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Evidence for the delayed photoactivation of electrogenic electron transport in chloroplast membranes

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Time-courses of the electric potential at chloroplast thylakoid membranes and of a chlorophyll fluorescence were measured simultaneously on individual chloroplasts of *Anthoceros* under the action of intensive 1.5 s light pulses. There were two distinct peaks of the electrical potential in the induction curve and a transient minimum that occurred at about 200 ms from the onset of illumination. The decay of the potential after an initial peak occurred concomitantly with an increase of the fluorescence up to a maximum P. The location of the transient minimum of the potential varied, depending on the preceding dark period, but it coincided with the location of the fluorescence maximum P. A delayed rise of the potential in dark-adapted chloroplasts was accompanied with a transient decline of the fluorescence. Both the secondary rise of the potential and the fluorescence decline from P to S_1 were suppressed by preillumination and abolished completely by DBMIB or dithionite. It is concluded that the second peak of the potential and a concomitant decline of the fluorescence are different manifestations of a delayed acceleration of PS-I-associated electron transport, which is coupled with electrogenic proton displacements across the thylakoid membrane.

Introduction

The electric-potential difference arising across the chloroplast thylakoid membrane upon illumination is being actively examined with respect to its ability to drive ATP synthesis as well as to control the activity of the coupling factor and photochemical centers [1–4]. Various techniques invented to measure the time-course of the membrane electrical potential ($\Delta\psi$) in chloroplasts upon a dark-to-light transition revealed a rapid rise of $\Delta\psi$ followed by a slow decay to a steady level [5–7]. The initial peak of $\Delta\psi$ attained within 20–50 ms of illumination is typical both for intact plastids and isolated thylakoids. Recent studies on the formation of $\Delta\psi$ in *Anthoceros* chloroplasts pointed out the existence of an additional peak of $\Delta\psi$ located at 0.4–0.5 s after the onset of illumination [8,9]. A similar conclusion on the build up of a second wave of $\Delta\psi$ has been drawn from measurements of light-induced electrochromic absorbance changes of isolated *Bryopsis* chloroplasts at 560 nm [10]. Such a delayed peak of $\Delta\psi$ has not been found so far in isolated thylakoids.

The occurrence of the delayed peak of $\Delta\psi$ was tentatively interpreted as due to a sudden acceleration after a certain lag of electron flow driven by PS I [9]. An independent evidence for the delayed photoactivation of electron transport at the level of PS I came from studies on the induction kinet-

Abbreviations: $\Delta\psi$, transmembrane electric potential; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; PS I, Photosystem I; PS_1-transient, transition from the initial peak P through a minimum S_1 toward a secondary maximum M.

ics of a chlorophyll fluorescence in *Bryopsis* chloroplasts [11,12]. It appears that the proposed relation of the delayed peak of $\Delta\psi$ to a photoactivated electron transport could be verified by comparing the photoinduction kinetics of $\Delta\psi$ and of a chlorophyll fluorescence in the same chloroplast. Both of these two parameters reflect the rate of electron transport, although in different manners. The electrogenic component of $\Delta\psi$ (as opposed to its diffusion component [13]) provides an information on the rate of redox reactions coupled with a binding or release of protons at the membrane–water interfaces [7,14]. Under short exposures to a strong light, the change in the chlorophyll fluorescence reflects the redox state of the primary acceptor Q of PS II which depends on the balance between the rates of Q reduction by PS II and an oxidation of Q by PS I [15].

In this paper we report on simultaneous measurements of the photoinduction kinetics of $\Delta\psi$ and a chlorophyll fluorescence in individual *Anthoceros* chloroplasts. It is shown that the secondary rise of $\Delta\psi$ and a transient decrease of the chlorophyll fluorescence are initiated synchronously, and are similarly affected by a preillumination and inhibitors. The direct relation of the delayed peak of $\Delta\psi$ to a photoactivation of electron transport driven by PS I is thus evidenced.

Material and Methods

Experiments were done on chloroplasts of a liverwort *Anthoceros*. Each cell in a thallus of this plant contains one giant lens-shaped chloroplast, 20–40 μm in diameter. Sections of thalli were placed in a medium containing 10 mM KCl/1.0 mM NaCl/0.5 mM $Ca(NO_3)_2$ and 5 mM Hepes-NaOH buffer (pH 7.0).

