*Biochimica et Biophysica Acta* 850 (1986) 218–225
Elsevier

BBA 42027

# Electro-induced changes of chlorophyll fluorescence in individual intact chloroplasts

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(Received December 24th, 1985)

Key words: Electron transport; Photosystem II; Membrane potential; Chlorophyll fluorescence; (*Anthoceros, P. metallica*)

**The intensity of chlorophyll fluorescence in isolated chloroplasts of *Peperomia metallica* and in situ chloroplasts of *Anthoceros sp.* was found to change by 5–10% on passing the electric current through the microelectrode inserted in the intrathylakoid space. The dependence of fluorescence deflections on the direction of a current flow and the absence of electro-induced fluorescence changes in chloroplasts made ion-permeable with gramicidin indicate that the emission was affected by the membrane potential created during the passage of current. A hyperpolarizing displacement of the membrane potential, positive on the inside, produced an increase in the emission in the absence of inhibitors, but has no effect on the fluorescence in chloroplasts treated with DCMU. A stimulation of a variable fluorescence by a positive membrane potential was interpreted as due to slowing down of charge separations and a respective decrease of excitation trapping in Photosystem II. A displacement of the membrane potential to the negative direction resulted in a decrease of chlorophyll fluorescence in both the absence and presence of DCMU. The fluorescence emission seems to be sensitive to variations of the membrane potential of about 100 mV or even less. Possible effects of the light-induced membrane potential on charge separations and the fluorescence emission in Photosystem II are discussed.**

## Introduction

The light-induced membrane potential in chloroplasts originates from a combined operation of several spatially separated electrogenic processes associated with PS I and PS II [1]. Variations of the membrane potential may influence the rates of forward and backward electron transport across the membrane at different sites of photosynthetic chain [2–5]. The potential-sensitive steps of electron transport are often studied through the application of an external electric field to a suspension of osmotically swollen chloroplasts. Particularly, a delayed light emission from photosynthetic membranes is highly stimulated by an external electric field [6–10]. This phenomenon, called electrophotoluminescence [7], is due to a strong effect of the membrane potential on the reverse electron transport in reaction centers of PS II. Meiburg et al. [11] reported recenlty that an application of an external electric field to a suspension of large spherical vesicles (swollen chloroplasts) brings about either an increase or a decrease in the yield of chlorophyll fluorescence, depending on the state of reaction centers of PS II. It has been suggested that excitation trapping and the light-induced

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; PS, Photosystem; P-680 and Q, the primary electron donor and the primary electron acceptor in the reaction center of Photosystem II, respectively.

electron transfer in PS II are only influenced by a large membrane potential opposing photochemical charge separations [11].

One obvious disadvantage of an external application of an electric field is that it induces membrane potentials with opposite polarities in each half of a spherical vesicle. The fluorescence changes induced in each hemisphere may superimpose which complicates the interpretation of the results. Therefore it seems appropriate to find out whether the yield of chlorophyll fluorescence is sensitive to an electric field imposed between the inside of thylakoids and the external solution. In this paper we describe changes of chlorophyll fluorescence of individual intact chloroplsts, both isolated and located within the cell, induced by applying an electric field across the thylakoid membrane by means of current injections through the microcapillary electrode. It is shown that chlorophyll fluorescence in intact chloroplasts is enhanced or quenched, depending on the polarity of the membrane potential displacement. The results are discussed in terms of a regulation by a membrane potential of electron transfer reactions in PS II.

## Materials and Methods

Experiments were done on in situ chloroplasts of *Anthoceros* and isolated chloroplasts of *Peperomia metallica.* Growth conditions of plants and procedures for chloroplast preparations were essentially the same as described in Refs. 12 and 13. Thin sections of *Anthoceros* thalli were placed in a medium containing 10 mM KCl/1.0 mM NaCl/0.5 mM $Ca(NO_3)_2$/5 mM Hepes-NaOH buffer (pH 7.0). Isolated chloroplasts were immersed into the medium containing 0.25 M sucrose/25 mM Tris-HCl buffer (pH 7.5)/10 mM KCl/25 $g \cdot l^{-1}$ phycoll and bovine serum albumin (1 mg/10 ml solution).

