a flash in the presence of methylviologen has been observed in PS-I-enriched particles [9]. This implies that at 10 ms after the flash (the shortest dark-time allowed by our electronic shutter) the amount of precursors which can recombine under the influence of the electric field has diminished to an extent which is determined by the methylviologen concentration.

The effect of methylviologen on the R phase was reproduced by other viologens. The three viologens available to us were diquat (midpoint redox potential −354 mV [24]), benzylviologen, (−359 mV [24]), and methylviologen (−446 mV [24]). The concentration dependence of the effect of the viologens on the R phase is compared in Fig. 4. In correspondence to its higher midpoint potential it is seen that diquat is about twice as effective as methylviologen. Benzylviologen has exactly the same effect as methylviologen, although one would expect from its higher midpoint potential that it would be more effective.

For the experiments in Fig. 4 the blebs were prepared in an Mg^{2+}-containing medium. In the absence of salt, the effect of the viologens is noticed in concentrations which are one or two orders of magnitude lower (not shown). This higher effectiveness can be explained by the negative surface charge of the thylakoid membrane which, at low ionic strength, establishes a negative surface potential and concentrates the positive viologens in the double layer next to the membrane surface [26]. The experimental results, at low ionic strength, are less reproducible however.

We also tested the effect of other artificial

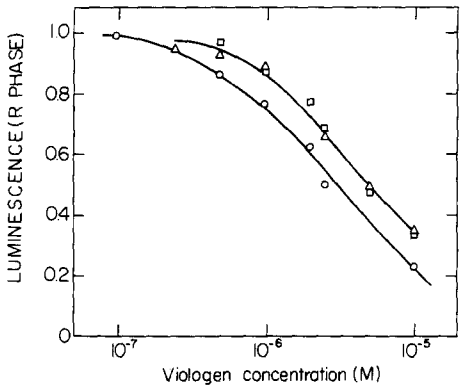

Fig. 4. Concentration dependence of the inhibition of the R component of the field induced luminescence by different viologens. Applied field strength was 1.6 kV·cm^{-1}. t_d after flash was 10 ms. Suspension medium contained 10 mM Tris (pH 5)/4 mM $MgCl_2$. Bleb formation time was 25 min. ○——○, diquat; □——□, benzyl viologen; △——△, methyl viologen.

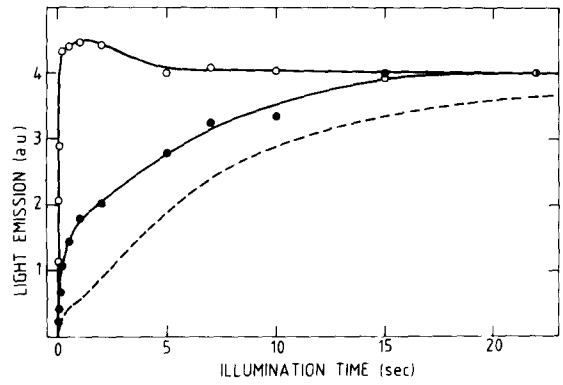

Fig. 5. Fluorescence induction and preillumination time dependence of R and S components of electric-field induced luminescence. t_d was 21 ms. External field was 1.6 kV·cm^{-1}. Illumination intensity was 0.7 nE·cm^{-2}. Blebs were formed by suspension of the broken chloroplasts in distilled water for 30 min. ○——○, R component; ●——●, S component; broken line, variable part of fluorescence.

electron acceptors such as DCIP, ferricyanide, diaminodurene and silicomolybdate. It is known that these compounds can accept from both photosystems in a preparation-dependent manner [26–28]. We found that after addition of 20 μM DCIP, at pH 5–6, both R and S were inhibited, although to different extents, about 80% and 30%, respectively. This is in line with the findings of Kok et al. [25] who observed a very efficient reduction of DCIP by PS I and a much slower one by PS II. Ferricyanide (1 mM) strongly inhibited R and did not affect S. Oxidized diaminodurene, 100 μM, appeared to have little effect on R and inhibited S by 30%. Silico molybdate at 1 μM inhibited both R and S by about 70%.