The experimental arrangement for simultaneous measurements of $\Delta\psi$ and the chlorophyll fluorescence on individual chloroplasts was constructed over a luminescent microscope LUMAM I-3 (U.S.S.R.) equipped with an epifluorescent illuminator and a microscope photometer. A schematic diagram of the measuring system is shown in Fig. 1. Chlorophyll fluorescence was excited with a strong broad-band blue light. An excitation light from a 70 W halogen lamp passed through a

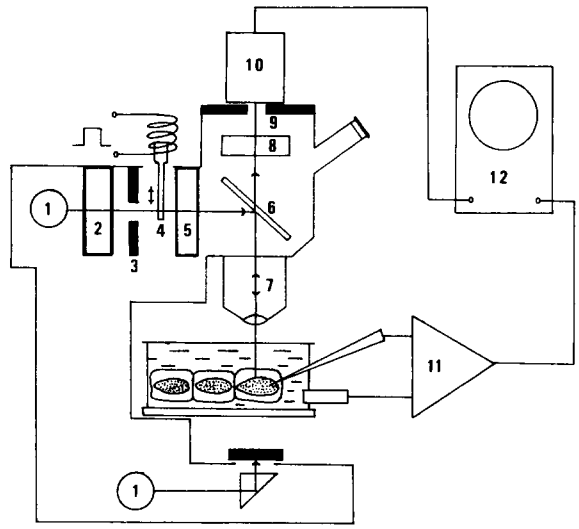

Fig. 1. Schematic diagram of the experimental arrangement for measuring the photoinduction kinetics of chlorophyll fluorescence and membrane potential on individual chloroplasts. 1, halogen lamps; 2, water heat filter; 3, aperture; 4, shutter; 5, blue filter; 6, dichroic mirror; 7, an objective lens; 8, red filter; 9, measuring aperture; 10, photomultiplier; 11, electrometer amplifier; 12, oscilloscope.

4 cm layer of water and a glass blue filter cutting off light with wavelengths above 550 nm. The light beam was reflected by a dichroic mirror and was directed through the objective lens on to a plant preparation. The area of excitation of a specimen was delimited by a flexible aperture and was about 0.1 mm^2. Light intensity at the surface of a preparation was approx. $5 \cdot 10^2$ $J \cdot m^{-2} \cdot s^{-1}$. The fluorescence emission of a chlorophyll was collected by the objective lens and passed through the dichroic mirror and a red filter cutting off the reflected light (bandpass, above 650 nm). The microscope was equipped with a set of variable apertures designed to measure light emission from a delimited area of a preparation. A light-collecting spot of 50 μm in diameter was appropriate for the accommodation of one single chloroplast. Chlorophyll fluorescence was detected by a photomultiplier. The output voltage from a photomultiplier was filtered to reduce noise as to provide the time resolution of 10 ms.

Light-induced changes of $\Delta\psi$ were measured with glass microcapillary electrodes filled with 1 M choline-chloride as described before [8,9]. The

electrical potential difference between the microelectrode inserted into the chloroplast and the reference electrode in the outer medium was measured with a high-impedance amplifier. The potential difference across the plasmalemma was low and did not change upon illumination [9]. Light-induced changes of $\Delta\psi$ and a chlorophyll fluorescence were displayed on a two-channel oscilloscope. A signal from a photomultiplier was partially off-set by feeding into a differential input of the oscilloscope a calibrated DC voltage up to 1.0 V in magnitude. The initial part of the induction curve of the fluorescence (within about 20 ms) was not resolved in our experiments.

An adjustment of the chloroplast in the area of photometric assay and microelectrode impalements were done under weak transmitted light with the photomultiplier shielded from illumination. After an impalement of the chloroplast with microelectrode, the transmitted light was blocked and a protective shield in front of the photomultiplier was removed. Light flashes of controlled length were provided through the epi-illuminator with a shutter driven by an electrical pulse generator. The duration of light pulses was usually 1.5 s.

