ELECTROSELECTION IN THE PHOTOSYNTHETIC MEMBRANE: POLARIZED LUMINESCENCE INDUCED BY AN EXTERNAL ELECTRIC FIELD

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1. Introduction

Delayed luminescence emission (a general property of photosynthetic systems) is closely related to primary electron flow and energetic events in photosynthesis [1,2]. In particular, electrical phenomena at the membrane level have a marked influence on the emission; thus, for example, an artificially induced diffusion electric potential (positive inside) [3] or a change in the dielectric constant of the membrane [2] have been shown to stimulate delayed luminescence.

An efficient and kinetically unique method to induce transmembrane potentials in a suspension of membrane-bound vesicles is the application of an external macroscopic electric field [4]. In this way, delayed luminescence can be significantly enhanced (by 1–3 orders of magnitude) [5,6], especially in hypotonically extensively swollen particles originating from the chloroplasts (blebs [7]). This phenomenon, termed electrophotoluminescence (EPL) [6] has interesting kinetic features, not yet understood. The role of the electric field is explained [6,8] generally in terms of the primary photosynthetic charge separation in photosystem II, its vectorial nature in the membrane [9] and an additional term in the activation energy for its back reaction introduced by the transmembrane field [8]. The resulting charge recombination produces the chlorophyll excited singlet state, giving rise to luminescence.

In order to obtain additional information on the mechanism of EPL production and its relation to membrane topology, one can make use of the directional nature of the external electric field as a triggering agent. Since the electric field induced within the membrane by the external field has a strong angular dependence on the external field direction [6], one expects different contribution to EPL by various regions of a vesicle's membrane. On the other hand, the chromophore responsible for the emission (chl *a*) has a preferential orientation within the membrane [10,11]. In view of this, a relatively high degree of polarization of the field induced emission is to be expected. We report here a study of EPL emission induced by DC electric pulses using polarized detection. The results show a significant electroselection in the membrane, and are consistent with an orientation of the long-wavelength transition moment Q_y of chlorophyll *a* in photosystem II parallel to the plane of the membrane [12,13]. The kinetics and field dependence of the emission polarization are quite complex. Some of its features can be explained by assuming the existence of two mechanistically different components of EPL emission, and a rapid adjustment of the membrane and its ionic environment to the external electric field.

2. Materials and methods

Chloroplasts were prepared from lettuce, pea and tobacco leaves (with no significant differences in the results), essentially according to [14], and stored for long periods of time at low temperatures [15] with complete preservation of all relevant photosynthetic activities. 'Blebs' [7] were obtained by resuspending the stock chloroplasts in distilled water, at final conc. 100 μM NaCl and 10–50 μg/ml chl, in most cases. A pronounced swelling, completed within minutes, was induced by the hypotonicity of the medium, yielding quasi-spherical objects of 3–10 μm diam. and 10^2–10^3 μm^3 vol. (blebs) as in [7,16–18]. In isotonic media, no 'blebbing' occurred. The samples

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used in the experiments were characterized (fig.1a,c) by fluorescence microscopy coupled to an image-intensified video camera [29].

The experimental set-up included an E-jump cell (similar to that in [19]) a timing circuitry and a high-voltage pulser (Cober 606) capable of delivering rectangular DC pulses of up to 2500 V (corresponding field value E = 3200 V/cm), with very fast rise and fall times (<1 μs) and a variable duration of 1 μs–10 ms. Preillumination of the sample was provided by either saturating 10 μs flashes or continuous light passed through a Corning 4-96 filter. The luminescence emission was detected by two photomultipliers (EMI 9558 B and Hamamatsu R376) situated at 180° from one another, perpendicularly to both the preillumination and the external electric field direction. In front of the photomultipliers two Schott RG 5 ($\lambda > 665$) cut-off filters and KS-DEM (Käsemann, Oberaudorf) polarizers were placed. Appropriate checks for the absolute directions of