The experimental arrangement for microelectrode and fluorometric recordings on single chloroplasts was similar to that previously described [14]. Chlorophyll fluorescence was excited with a broad band blue light through the microscope epi-illuminator. The actinic light was focused on the preparation at a spot of 50 $\mu$m in diameter. Fluorescence transmitted by a red filter with a bandpass above 650 nm was detected by a microscope photometer. A set of apertures installed in front of a photomultiplier allowed to delimit the area of photometric assay to a diameter of 50 $\mu$m which was sufficient for collecting light emission from a single chloroplast.

Measurements of light-induced membrane potentials and the passage of current in the same chloroplast were accomplished with microcapillary electrodes having a resistance 100–150 M$\Omega$ when filled with 1 M choline chloride. By means of a switch with a remote control, the lead from the microelectrode assembly was connected either to an input of a high-impedance amplifier for potential measurements or, alternatively, to an output of a pulse generator through a 1.5 G$\Omega$ load resistor for passing current. Depending on the commutation of the microelectrode, either light-induced membrane potential or the current flowing through the membrane was displayed at one channel of a dual-beam oscilloscope. A signal from photomultiplier was continuously displayed at the other channel of an oscilloscope.

Microelectrode impalements were done under weak transmitted light with a photomultiplier shielded from illumination. After the insertion of a microelectrode, the transmitted light was switched off and light-induced changes of the membrane potential and chlorophyll fluorescence were recorded. This procedure served as a test for a successful penetration of the microelectrode tip in thylakoids and for proper scaling the fluorescence signal. After that, microelectrode was switched to the current circuit and the experiment proceeded on the same chloroplast. Within the course of one experiment, the connection of the microelectrode could be repeatedly switched between measuring and current circuits.

## Results

### *Effect of imposed membrane potential on chlorophyll fluorescence*

By recording a characteristic light-induced membrane potential it was possible to verify the intrathylakoid location of a microelectrode tip in each particular experiment. The time-course of the membrane potential formation was multiphasic in dark-adapted *Anthoceros* chloroplasts [13,14], but

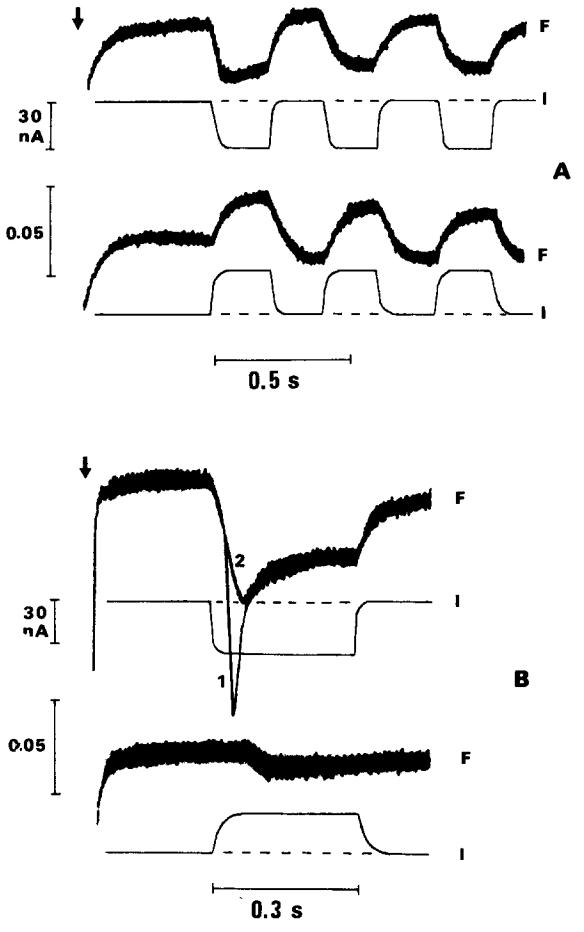


Fig. 1. Changes of chlorophyll fluorescence (traces F) of individual *Anthoceros* chloroplast induced by injecting pulses of current (traces I) through the microelectrode. Deflections of I from a baseline downward correspond to an inward current and a negative displacement of the membrane potential, while upward deflections correspond to an outward current and a positive shift of the potential. (A) Control; (B) in the presence of 10 $\mu$M DCMU. The fluorescence trace denoted 1 shows a response to a first application of a current and the trace denoted 2 shows reproducible responses to seven subsequent pulses of a current applied in succession with pulses of opposite polarity at a frequency of one per min (1/60 Hz). Arrows indicate the beginning of illumination.

