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EXCITATION TRAPPING AND CHARGE SEPARATION IN PHOTOSYSTEM II IN THE PRESENCE OF AN ELECTRICAL FIELD

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To investigate the effects of a membrane potential on excitation trapping and charge separation in Photosystem II we have studied the chlorophyll fluorescence yield in osmotically swollen chloroplasts subjected to electrical field pulses. Significant effects were observed only in those membrane regions where a large membrane potential opposing the photochemical charge separation was built up. When the fluorescence yield was low, close to F_0, a much higher yield, up to F_{max}, was observed during the presence of the membrane potential. This is explained by an inhibition by the electrical field of electron transfer to the quinone acceptor Q, resulting in a decreased trapping of excitations. A field pulse applied when the fluorescence yield was high, Q and the donor side being in the reduced state, had the opposite effect: the fluorescence was quenched nearly to F_0. This field-induced fluorescence quenching is ascribed to reversed electron transfer from Q^- to the intermediate acceptor, pheophytin. Its field strength dependence suggests that the midpoint potential difference between pheophytin and Q is at most about 300 mV. Even then it must be assumed that electron transfer between pheophytin and Q spans 90% of the potential difference across the membrane.

Introduction

In the reaction centers of photosynthesis, excitation energy collected by antenna pigments normally leads to a vectorial electron transfer across the photosynthetic membrane, resulting in an electrical potential difference between the aqueous phases separated by the membrane [1]. Variations of the membrane potential influence the rate of the reversed process. Especially in PS II such a variation is easily detected because reexcitation of chlorophyll causes delayed fluorescence emission [2–4].

A convenient system to study the effects of a membrane potential is a suspension of osmotically swollen chloroplasts, so-called blebs. Externally applied electrical field pulses can generate potential differences of up to 1 V across the bleb wall, which consists of a single membrane [5]. The bleb wall acts as a capacitor, which is charged by movement of ions in the internal and external aqueous media. The electrical field inside the hydrophobic layer is thereby enhanced, up to several thousand-fold, and responds to the applied field pulse with an *RC* time of the order of 10–100 μs, depending on the conductivity of the suspension [5]. The field causes a very strong enhancement of delayed fluorescence emission [6–10].

A quantitative correlation of this 'electroluminescence' with reversed electron transport in the reaction center of PS II was demonstrated in a previous publication from this laboratory [10].

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; PS, photosystem; P-680, pheophytin and Q, the primary electron donor, the intermediary electron acceptor, and the first stable electron acceptor in the reaction center of PS II, respectively; Tricine, *N*-tris(hydroxymethyl)methylglycine.

Blebs were preilluminated in the presence of DCMU so that the fluorescence yield was high. Stimulation of the back-reaction during an electrical field pulse resulted in a lower fluorescence yield after the pulse. The kinetics of the back-reaction were determined by varying the pulse duration.

With the aid of rapidly modulated excitation light we have now been able to measure the fluorescence yield changes during the pulse. In addition to the 'permanent' fluorescence decrease as a result of charge recombination, these measurements revealed other fluorescence changes of similar amplitude, which disappeared immediately after the pulse. These changes are interpreted in terms of an inhibition and a reversal of the light-induced electron transfer across the membrane from excited P-680, the primary electron donor, via pheophytin *a*, the intermediary electron acceptor [11], to Q, the quinone known as the 'primary' electron acceptor of PS II.

Materials and Methods

Spinach leaves, obtained from local shops, were ground in a cooled blender in 50 mM Tricine buffer (pH 7.8) containing 0.4 M sucrose, 10 mM KCl and 2 mM $MgCl_2$. After filtration through nylon cloth the chloroplasts were sedimented by brief centrifugation at $8000 \times g$ and stored on ice. Just before measurement the pellet was diluted about 200-fold in distilled water in order to obtain 'blebs', and DCMU and an electron donor (where indicated) were added.

An automatic stopped-flow system brought the sample into a cubical plexiglass vessel with the sample in- and outlet on one side. Two opposite walls were covered inside by platinum electrodes, connected to a home-made power supply which generated pulses up to 1200 V with rise and decay times of about 1 μs (90% completion). The remaining three sides of the vessel allowed illumination, fluorescence measurement at 90°, and absorbance measurements.

