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Fluorescence induction kinetics as a tool to detect a chlororespiratory activity in the prasinophycean alga, *Mantoniella squamata*

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Pulse-modulated fluorescence measuring systems were used to study the fluorescence induction kinetics of the primitive prasinophycean alga, *Mantoniella squamata*. DCMU-treated cells show a very strong non-photochemical quenching which is insensitive to uncouplers and can not be attributed to state transitions or photoinhibition. In order to analyze the origin for this quenching, different inhibitors of the photosynthetic electron flow were applied. It was found that DCCD, an inhibitor of the ATP-synthase as well as of the cytochrome-*c* oxidase, enhances the fluorescence quenching, whereas antimycin A, which blocks the cyclic electron flow around PS I, stimulates it. The effect of both inhibitors can be abolished only under the condition of anaerobiosis, when all components of the electron transport chain were reduced. As a consequence of this, we postulate that this non-photochemical quenching is attributed to the redox state of the plastoquinone pool. In DCMU-treated cells it becomes oxidized under saturating light intensities, but will be reduced by the activity of an electron influx from NAD(P)H. This electron flow is considered to create a pH gradient in the dark, which is probably used for ATP synthesis under dark and extremely low incident light conditions. This chlororespiration requires the existence of an intrathylakoid bound cytochrome-*c* oxidase, which may be a conserved phylogenetic relict in primitive chloroplasts.

Introduction

The pulse-amplitude modulated technique has become a powerful tool to measure changes in the fluorescence induction characteristics of photosynthetically active cells [1,2]. It has been shown that the fluorescence intensity does not only depend on the redox state of Q_A^- (the so-called photochemical-quenching, q_Q) but also on pH gradient (energy-dependent quenching, q_E), on state transitions (q_T), on the injury of photoinhibition (q_I) and some other factors, which are not well understood [3–5]. Some authors have introduced the idea that the fluorescence level can be altered by the redox state of the plastoquinone pool [6,7]. Nevertheless, the experimental basis of these assumptions had not been fully convincing. In this paper we give some new data that under physiological conditions a quenching seems very likely, being controlled by the redox-state of the plastoquinone pool.

The unicellular prasinophycean alga *Mantoniella squamata* was shown to be very unusual with respect to its molecular architecture of the thylakoid membrane. It contains chlorophylls *c* and *b* in its light-harvesting antenna [8,9] and the thylakoids are not truly stacked [10]. There is indication neither of strong lateral heterogeneity nor of state transitions at all [11]. In summary, this alga may be considered as extremely primitive. Recently it was reported that the fluorescence induction curve does not show an energy-dependent quenching starting from dark-adapted cells. This observation led to the assumption that the thylakoid membrane is energi-

Abbreviations: Chl, chlorophyll; DCCD, dicyclohexylcarbodiimide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonyl cyanamidetrifluormethyoxyphenylhydrazone F_o, fluorescence intensity under non-actinic light intensity; FNR, ferredoxin–NADPH oxidoreductase; F_{max}, maximal fluorescence intensity; GO, glucose oxidase, pH, proton gradient; PQ, plastoquinone pool; q_E, energy-dependent quenching coefficient; q_{PQ} non-photochemical quenching due to the redox state of the plastiquinone pool; q_Q, photochemical quenching coefficient.

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zised, even in the dark [11]. This paper will give more information as to whether this unusual phenomenon is attributed to the so-called chlororespiration according to Bennoun [12]. It is considered to be an electron flow oxidizing NAD(P)H at the expense of oxygen by the intermediary of plastoquinone. This pathway of electrons was recently appreciated in the green alga *Chlamydomonas* [13] and in a diatom [14].

Materials and Methods

Mantoniella squamata (Plymouth No LB 1965/5) was cultivated under low light conditions (3.5 W/m^2) as previously published [8]. The cells were taken for measurement directly from logarithmically growing cultures at a chlorophyll content of 3 mg Chl $a+b+c$ l^{-1}.