it looked simpler in preilluminated *Anthoceros* chloroplasts and isolated plastids of *P. metallica*. A positive electric potential attributed to the inner volume of the thylakoid system [15] was rapidly generated in the light and decayed gradually within one or several seconds to a stationary level. After verifying the internal location of a microelectrode tip, light exposures were applied in a combination with pulses of an electric current.

Fig. 1 shows typical changes of chlorophyll fluorescence induced in *Anthoceros* chloroplasts by 30 nA current pulses of opposed polarities in the absence and in the presence of DCMU. Under control conditions (Fig. 1A), the displacement of the membrane potential by an inward current to the negative, i.e., in the direction opposed to the polarity of light-induced membrane potential, resulted in a decrease of a chlorophyll fluorescence, and the positive displacement of the membrane potential in the direction coinciding with the light-induced potential produced an increase of chlorophyll fluorescence by a similar or somewhat smaller extent. The fluorescence changes were reversible and constituted 5–10% with respect to the overall intensity of a fluorescence. The rates of electro-induced fluorescence changes were roughly similar to that of discharging the membrane capacity of thylakoids after a flash [15].

On addition of 10 $\mu$M DCMU, a total intensity of the fluorescence increased almost twofold indicating a complete reduction of a primary acceptor of PS II, Q. Under these conditions (Fig. 1B), the negative displacement of the membrane potential by an inward current produced a decrease of the emission with a sharp minimum, but the positive displacement of the potential had no effect on the fluorescence or – occasionally – produced a small decrease of the fluorescence after a long delay. Biphasic changes of the emission with a transient minimum could also be induced in untreated chloroplasts by applying inward currents of higher strength.

When a treatment of chloroplasts with 1 mM hydroxylamine and 0.1 mM methyl viologen was used to keep the primary acceptor Q largely oxidized in the light, we found a strong expression of the current-induced fluorescence increase, but a weak one if any current-induced fluorescence was quenching. From the above experiments it appears that the chlorophyll fluorescence can be stimulated or suppressed by the imposed voltage only within the range between $F_0$ and $F_{max}$, where $F_0$ and $F_{max}$ are the fluorescence yields in chloroplasts with fully open and completely closed PS II reaction centers, respectively.

Apart from a shift of the membrane potential

by a current flow, the interfering concomitant factors were considered and rejected as a possible source of electro-induced fluorescence changes. These changes of emission were not caused by a local heating of a chloroplast as evidenced by their dependence on the polarity of an applied voltage. Passing of a current did not produce any discernible change in the position or geometry of a chloroplast, both isolated and located within the cell. Electrophoretic injection of choline from a microelectorde tip was not involved, since similar fluorescence changes were recorded with KCl-filled microcapillary electrodes. By applying short bipolar current pulses known to minimize the electrophoretic injection of an electrolyte, we observed an intermittent stimulation and a decrease of a fluorescence.

Additional tests were done on chloroplasts treated with gramicidin D (Boehringer Mannheim GmbH). Since this ionophore increases the conductance of lipid bilayers and biological membranes it was expected that the membrane voltage produced by the passage of an electric current would be diminished. In accord with the expectation, in *Anthoceros* chloroplasts treated with gramicidin at 1 $\mu$m 'overall' concentration, there were no detectable electro-induced changes of the fluorescence or were only faint changes, less than 0.5% with respect to a total emission, although light-induced changes of the membrane potential in the same chloroplasts remained uninhibited or were even stimulated. The inhibitory effect of gramicidin suggests that electro-induced fluorescence deflections were caused by the imposed membrane potential, not by altered ionic composition of a chloroplast. A weak sensitivity of the light-induced membrane potential for gramicidin was probably due to counteracting effects of an increased membrane conductance and an uncoupler-stimulated electron transport.