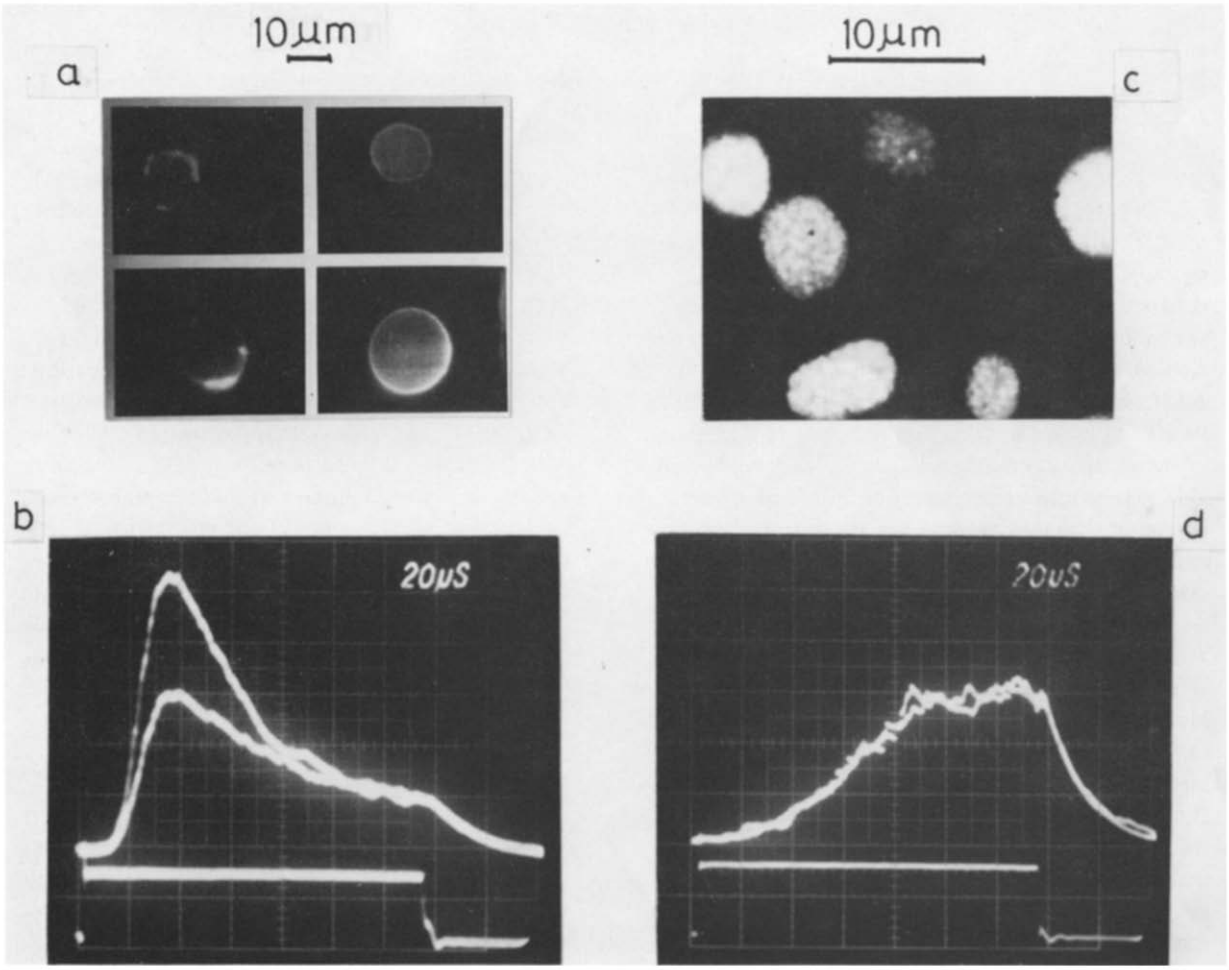

Fig.1. Polarization of EPL emission and its dependence on sample preparation. (a) Typical 'blebs': These large spherical particles are bounded by one pigmented membrane, with occasional 'patches' of higher fluorescence (left) probably representing aggregated or partially stacked membranes. Often these patches are absent (right). Conditions: pea chloroplasts resuspended in distilled water, at 100 μM NaCl and 25 μg/ml chl (pH 7.8). Fluorescence microscopy with image intensification [29]. (b) EPL emission from blebs: upper trace, $EPL_{\perp}$ (see text); middle trace, $EPL_{\parallel}$; lower trace, the applied electric field pulse shape (E = 2200 V/cm); time scale, 20 μs/division. (c) Chloroplasts in (quasi) isotonic buffer: 10 mM Tricine, 0.2 M sucrose, 10 mM NaCl, (pH 7.8); the highly fluorescent 'granules' inside the large particles represent the thylakoids. (d) EPL emission from chloroplasts (as shown in (c)): The two upper traces are $EPL_{\perp}$ and $EPL_{\parallel}$, respectively (detection sensitivity 5-times higher than in (b)). The lower trace is the electric field pulse (identical to (b)). Time scale 20 μs/division.

polarization and the intrinsic polarization of the detection system were made [20]. The signals from the photomultipliers were monitored on fast-storage oscilloscopes (Tektronix 7313, 7623 A and Nicolet Nic 1170) and photographed.