Results

The induction curves of a chlorophyll fluorescence measured in situ on one *Anthoceros* chloroplast were similar to those measured in intact chloroplasts of some other species, particularly in *Bryopsis* [11,12]. Under continuous light, there was a large slow quenching of chlorophyll fluorescence from the peak level, P, to the steady state, S, which took several tens of seconds to complete. This slow quenching attributed to a formation of a transmembrane pH gradient [16] was not a homogeneous process. Within about 2 s of illumination, there was a distinguished transition of fluorescence from the initial peak, P, through a minimum, S_1, toward a secondary maximum (or shoulder), M. The fluorescence changes of this type, called PS_1-transients, were extensively studied on *Bryopsis* chloroplasts, and were interpreted in terms of redox changes of the primary acceptor Q of PS II [11,12]. In this study, the attention was concentrated on fluorescence changes occurring within a time range of 1–2 s whereupon the most pronounced changes of $\Delta\psi$ took place.

In Fig. 2 are shown typical photoinduction kinetics of $\Delta\psi$ and the chlorophyll fluorescence in an individual chloroplast under the action of three consecutive light pulses. The following experimental procedure was adopted. After an insertion of a microelectrode into the chloroplast, the plant preparation was illuminated with a flash of 1.5 s duration, and then it was left in darkness for 2 min. Following this period of dark adaptation three flashes were fired that were separated with dark intervals of 15 s and 5 s (first and the second dark intervals, respectively). The first light pulse admitted after 2 min dark adaptation (Fig. 2a) induced changes of $\Delta\psi$ with two maxima [9] and a

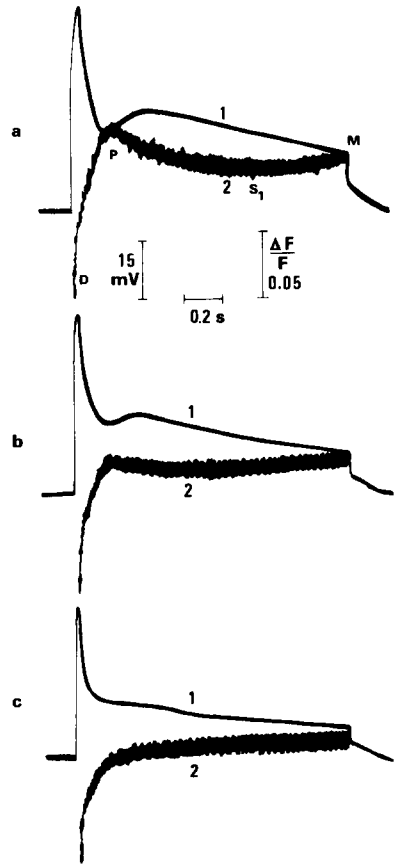

Fig. 2. Simultaneous recordings of the light-induced $\Delta\psi$ (1) and the chlorophyll fluorescence (2) from a single *Anthoceros* chloroplast. (a), After 2 min dark adaptation; (b), 15 s after (a); (c), 5 s after (b). Sequential changes of the fluorescence are marked with symbols D, P, S_1, M. An initial level and a dip of a fluorescence (D) were not resolved.

fluorescence change with an obvious PS_1-transition (temporary decline of the fluorescence). The delayed peak of $\Delta\psi$ and the fluorescence decline from P to S_1 became less pronounced upon a second illumination (Fig. 2b) and were absent on the third flash (Fig. 2c). These modifications of the induction curves were completely reversible. On subsequent 2 min dark adaptation of the chloroplast, the initial time-courses of light-induced $\Delta\psi$ and the fluorescence change were restored.

It is evident from Fig. 2 that within the time-span of the experiment, the induction curves of $\Delta\psi$ and the fluorescence exhibit some similar features. These induction curves look, at a rough inspection, like mirror reflections of each other. The decline of $\Delta\psi$ after the first peak is paralleled by an increase of the fluorescence up to a maximum P. A subsequent initiation of the secondary rise of $\Delta\psi$ coincides in time with the beginning of the fluorescence decline PS_1. The position of the transient minimum of $\Delta\psi$ shifted along the time-scale upon repetitive illuminations (cf. Ref. 9), and a similar shift was noticed in the location of the fluorescence peak P.