In some experiments, the load resistor was removed from a current circuit so that the output voltage of a pulse generator was totally applied to a microelectrode and chloroplast membranes in series. In the absence of the load resistor, fluores-

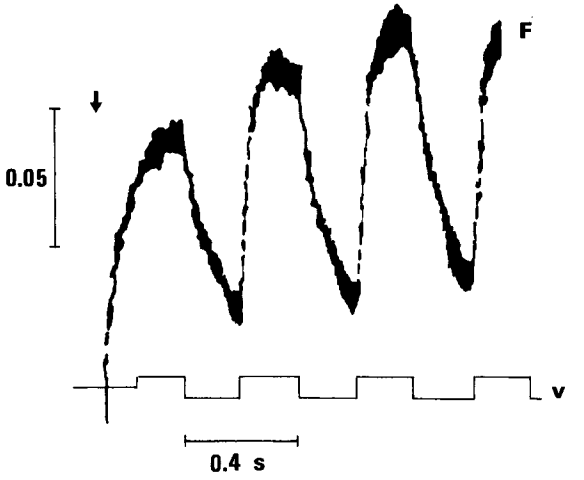


Fig. 2. Fluorescence changes of *Anthoceros* chloroplast induced by application of bipolar 1 V pulses of voltage to a series connection of a microelectrode and a chloroplast. Downward and upward deflections in the bottom trace indicate the application of a negative and a positive voltage, respectively, to a thylakoid interior. The arrow marks the beginning of illumination.

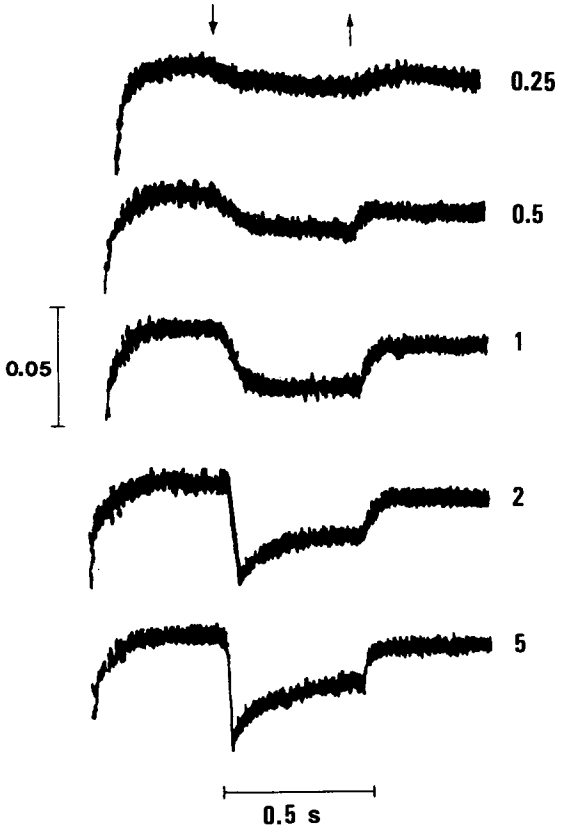


Fig. 3. Fluorescence changes of one isolated *P. metallica* chloroplast induced by various negative voltages applied to a microelectrode and a chloroplast in series. Figures along the curves indicate the voltage applied. The beginning and the end of current pulses are marked with arrows.

cence changes of about 10% in magnitude were observed under application of 1 V voltage pulses (Fig. 2). With a resistance of a microelectrode 100–150 MΩ and an input resistance of a chloroplast 20–50 MΩ [12,16], a voltage drop at the chloroplast membrane would not exceed one third of the applied voltage and could be much smaller. Hence the fluorescence changes were caused by membrane potential displacements not exceeding 0.3–0.4 V.