A typical experiment consisted of a 10 μs flash, followed by a 5–500 ms dark time in which the natural luminescence decayed, a 20–5000 μs electric field pulse (E = 500–3000 V/cm) and monitoring of delayed luminescence, the field pulse and the resulting electrophotoluminescence (EPL), with one of the polarizers perpendicular and the other parallel to the external electric field direction.

3. Results

Upon application of the rectangular electric field pulse to the preilluminated suspension of 'blebs', a burst of luminescence (EPL) was observed, 1–3, orders of magnitude more intense than the original delayed luminescence corresponding to the same dark time. Two typical signals of this kind are shown in fig.1b, corresponding to detection through polarizers with their axes perpendicular and parallel to the external field direction ($EPL_\perp$ and $EPL_\parallel$, respectively). The general kinetic behaviour of both signals was similar in that they were produced after a certain lag time, increased to a maximum and decreased towards a steady-state during the field pulse. $EPL_\perp$ showed much more pronounced maximum than $EPL_\parallel$. Upon termination of the field pulse both polarization components decayed simultaneously to the background level of regular delayed luminescence. The relative magnitude and kinetic features of these phases were strongly dependent on the intensity of the applied field, the dark time t_d elapsing between preillumination and electric stimulation, the parameters of the medium (osmolarity, viscosity, ionic strength, pH, temperature) and the state of the photosynthetic membrane (in preparation).

Regarding polarization, the following characteristics can be distinguished:

(1) *Phenomenology and kinetics (fig.1b):* The (field-dependent) lag time is similar for both polarizations. Immediately after the lag phase $EPL_\perp$ is higher than $EPL_\parallel$. We define a (time-dependent) polarization ratio q as:

$$q = EPL_\perp/EPL_\parallel \quad (1)$$

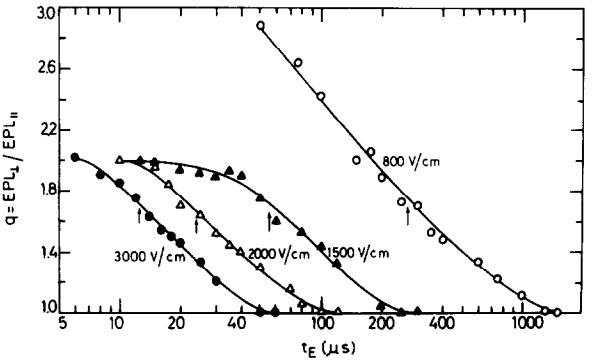

Fig.2. Polarization ratio variation during the electric field pulse. t_E, time elapsed during the pulse, from its application (logarithmic scale). Numbers on the curves indicate the intensity of the external electric field. The arrows show the position of the emission peak of EPL. Conditions: 'Blebs' from pea chloroplasts, t_d = 200 ms, 100 μM NaCl (pH 7.8).

This ratio changes during the field pulse, as illustrated in fig.2. It is initially maximal through the rising phase of EPL and decays afterwards, tending towards 1 (i.e., no polarization), reaching ultimately this value for experiments where the pulse length is sufficiently long. This steady state of no polarization coincides with the steady state of the EPL intensity, which is still much higher than the natural delayed luminescence. If the exciting field pulse is interrupted before the steady state is reached ($q > 1$) there is a parallel decay of both the EPL intensity to the level of the original luminescence and to $q = 1$, occurring usually within a few tens of μs.

(2) *Electric field dependence:* The initial maximal value for q, obtained during the rising phase of EPL depends on the field intensity in a rather interesting manner. It reaches quite high values ($q \approx 3$) at relatively low fields (800 V/cm, the lowest field value at which measurements were not distorted by noise), and diminishes at higher fields, tending apparently to a field-independent limit around $q = 2$, which is observed in the range 1500–3000 V/cm. The field at which the behaviour changes from the high to low limit values is somewhat variable from experiment to experiment and seems to be dependent mainly on the size and condition of the blebs. The decay of q during the field pulse is faster the higher the field intensity.