We tested the effects of chemical agents that were known to inhibit a second peak of $\Delta\psi$ or a PS_1-transient of the fluorescence attributed to a delayed activation of electron transport. In Fig. 3a are shown changes of $\Delta\psi$ and of the chlorophyll fluorescence of an individual chloroplast after addition of 20 μM DBMIB which is known to inhibit noncyclic electron transport and a ferredoxin-dependent cyclic electron transport by preventing a plastoquinone oxidation [17,18]. It was found previously that DBMIB eliminated the fluorescence decline PS_1 [10]. In the presence of DBMIB, the steady level of $\Delta\psi$ was greatly diminished in accordance with the expected inhibition of electron transport at the plastoquinone level. Both the second peak of $\Delta\psi$ and the transient decline of the fluorescence from P to S_1 disappeared concomitantly under the action of DBMIB.

Apart from modifications of $\Delta\psi$ and fluorescence inductions, a continuous decrease in the overall fluorescence yield was noticed within 5–15 min after an addition of DBMIB without exogenous reductants. The intensity of the fluorescence decreased gradually by a factor 2–3, in accordance with the fact that the oxidised form of DBMIB is a strong quencher of chlorophyll fluorescence [19,20]. Along with the progress in fluorescence quenching by DBMIB, there appeared a slow rising phase in fluorescence induction (Fig. 3a) which might be due to a reduction of DBMIB by PS II [19].

In Fig. 3b are shown the induction curves of $\Delta\psi$ and of the chlorophyll fluorescence that were observed in 5 min after the addition into the medium of sodium dithionite (0.25 mg/ml). Sodium dithionite was supposed to inhibit a photoactivated electron flow in the region of PS I by means of an oxygen depletion [12]. With dithionite added, both the second peak of $\Delta\psi$ and the fluorescence decline from P to S_1 were abolished. A similarity in the time-courses of the fluorescence change and $\Delta\psi$ was completely disturbed under these conditions. There was an apparent sigmoidal phase in the time-course of the fluorescence change which coincided in time with a decay of $\Delta\psi$. A subsequent slow rise of the chlorophyll fluorescence was not accompanied by a change of $\Delta\psi$. The effect of dithionite was presumably mediated by a depletion of O_2. A direct reduction of components of electron-transport chain was unlikely due to a restricted permeation of dithionite through the cell wall and plasmalemma and the envelope membrane of a chloroplast.

Discussion

The results of this study have shown a correlation between the appearance of the delayed rise of $\Delta\psi$ and of the fluorescence decline PS_1. Suppression of the delayed peak of $\Delta\psi$ by preillumination or chemical agents was paralleled with a suppression of a transient decline of the fluorescence PS_1. The data of Figs. 2 and 3 contain the evidence that the second peak of $\Delta\psi$ is associated with events in electron transport and is neither a consequence of a possible decrease of the ionic conductance of the membrane nor the result of ATP-dependent H^+ transport into the thylakoid by a light-activated ATPase (the latter would have been accompanied by a reduction of Q due to a backward electron flow [21]). As an alternative, one might also have proposed a decrease of the proton permeability at a certain pH gradient. This would lead to an increase of $\Delta\psi$ and to fluorescence quenching (due to a higher rate of H^+ accumulation in thylakoids).