In Fig. 3 are shown fluorescence changes induced in an isolated *P. metallica* chloroplast by applying negative voltages of various magnitude. Discernible fluorescence changes, about 1% of the overall emission, were detected under application of 250 mV voltage to a series circuit consisting of a micropipette, a chloroplast envelope and thylakoid membranes. This fact indicates that a displacement of the membrane potential by 100 mV or even less would be sufficient to affect the fluorescence yield. On increasing the applied voltage the fluorescence change increased up to a saturation, and a sharp transient minimum appeared. Current pulses of increasing strength produced similar effects on chlorophyll emission both in isolated and in situ chloroplasts. Unfortunately, a meaningful relationship between the imposed membrane potential and the fluorescnece change could not be determined with the method employed because of nonlinear voltage-current characteristics of chloroplast membranes [12,16] and nonhomogeneous density of the transmembrane current injected through the microelectrode into a folded membrane system of interconnected thylakoids.

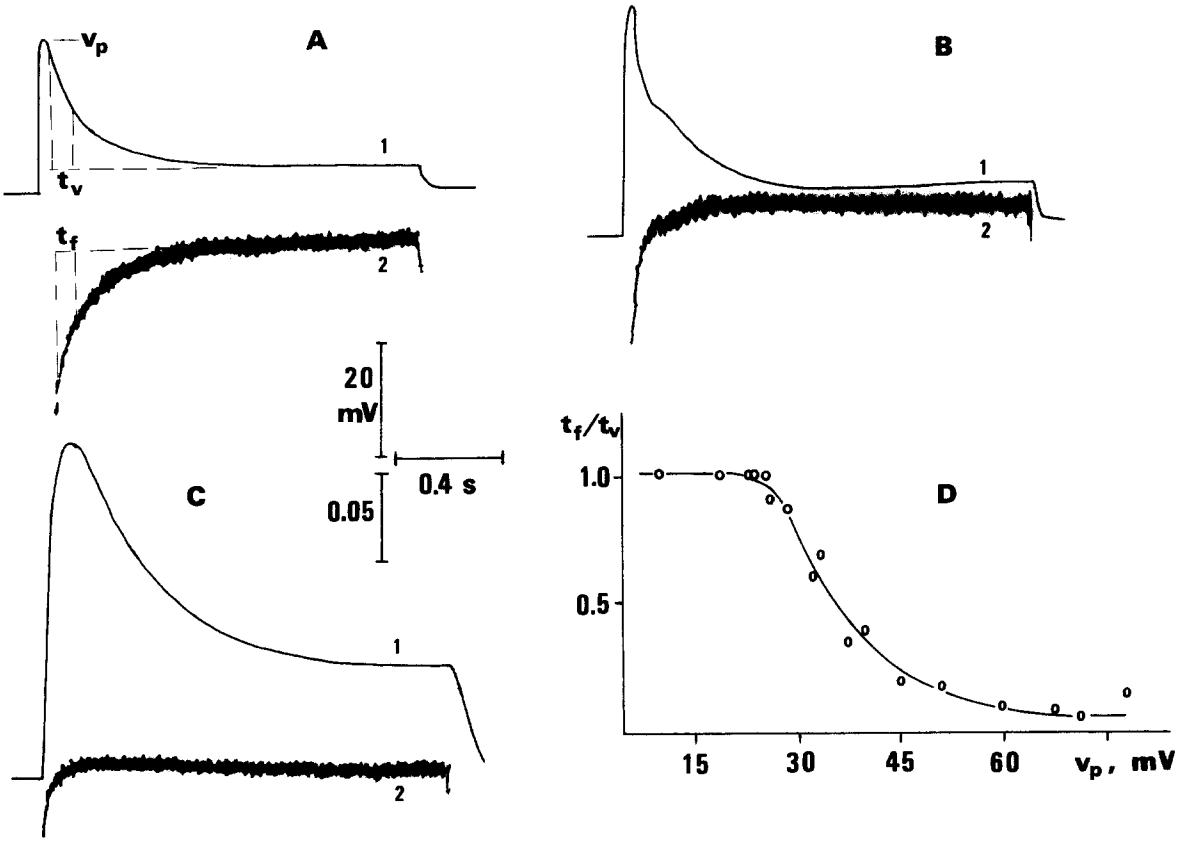


Fig. 4. Fluorescence induction time curves under variable membrane potential. (A–C) Simultaneous measurements of light-induced membrane potential (1) and the induction curves of chlorophyll fluorescence (2) in isolated chloroplasts of *P. metallica* from various preparations. (D) The ratio of half-times, $t_f$ (slow phase of the fluorescence rise) and $t_v$ (a decay of the membrane potential to a stationary level), plotted against the peak value, $V_p$ of the light-induced membrane potential.