Fluorescence was excited with a 250 W tungsten halogen lamp connected to a current-stabilized d.c. power supply. The light beam passed a mechanical shutter (6 ms opening time), a Calflex C and a Corning CS 4-96 filter, and was modulated at 100 kHz by a photoelastic modulator (Hinds International PEM 03) placed between two crossed polarizers. Fluorescence transmitted by Schott AL 680 and RG 645 filters was detected at 90° to the excitation beam. The photomultiplier signal was demodulated by a home-built lock-in amplifier and fed into a transient recorder (Datalab 905), connected to a microcomputer for signal averaging.

Results

It was shown earlier [10] that an electrical field pulse applied to preilluminated blebs (osmotically swollen chloroplasts) strongly enhanced the rate of the back-reaction between Q^- and an oxidized donor, resulting in a burst of luminescence during and a lower fluorescence yield after the pulse. Fig. 1A shows a similar experiment. A suspension of blebs was illuminated by a saturating flash in the presence of DCMU to obtain a one-step oxidation of the oxygen-evolving enzyme and a reduction of Q, with concomitant increase in the chlorophyll fluorescence yield. Two successive pulses of the same polarity were then applied. The fluorescence yield was measured with 100 kHz modulation and a high intensity of excitation light, to avoid any contribution of the luminescence burst to the signal.

The result shown in Fig. 1A confirms that the fluorescence yield is lower after the pulses than before. This 'irreversible' decrease is nearly complete already after the first pulse and could be induced again only after the actinic effect of the measuring beam had restored the fluorescence yield

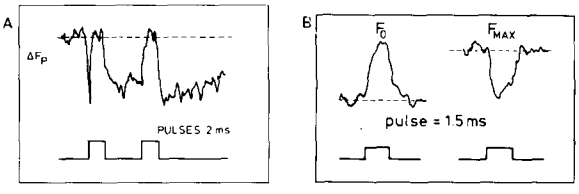

Fig. 1. (A) Changes of the chlorophyll fluorescence yield in a suspension of blebs, induced by two pulses of 1100 V/cm with the same polarity, 40 ms after a saturating light flash in the presence of 5 μM DCMU. (B) Kinetics of the fluorescence changes induced by a pulse of 1100 V/cm before (F_0) and after (F_{max}) photoreduction of Q in the presence of 5 μM tetraphenylboron and 10 μM DCMU.

(the increase observed at the end of the trace), or – once – by a pulse of reversed polarity (not shown). The initial fluorescence decrease at the onset of the first pulse probably reflects the kinetics of the irreversible decrease [10] and is therefore ascribed to the charge recombination of Q^- with an oxidized donor D^+ (Phe, pheophytin):

$$D^+ \text{ P-680PheQ}^- \rightarrow \text{DP-680}^+ \text{ Phe}^- \text{ Q} \rightarrow \text{DP-680PheQ}$$

The amplitude of the change reflects the total amount of PS II that is subjected to a large membrane potential of the appropriate polarity [10]. It was less than 10% in our preparations. Most of the chloroplast material remains concentrated in a few small clumps on the surface of the bleb and is not exposed to a large membrane potential [5].

In addition to the irreversible fluorescence decrease a rapidly reversible fluorescence increase was observed during both pulses. In order to decide whether this temporary increase was due to the centers opened by charge recombination, or to the other centers on the opposite side of the bleb, where Q was still in the reduced state, the experiments of Fig. 1B were carried out. The first recording shows the fluorescence change induced in a dark-adapted sample, when all centers were still open. It was the same as that induced by the second pulse in Fig. 1A, indicating that there, too, it originated in the open centers. In Fig. 1A this reversible increase is seen to restore the fluorescence yield to the level observed before the pulses. Apparently, the membrane potential ultimately prevents the trapping of excitations by PS II centers as effectively as if Q were still in the reduced state:

$$\text{P-680*PheQ} \not\rightarrow \text{P-680}^+ \text{ PheQ}^-$$

The opposite phenomenon was observed when an electrical pulse was applied after complete photoreduction of Q, if the endogenous donor was kept in the reduced state (second recording of Fig. 1B). Since an electron donor to PS II (tetraphenylboron) was present in the experiments of Fig. 1B, charge recombination could not occur in this case but nevertheless a fluorescence decrease of magnitude similar to that seen in Fig. 1A was observed during the pulse. In contrast to the irreversible fluorescence decrease shown in Fig. 1A, this phenomenon could be repeated in subsequent pulses. We attribute this quenching to pheophytin$^-$, reduced by reversed electron transport:

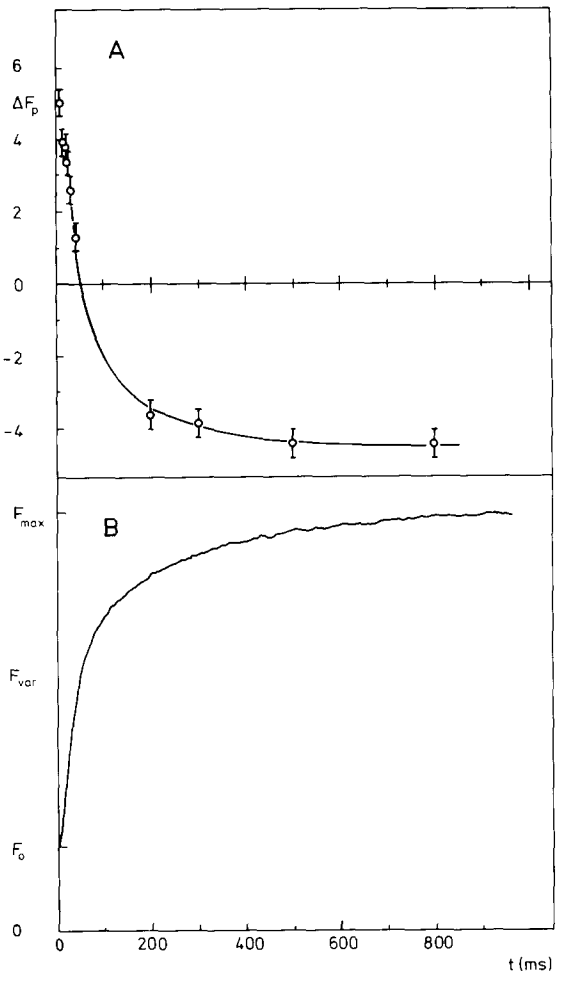

Fig. 2. (A) Field-induced fluorescence changes (1100 V/cm) in percent of the variable fluorescence as a function of the time after the complete opening of the actinic light shutter in the presence of 10 μM DCMU and 5 μM tetraphenylboron. (B) Fluorescence induction under the same light conditions in the presence of 10 μM DCMU.

$$\text{DP-680PheQ}^- \rightarrow \text{DP-680Phe}^- \text{ Q}$$

Although the charge separation cannot take place, the fluorescence yield is low when pheophytin is reduced [12], probably as a result of excitation transfer to pheophytin$^-$ [13].

The reversible quenching cannot be ascribed to centers subjected to a membrane potential of op-

posite polarity, because it should then have occurred in Fig. 1A as well as should have cancelled the fluorescence increase. A reversible fluorescence yield decrease should have occurred on the opposite side of the bleb as well, if the field could stabilize the state P-680$^+$Phe$^-$Q$^-$. A significant decay via recombination to the triplet state of P-680 should then occur. This quenching mechanism is expected to be very sensitive to magnetic fields [14]. Such an effect was not observed. The quenching induced, after reduction of Q, by non-saturating electrical pulses of 450 V/cm was not significantly, i.e., by less than 5%, affected by the presence of magnetic fields up to 0.2 T. This negative result was not due to compensation by the magnetic field-induced fluorescence increase in the presence of Q$^-$ [15], which was found to be less than 0.1% of F_{max} under our conditions.

It may be concluded that all fluorescence changes shown in Fig. 1 amount to the total variable fluorescence of the same fraction of PS II, where a large membrane potential of the same polarity as the light-induced one is built up by the externally applied field, i.e., the fraction of PS II that is located in that half of the bleb wall that faces the negative electrode [16].