It has been shown for a variety of experimental conditions that the decisecond component of the delayed luminescence has a linear relationship with the variable fluorescence yield [31–33]. The variable fluorescence is an indicator of the reduction of the primary acceptor Q of PS II [23]. We therefore expected to find a correlation between the variable fluorescence rise and the illumination time dependence of the S phase. As shown in Fig. 5 the S component grows in time in two kinetic phases with the slow one displaying a close correlation with the variable fluorescence rise. The initial rise kinetics of S might reflect the building up of an oxidized electron donor. This is a much faster process due to its limited pool size, compared to the large plastoquinone pool at the reducing side. A similar kinetic behaviour was observed when delayed light was compared with the variable fluorescence which was explained by a direct involvement of Z (the electron donor to PS II) as a precursor in the production of delayed light [32]. The R component reaches saturation at very early time and is not correlated at all to the variable fluorescence. Upon addition of DCMU the induction of the fluorescence and the S phase of the stimulated luminescence kinetics are accelerated to a similar extent, whereas the induction of the R phase is slightly retarded (not shown).

Further evidence for the origin of R and S came from heat treatment experiments. It is well known that PS II is more heat-sensitive than PS I [34]. This is essentially what is demonstrated in Fig. 6, depicting the behaviour of the amplitudes of R and S. It can be seen that upon preheating the chloroplasts from 20°C on, the S phase starts to be inhibited at 40°C, whereas R is stimulated at this temperature and reaches a maximum around 50°C from where it declines as the temperature still increases. R vanishes at temperatures above 55°C. This is lower than the inactivation temperature of PS I activity, which is about 65°C [9,35]. This is

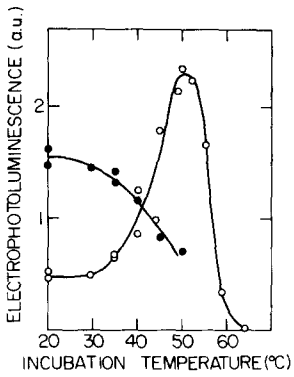

Fig. 6. Temperature sensitivity of R and S components of electric-field induced luminescence. A concentrated suspension of chloroplasts (3 mg/ml chlorophyll) was incubated at the indicated temperature for 5 min. Subsequently blebs were formed in 10 mM Tris (pH 4.5) for 10 min. t_d after flash was 10 ms. Applied field strenght was 1.6 kV·cm^{-1}. O———O, R component; ●———●, S component.

probably due to the additional effect that bleb formation, as monitored by phase contrast microscopy, is inhibited at high temperatures, being inexistent after incubation of the chloroplasts at 60°C. The remarkable stimulation of the R phase by heat treatment appears to be correlated with an increase in average bleb size, as inspected by phase contrast microscopic (not shown), but it is quite difficult to quantify this phenomenon. On the other hand the increase in R might also be related to changes in PS I, as was observed in PS I particles [9,22]. In those preparations it was shown that a 20 ms luminescence component could be induced by heating the sample. The induction of this luminescence phase paralleled the inactivation of P-430, the acceptor of PS I. This 20 ms luminescence was maximal after incubation at 65°C. At higher temperatures the luminescence decreased in parallel with the inactivation of P-700, the primary electron donor.

We studied in a preliminary way some other inhibitory treatments and inhibitors which act on the water oxidizing site of PS II. Blebs originating from Tris-treated chloroplasts showed a considerable increase in the S phase. This effect was most marked at pH 6, where S increased by about a factor 3. R was unaffected by Tris treatment. Similar results were obtained by addition of 2 mM hydroxylamine. On the other hand, ANT-2p, which is the most effective ADRY reagent [35] and therefore is expected to affect only PS II, inhibited both R and S phases in a very similar way: 0.5 μM decreased the two phases by more than 50%. At the moment the only explanation which can be forwarded stems from a report [36] that the ANT-2p also acts as an acceptor from PS I in *Chlamydomonas reinhardtii,* which may be quite a general effect.

Concluding remarks

In this paper we give evidence that the rapid (R) phase of the electric-field induced luminescence is associated with PS I. The slow (S) phase is evidently created by precursors elicited by PS II. We have suggested previously that the precursors of the R component are located in the bleb wall and S in the patches attached to this wall [18]. Most likely those patches are formed by stacked grana membranes [37] and the bleb wall by the stroma region of the thylakoid membrane. This is in line with the now extensively documented findings that PS I is predominantly located in the stroma lamellae and PS II in the grana region [38–40].

Acknowledgements

We would like to acknowledge Mordhai Avron and Yosepha Shahak for the many stimulating discussions. This research was supported by the United States – Israel Binational Science Foundation.

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