*Time-course of the fluorescence induction in chloroplasts showing different membrane potentials*

In view of the described sensitivity of chlorophyll fluorescence to variations of the membrane potential we compared the kinetics of the fluorescence induction in chloroplasts exhibiting light-induced membrane potentials of various extnet. Observations made on *Anthoceros* and *Peperomia* chloroplasts were essentially similar.

Typical results obtained with isolated plastids are shown in Fig. 4. In chloroplast preparations showing light-induced membrane potentials of low extent (peak level below 30 mV), a slow rise of the fluorescence and the potential decay to a steady level occurred with similar half-times (Fig. 4A). In contrast to this, a rise of the fluorescnece to a maximum occurred essentially faster in chloroplasts showing high photopotentials (Fig. 4C). The observed relation is additionally illustrated by the ratio of half-times of the fluorescence rise (a slow phase) and the potential decay in the light, plotted against the peak level of the light-induced membrane potential (Fig. 4D).

Although the accelerated fluorescence rise accompanied with a high-level electric potential could be interpreted in various ways (e.g., a higher rate of PS II turnovers or stimulation of PS I-dependent electron flow after a short delay), these results are consistent with the assumed direct effect of the membrane potential on the fluorescence.

## Discussion

The present results show that the yield of chlorophyll fluorescence in intact chloroplast is influenced by an imposed transmembrane electric field. Two main effects were discovered: a stimulation of the emission by a positive membrane potential ('plus' inside the thylakoid) and the quenching of the fluorescence by a negative imposed potential.

These findings are consistent with observations by Meiburg et al. [11] made on swollen thylakoid vesicles, called blebs, in that a positive membrane potential induces a fluorescence increase if the primary acceptor of PS II, Q is partially or totally oxidized. Our results also support the observation of field-induced fluorescence quenching under conditions of complete reduction of Q (that is in the strong light in the presence of DCMU) [11], but suggest clearly that this fluorescence quenching should be ascribed to a negative, not positive membrane potential. In contrast to experiments with blebs exposed to an external electric field, in our experiments there is no uncertainty regarding the polarity of the imposed membrane potential to which the field-induced fluorescence changes should be ascribed.

Because of a certain similarity of potential-dependent fluorescence deflections in individual chloroplasts and swollen thylakoids it appears that changes in the emission of intact chloroplasts are determined by a direct effect of the membrane potential on the electron transport in reaction centers of PS II [11] rather than by the field-induced redistribution of $Mg^{2+}$ between thylakoids and stroma compartments and the associated change in spill-over [17].

It can be assumed, as in Ref. 11, that in the presence of a sufficiently large positive membrane potential, charge separations between the primary donor P-680 and the primary quinone acceptor Q are prevented which leads to a decrease of excitation trapping and a fluorescence increase as if Q were in the reduced state. This effect of the membrane potential is only possible if Q is present in an oxidized form. When Q is completely reduced in the light in the presence of DCMU, then the rate of charge separations becomes negligible, and the applciation of a positive membrane potential has no additional effect on excitation trapping and fluorescence yield.

Electron is transported from P-680 to Q at least in two steps via an intermediary acceptor pheophytin [18–20]. The sensitivity of the initial and the secondary step of charge separation to variations of membrane potential would depend on several factors such as the difference of midpoint redox potentials for primary reactants (excited state of P-680, pheophytin and Q), the distances between these reactants in their proportion to the total membrane thickness and the heights of electrostatic barriers for electron transfer across the hydrophobic layer of the membrane [21]. Remarkably, fluorescence changes could be evoked by a relatively small variations (of about 100 mV or even less) in a membrane potential. This fact

may indicate that the difference between energy levels of the donor and the acceptor in the potential-sensitive step is relatively small. In particular, the electron transfer from the excited state of P-680 to pheophytin (the midpoint potential difference 0.08 V [18]) might perhaps be very sensitive to variations of membrane potential on the condition that the distance between P-680 and pheophytin is not too small with respect to the total thickness of the membrane. In bacterial photosynthesis, charge separation between the primary donor and the intermediary acceptor was reported to be electrogenic and controlled by the membrane potential [22].