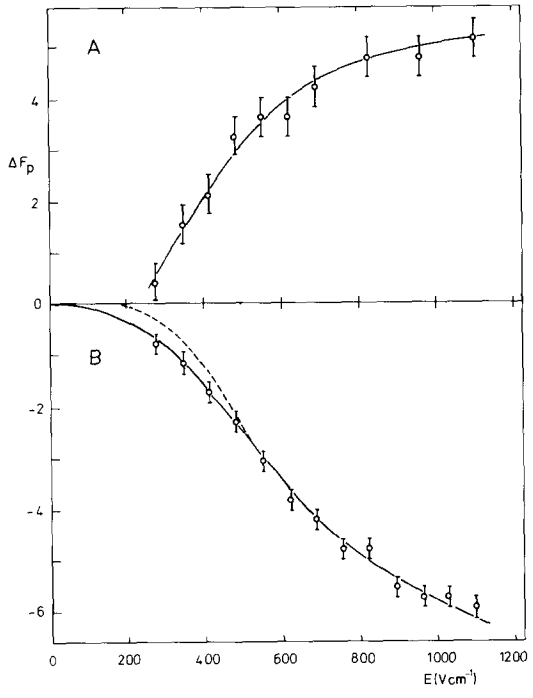

Fig. 3. (A) Electric field dependence of the fluorescence increase measured 5 ms after complete opening of the shutter as in Fig. 2A. (B) Electric field dependence of the fluorescence decrease measured in continuous light, other conditions as in Fig. 2A. The dashed line is a theoretical fit assuming 300 mV midpoint potential difference between pheophytin and Q. The continuous line would imply 130 mV (see Discussion).

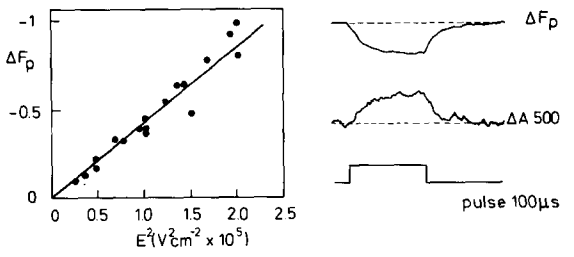

Fig. 4. (A) Kinetics of the electric field-induced fluorescence decrease (ΔF_p) and the concomitant electrochromic absorption change at 500 nm ($\Delta A500$) measured in continuous light with pulses of 450 V/cm in the presence of 5 mM hydroxylamine and 5 μM DCMU. (B) Extent of the fluorescence quenching during the pulse plotted vs. the square of the applied field strength. Measurements performed in the presence of 5 mM hydroxylamine and 5 μM DCMU in continuous light.

In Fig. 2A the amplitude of the reversible, field-induced fluorescence change is shown as a function of illumination time. The transition from the field-induced fluorescence increase to the reversible decrease is clearly due to the photoreduction of Q, as indicated by the corresponding fluorescence induction curve (Fig. 2B).

Fig. 3A shows the field strength dependence of the fluorescence increase induced by a pulse in dark-adapted samples. Fig. 3B shows that of the reversible fluorescence quenching induced by a pulse when Q was in the reduced state and charge recombination was prevented by addition of an electron donor. Both curves indicate a saturation at lower field strength than can be explained by the limitation of the membrane potential by dielectric breakdown [5].

The field-induced quenching, which could be measured with high repetition rate and high light intensity with a single sample, was studied in more detail. Fig. 4A shows that it depends quadratically on the field strength at low values of the field. Its time-resolved kinetics (Fig. 4B) were very similar to those of the electrochromic carotenoid absorbance changes, the amplitude of which depends

also quadratically on the field [5]. No significant delay was observed in rise or decay time.

Discussion

Three different effects of a large membrane potential, of the same polarity as the light-induced one, on the chlorophyll fluorescence yield in PS II were observed. The irreversible decrease induced by an electrical field pulse after illumination is due to the oxidation of Q^- by reversal of the light-induced electron transfer across the membrane [10]. The fluorescence yield decreases because after the back-reaction has taken place the reaction centers are open again and effectively trap excitations from the chlorophyll antenna.

When the reaction centers were open, after dark adaptation or after field-induced charge recombination, the fluorescence yield was reversibly increased, up to F_{max}, during the presence of the membrane potential. Apparently, when the membrane potential reaches its maximum value, it prevents the trapping of excitations by PS II centers as effectively as if Q were in the reduced state. It is likely that electron transfer from excited P-680 to Q becomes impossible when the electron has to overcome a membrane potential of about 1 V.

The third effect, a reversible fluorescence quenching during the presence of the membrane potential, was observed only when both Q and the oxygen-evolving enzyme were reduced before the pulse. Charge recombination then is impossible and reversed electron transfer from Q^- would cause a reduction of the intermediate electron acceptor, pheophytin. Pheophytin$^-$ is an efficient fluorescence quencher.

Remarkably, no significant effects could be detected of the equally large membrane potential of opposite polarity at the other side of the bleb. With open PS II traps, this membrane potential might have decreased F_0 by enhancing the trapping efficiency. With closed traps (Q^-), it might have decreased F_{max} by stabilizing the primary charge pair P-680$^+$Phe$^-$, resulting in a decay via recombination to the triplet state of P-680.