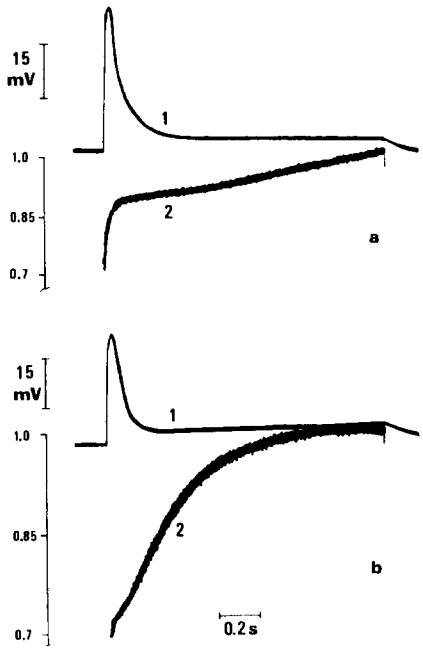

Fig. 3. Effects of DBMIB and dithionite on the kinetics of light-induced $\Delta\psi$ (1) and chlorophyll fluorescence (2) in dark-adapted chloroplasts. (a), In the presence of 20 μM DBMIB; (b), in the presence of 0.25 mg/ml dithionite. The photoinduction kinetics of $\Delta\psi$ and chlorophyll fluorescence under control conditions were similar to those shown in Fig. 2a. The intensity of fluorescence is expressed in arbitrary units.

However, this interpretation is hard to reconcile with observations that the second maximum of $\Delta\psi$ and the fluorescence transition PS_1M remained uninhibited in the presence of NH_4Cl (1 mM) or ionophore A23187 (1 μM), agents supposed to decrease a pH gradient in chloroplast thylakoids (data not shown).

A partial kinetic similarity between $\Delta\psi$ and the fluorescence change under physiological conditions is apparently explained by the dependence of both parameters on the rate of electron transport along the photosynthetic chain. In the light, an accumulation of the reduced electron acceptor Q of PS II and a corresponding increase in the fluorescence takes place due to existence of a rate-limiting step located either between two photoreactions [22] or at the acceptor side of PS I [11,12]. The accumulation of the reduced acceptor Q is kinetically linked with a retardation of the linear electron transport and a respective diminution of the electrogenic component of $\Delta\psi$.

The most probable explanation for the secondary rise of $\Delta\psi$ and the corresponding decrease of the fluorescence PS_1 is that an electron flow driven by PS I accelerates rapidly after a lag of about 200 ms from the onset of illumination [9,11,12]. It has been supposed that the rate limitation in the acceptor side of PS I disappears upon a reduction of a certain component in the region of ferredoxin-NADP-reductase [11,12]. Such a photoactivation of PS-I-associated electron flow coupled with the translocation of protons across the thylakoid membrane would result on the one hand in an increase of $\Delta\psi$ and on the other hand in a reoxidation of the quinone acceptor Q and a decrease of the chlorophyll fluorescence (PS_1 transition). The data presented in Fig. 2 and 3 point to the conclusion that the photoactivated electron transport is electrogenic, i.e., it comprises stages of electron translocation across the hydrophobic layer of the membrane [7].

Although there is a rough kinetic similarity (complementarity) between certain phases in the induction curves and the fluorescence under physiological conditions, one obvious discrepancy is that the delayed peak of $\Delta\psi$ occurs earlier than the transient minimum of the fluorescence S_1 (Fig. 2). The main cause of this discrepancy is that the actual rate of PS-I-dependent oxidation of Q is much higher than the rate of a transient PS_1 [11]. The reoxidation of the acceptor Q monitored as a fluorescence decrease reflects the resulting difference between the rates of Q reduction by PS II and Q oxidation by PS I. It appears therefore that changes of $\Delta\psi$ may provide more coincise reflection of the actual rate of the photoactivated electron transport as compared with a fluorescence transient from P to S_1.

The inhibitory effect of dithionite on the second peak of $\Delta\psi$ indicates the requirement in O_2 for the delayed activation of electrogenic redox steps. Oxygen is required as a terminal acceptor of pseudocyclic electron flow which is presumably activated in the light after a certain time lag [12]. On the other hand, a requirement in O_2 for cyclic electron flow was documented [23]. It is possible therefore that a cyclic electron flow is activated after a lag period in association with a transient oxidation of components between PS II and PS I including a component Q. As was discussed in

Ref. 24, an activation of PS I turnovers might exert a control over the stoichiometry of H^+/e translocation in the cytochrome *bf* complex, so that the electrogenic operation of this complex would become most pronounced.

The described similarity in the time-courses of $\Delta\psi$ and the fluorescence is mediated by alterations in the rate of PS-I-associated electron transport. However, the other relations between the chlorophyll fluorescence and $\Delta\psi$ could also take place in functionally active chloroplasts. The direct effect of $\Delta\psi$ on the fluorescence intensity [4] is of particular importance in this respect.

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