It is conceivable therefore that the positive membrane potential superimposed over the light-induced electric potential in intact chloroplast leads to a lower rate of charge separations or/and to a stimulation of a back reaction. Such an explanation, based on the assumed electrostatic control of electron transfer through a hydrophobic region of the membrane, is, however, insufficient to account for the electro-induced fluorescence quenching observed in the presence of DCMU, i.e., when the rate of charge separation is negligible. A stimulation by a negative potential of DCMU-insensitive electron transport around PS II is excluded, since electro-induced fluorescence quenching was also observed in the presence of DCMU and 1 mM hydroxylamine, known to inactivate oxygen evolution. Our tentative explanation of the field-induced fluorescence quenching is that a negative membrane potential ('minus' inside the thylakoid) promotes an accumulation of reduced pheophytin which acts as an efficient fluorescence quencher [18,23]. Alternatively, one may assume that a strong electric field brings about reversible conformational changes of membrane proteins leading to alterations of light-harvesting in PS II.

It should be clear that the current injection through the microelectrode into a folded lamellar system produces a nonhomogeneously distributed membrane potential. A considerable fraction of PS II reaction centers is apparently not subjected to the imposed electric field, whereas other centers experience the electric field of various strength. It seems likely that the amplitude of electro-induced fluorescence changes could have been higher than reported here if the overall membrane surface of thylakoids were exposed to an electric field. Comparing our data with those obtained on blebs [11], we go to a proposal that the fluorescence yield in regions exposed to a high negative or positive membrane potential is close to levels $F_0$ and $F_{max}$, respectively, where $F_0$ and $F_{max}$ stand for fluorescence yields before and after photoreduction of Q. A decrease of an overall emission by 10% due to a change of a variable fluorescence indicates that a considerable fraction (at least 15% according to our estimate) of the total membrane surface was exposed to an electric field during a current injection. This is in line with the assumed interconnections of thylakoids into a continuous system [16,24].

A biphasic change of a fluorescence shown in Figs. 1A and 3 might be caused by a reversible breakdown of the thylakoid membrane. Then, an overshoot in the fluorescence response reflects an initial high negative membrane potential and its subsequent decline due to a sudden increase of the membrane conductance. A transient overshoot of the membrane potential was indeed noticed in *Peperomia* chloroplasts upon injection of high-intensity current [16] and was inferred also from studies of electrophotoluminescence of swollen thylakoid vesicles [9].

The relation of the biphasic fluorescence transients to a phenomenon of a reversible breakdown is further substantiated by a peculiar fluorescence response to an application of a positive membrane potential (bottom trace in Fig. 1B). In DCMU-treated chloroplasts, an applied positive voltage does not induce usually any fluorescence change, but sometimes a small reproducible fluorescence decrease was noted after a long lag period. We believe that this signal develops after a local breakdown of the thylakoid membrane. Indeed, after a breakdown a low resistance contact of the microelectrode with a chloroplast stroma is established so that additional pathways for current flow become operative. In addition to extrathylakoid shunting pathway, some current flows across the foldings of the thylakoid membrane system, thus giving rise to a local negative and positive membrane potentials at regions where it enters and leaves the thylakoid, respectively. Membrane regions exposed to positive membrane

potential give no fluorescence response, whereas the regions exposed to a negative potential produce a small decline of the fluorescence.

From Fig. 1 it appears that higher inward currents are required to induce a breakdown in undamaged chloroplasts than in DCMU-treated plastids. This may result from a superposition in untreated chloroplasts of artificial negative membrane potential with the light-induced positive electric potential.

The results of the present study indicate that the light-induced membrane potential may influence the excitation trapping and charge separation in PS II. Significant changes of the membrane potential in first seconds of illumination should then be considered as a factor affecting the induction curves of chlorophyll fluorescence in intact chloroplasts.

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