Electron transfer from P-680 to Q is the only part of PS II electron transport known to generate a membrane potential [17]. So, if P-680$^+$Phe$^-$ is insensitive to the field, the couple PheQ would have to span most of the potential drop across the membrane. The fraction of the membrane potential, to which electron transfer between pheophytin and Q is exposed, may be calculated from the field strength dependence of the reversible quenching (Fig. 3B).

The reversible quenching is attributed to a shift of the equilibrium $PheQ^- \rightleftarrows Phe^-Q$ by the fraction α of the membrane potential ΔV, according to:

$$\frac{Phe^-Q}{PheQ^-} = Ke^{\alpha\Delta V/kT}$$

where K is the equilibrium constant in the absence of a field and kT the product of Boltzmann's constant and temperature. The fraction of reaction centers in the state Phe^-Q in one bleb, obtained by integration over the spherical surface, is given by:

$$\frac{Phe^-Q}{Phe^-Q + PheQ} \approx \frac{\ln(Ke^n + 1) - \ln(Ke^{-n} + 1)}{2n}$$

$$\overset{K \ll 1}{\approx} \frac{\ln(Ke^n + 1)}{2n}$$

in which n is the value of $\alpha\Delta V/kT$ at the point nearest to the negative electrode, where $\Delta V = \frac{3}{2}RF$, R being the bleb radius and F the applied field strength [5].

Taking into account that the membrane potential is limited between +1 and −1 V by dielectric breakdown [5], we have calculated, for the bleb size distribution given in Ref. 5, the fraction of centers in the state Phe^-Q as a function of field strength and determined the value of α required to fit the data of Fig. 3B.

Expectedly, the best-fitting value of α was proportional to the midpoint potential difference assumed to exist between Q and pheophytin. It was found that $\alpha/(0.058 \log K) = -3.0$. Thus, the data seem to exclude a midpoint potential difference larger than 350 mV. Taking −300 mV for the midpoint potential of Q [18] and −610 mV for that of pheophytin [19], the electron transfer between pheophytin and Q would be exposed to 90% of the membrane potential. The deviation at low field strengths would not occur if the midpoint potential difference was 130 mV; α then is 0.4.

The field strength dependence of the field-in-

duced inhibition of excitation trapping (Fig. 3A) presumably reflects that of the probability that the electron transfer $P\text{-}680^+Phe^-Q \rightarrow P\text{-}680^+PheQ^-$ takes place at all within the lifetime of the charge pair. The back-reaction from Q^- would generate delayed fluorescence fast enough to pass the 100 kHz modulated detection, but the yield of this emission is very low. It was shown earlier that, when recombination occurs from Q^-, the probability that this charge recombination results in fluorescence emission is only 2.8% of the fluorescence yield observed upon excitation in the presence of Q^- [10]. It was suggested that recombination occurred via triplet formation mainly. If $P\text{-}680^+Phe^-$, formed by back-reaction, is assumed to recombine 3-times more often to the triplet state of P-680 than to the singlet excited state, and direct recombination to the ground state is neglected, one may calculate the probability p that the singlet excited state is retrapped. The total triplet yield T then is given by:

$$T = 0.75 + 0.25pT = 1 - 0.028$$

from which a value of p larger than 0.9 follows.

In a recent paper by Lavorel et al. [20], it was claimed that because of this retrapping the emission yield during charge recombination should be related to a much lower fluorescence yield than that observed upon excitation in the presence of Q^- (F_{max}). The calculations in Ref. 20 are equivalent to those in Ref. 10 but relate to actual molecular rate constants rather than to net observed rates. As a consequence, the result is experimentally useless as long as the actual reexcitation rate or the actual fluorescence yield of the reexcited chlorophylls have not been measured. As was pointed out in Ref. 3, charge recombinations followed by retrapping do not contribute to the net observed decay rate of Q^-. Therefore, even though 25% of the charge pairs presumably recombine to a singlet excited state at least once, only 2.8% are actually lost via that state.

The inhibition of trapping in a strong field should also apply to the retrapping of excitations produced by charge recombination. This would seem to contradict the observation that the emission yield of charge recombination from Q^- is not enhanced by a field [10]. However, the fluorescence kinetics of Fig. 1A show that reoxidation of Q^- is completed before a large enough membrane potential has been built up to cause a significant increase in fluorescence yield. Thus, our present finding that the fluorescence yield is increased to F_{max} during the pulse is compatible with the low yield of 'electroluminescence' only because the two phenomena are separated in time: apparently, the relevant membrane regions are swept free of charge pairs before the membrane potential reaches the values required for a significant inhibition of trapping.

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