Two different pluse-modulated fluorometers were used for the experiments. The model PAM 101 (Fa Walz, Effeltrich, F.R.G.) described in detail by Schreiber [2] allows the determination of F_0 by the application of pulsed, non-actinic light and the addition of continuous non-saturating actinic light (3 W/m^2) and saturating flashes (400 W/m^2, with a life-time of 300 ms and a frequency of 1 s). The second system used was that of Caron et al. [14], modified. We use an incident detector ligth (modulated at 500 Hz) of an intensity of 1 W/m^2 which was actinic but definitely subsaturating. The saturating continuous light was adjusted at 50 W/m^2 and was not strong enough to induce photoinhibition, as proved on the basis of light saturation curves. In both measuring systems the cells were maintained under controlled temperature (20 °C). The time of dark incubation before measurement was at least 20 min, but was observed to have no influence on the fluorescence behavior. It was ascertained that the cells are well supplied with CO_2, in order to avoid nonphysiological conditions as best possible. If not otherwise noted, the second measuring system was used.

Reagents were added in the following concentrations: DCMU, $2 \cdot 10^5$ M, NH_4Cl, $5 \cdot 10^{-3}$ M, DCCD, $1 \cdot 10^{-5}$ M, antimycin, $5 \cdot 10^{-6}$ M. Anaerobiosis was obtained by adding 40 units of glucose oxidase/ml, $2 \cdot 10^{-3}$ M glucose and 600 units/ml catalase.

Results and Discussion

In an earlier study [11] it was shown that the fluorescence induction curve of dark-adapted cells exhibits no decrease attributable to the formation of a membrane energization by the onset of actinic light. In order to test whether a pH gradient persists also in the dark, the

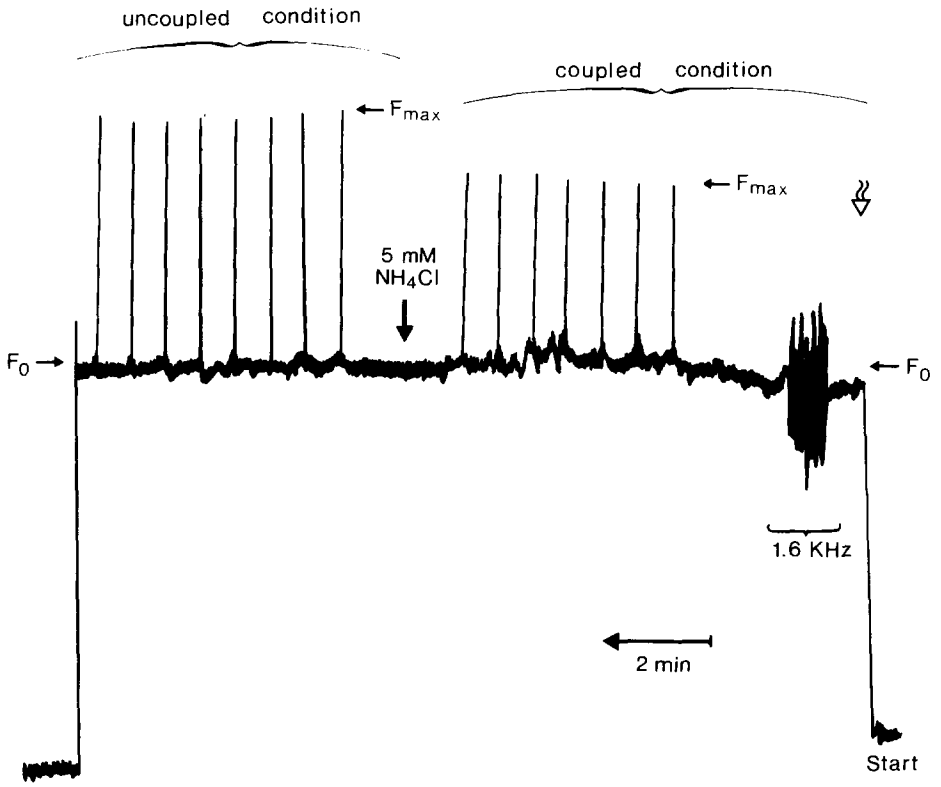

Fig. 1. The modulated fluorescence response (measured with the PAM 101) of the dark-adapted cells from *M. squamata* (right hand), and after the addition 5 mM NH_4Cl (left hand). The cells are maintained in temperature-controlled (20 °C) growth medium at a cell density of 3 mg Chl $a+b+c$ l^{-1}. ⇟, modulated light on.