(3) *Angular dependence:* We confirmed that the parallel and perpendicular polarization with respect to the field direction represent indeed the

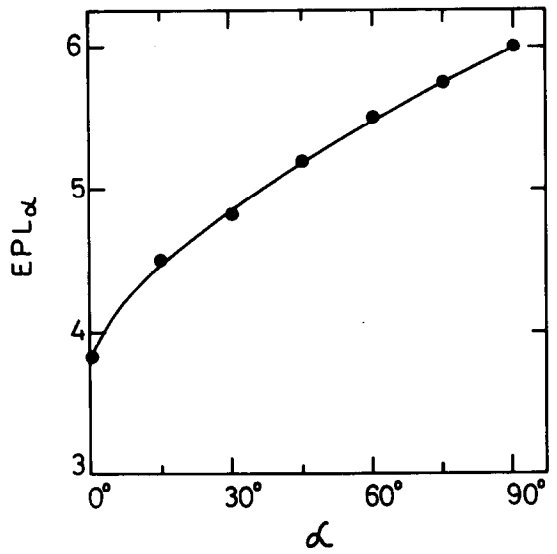

Fig.3. Angular dependence of EPL emission: α is the angle between the axis of the detection polarizer and the direction of the externally applied field. EPL was measured, at its peak, for different α values (EPL_α-relative units). Conditions: as for fig.2, E = 2000 V/cm.

minimum and maximum intensitites, respectively. Fig.3 represents an example for the angular dependence of the EPL (intensity as a function of the polarization angle α). Similar dependencies were observed for different field values and dark times.

(4) *Structural requirements:* In order to obtain polarization, 'bleb' formation seems to be a prerequisite (fig.1a), as chloroplasts under normal conditions (fig.1c), although exhibiting EPL (fig.1d), show practically no polarization of the emission. This is further substantiated by the dependence of the polarization on the conditions of the resuspension medium, as summarized in table 1.

(5) *Other features:* The polarization ratio and its variation during the field pulse are markedly influenced by the dark time (t_d). As a general rule, the shorter this dark time, the higher the polarization ratio and the faster it rises and decays during the pulse (for a given field value).

A sequence of several unidirectional pulses following a single preillumination are able each to excite EPL. However, EPL intensity decreases markedly as a function of the pulse number and in particular the sharp maximum tends to disappear already in the second pulse. This phenomenon is paralleled by a decrease of the initial maximal polarization ratio q. (e.g., a drop from q = 2 to q = 1.2 in the second pulse and no polarization in the third).

4. Discussion

Delayed luminescence from in vivo chl. *a* is currently explained as being a consequence of the back-reaction of the primary photosynthetic charge separation [1,2]. The recombination reactions require activation energy in order to produce the excited singlet state of chl *a*. The entities involved in this process (the primary donor and acceptor of photosystem II) have a precise location within the photosynthetic

Table 1
Effect of the suspension medium on the polarization of EPL

Resuspension medium[a]	'Blebs'	Av. 'bleb' size	EPL intensity at the peak of emission	Polarization ratio ($EPL_\perp/EPL_\parallel$) at the peak of emission[b]
H_2O	Present	Large	100	1.8
Isotonic buffer	Absent	–	40	1.0
Glycerol (20%, v/v)	Absent	–	20	1.0
H_2O → Isotonic buffer	Present	Large → Small	60	1.5 → 1.1[c]
H_2O → Glycerol	Present	Large → Small	30	1.4 → 1.1[c]
H_2O + $MgCl_2$ (3 mM)	Present	Large	150	1.4

[a] Chloroplasts from the stock (section 2) were resuspended in the media indicated in the first column, at pH 7.8 and the same [chl]
[b] This parameter has been chosen to facilitate comparison of signals with different kinetics
[c] This is a dynamic range, as the ratio changes in time following the addition of buffer or glycerol, in parallel to the slow shrinkage of the 'blebs'

membrane [9,21]. Assuming that the direction linking them is approximately perpendicular to the plane of the membrane [21], one can explain the ability of a properly oriented transmembrane electric field to enhance delayed light emission by assuming that the field destabilizes the photoinduced charge separation state, lowering the activation energy for the back-reaction(s) and facilitating recombination along the field direction. In the case of a diffusion-potential induced field [3] this is true for the whole membrane, while in the case of an external electric field only part of the membrane (for a spherical vesicle, a hemisphere) will have the local field oriented properly for enhancing emission.

As pointed out in [6], the magnitude of the enhanced signal (EPL) as compared to delayed luminescence indicates that it is induced in the membrane by an electric field much stronger than the externally applied one (10^5–10^6 V/cm *vs* 10^3 V/cm). In order to explain this local enhancement, they proposed the following simple model (fig.4):

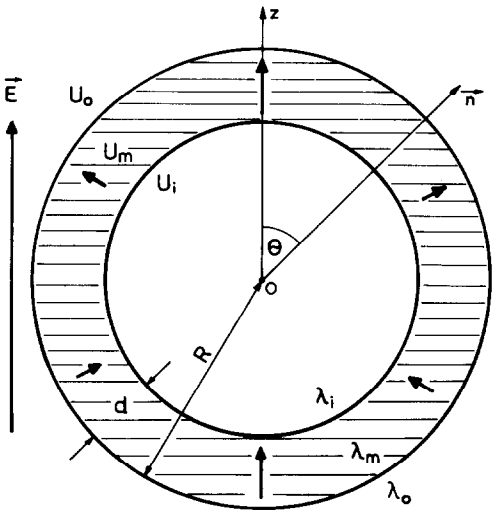

Fig.4. Model for a spherical membrane-bounded particle in an external electric field: E, the externally applied electric field (directed along the 0_z axis); θ, angle between the field direction and the normal to the plane of the membrane, at a certain point; U, electric potential; λ, specific conductivity; R, radius of the vesicle; d, membrane thickness; subscripts i, o and m stand for the inner, outer and membrane phase, respectively. Thick arrows within the membrane indicate the direction and angular dependence of the intensity of the intramembrane field (E_m, see text).

When a spherical particle of radius R, bounded by a membrane of thickness d and much lower conductivity than either the inner or the outer medium, is placed in an external electric field of strength E, the intensity of the radial field E_m at a certain point within the membrane can be written as [6,22]:

$$E_m = \frac{9}{2} \frac{1}{1-\left(\frac{R-d}{R}\right)^3} E \cos\theta \qquad (2)$$

where $\cos\theta$ is the angle between the external field direction and the normal to the membrane surface at that point.

If the membrane thickness d is much smaller than the radius R, eq. (2) becomes:

$$E_m = \frac{3}{2} \frac{R}{d} E \cos\theta \qquad (3)$$

Typical values to be inserted in eq. (3) are d = 5 nm [6,17,18,23] and R = 5 μm (average of our size distribution; [17]), yielding E_m^{max} = 1500 E for the pole of the spherical vesicle ($\cos\theta$ = 1).

The angular dependence of the local field and the presumably monotonous dependence of the emission on the field intensity result in more emission coming from regions near the pole (i.e., where the field is maximum). To obtain polarization the emitting pigment must have a definite orientation with respect to the membrane. The highest polarization in the perpendicular direction is consistent with the view that the chl *a* long wavelength transition moment Q_y is alligned mostly in parallel to the membrane surface, as concluded from linear dichroism [24], polarized fluorescence [25,26] and other methods [27].

We thought of several reasons why the polarization ultimately decays. The relative importance of membrane regions further away from the pole may increase during the field pulse because:

(i) Stronger initial depletion of precursors at the pole tends ultimately to equalize the contributions from different parts of the sphere;

(ii) The local strong field induces partial conductance in the membrane (dielectric breakdown [28]) and tends to decrease the field. This effect is presumably stronger at the pole;

(iii) EPL may consist of two mechanistically different components (the R and S components [6]).

To explain the kinetics, field dependence and E_d dependence of the polarization, one can make the following assumption regarding the kinetic components R and S: One component (R) is largely polarized and is more pronounced in the first part of the field pulse. This component contributes to the increase and decrease with a sharp maximum. The second component (S) is unpolarized and has shallower kinetics of mainly monotonous increase to a steady-state. The different properties of the polarization of the two components may stem from the field dependence of each. The S component is largely field-independent in the field range used by us (see also [6]) while the R component is strongly field dependent. The polarization changes as the ratio between the two components varies, either in time, or at different conditions including t_d. For example, the absence of polarization in chloroplasts and its lower values in smaller 'blebs' or in presence of $MgCl_2$ (table 1) could be due to the fact that in these cases the majority of the emission is S-type, unlike in distilled water. This seems to be of more importance for polarization than the actual intensity of EPL emission which is enhanced by magnesium.

(iv) A field-induced orientation effect [30].

However, the orientation time of the whole chloroplasts [12] and 'blebs' is in the order of seconds, while intramembrane orientation of the emitting chromophore should lead to polarization increasing with the strength of the applied field, which is not the case (fig.2). Moreover, the (unlikely) additional assumption of reciprocal perpendicularity of the electrical dipole moment and emission transition moment would have to be made.

The higher EPL emission polarization ratios observed for relatively low field values at the onset of the pulse can be explained by the electroselection mechanism as follows:

The rise of the transmembrane field takes time and since there is a threshold value of it for EPL production [6] it can be assumed that in the early stages of the pulse, only regions with low θ values (see fig.4) will contribute to emission.

The conclusions of this qualitative discussion are supported by detailed calculations on specific models, taking into account the actual field dependence of electrophotoluminescence (in preparation).

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