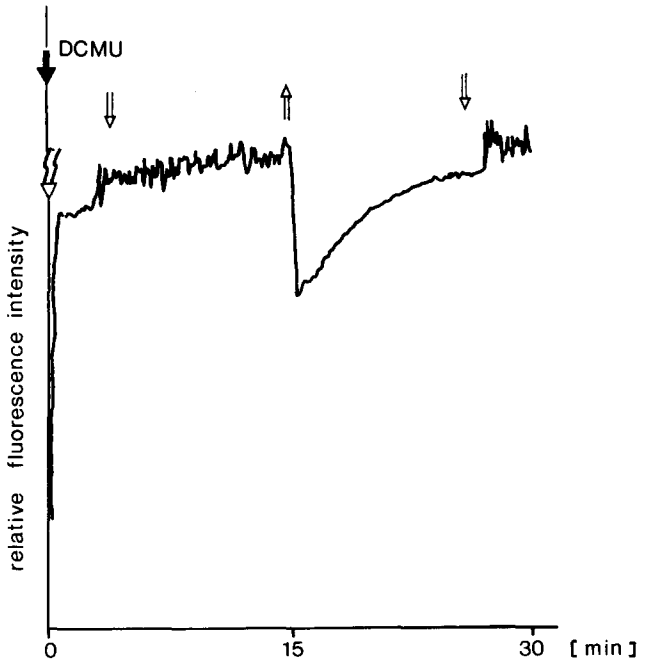

Fig. 2. The modulated fluorescence response of dark-adapted cells from *M. squamata* after the addition of DCMU. ⇓, modulated light on; ⇓, actinic light on; ⇑, actinic light off; ↓, addition of inhibitor. For light intensities see Materials and Methods.

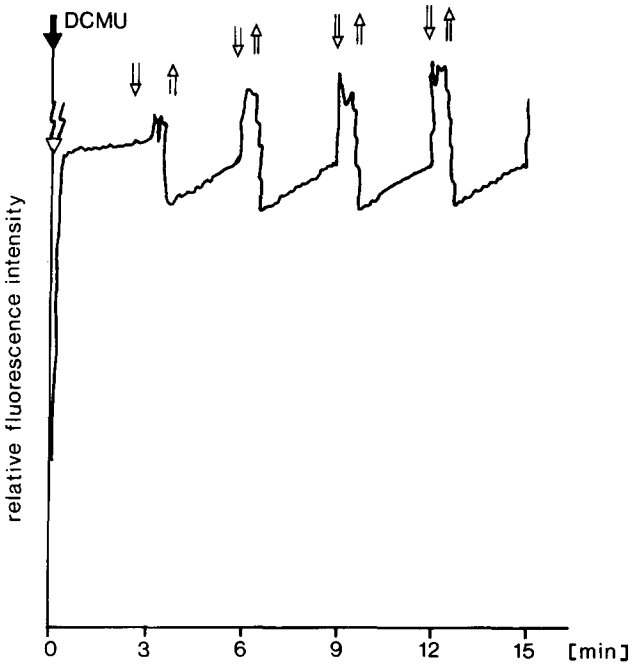

Fig. 3. The reversibility of the non-photochemical quenching of DCMU-treated cells of *M. squamata* after the application of short saturating light pulses (duration of the pulse: 1 min). Symbols, see Fig. 2.

levels of F_o and F_{max} were determined under coupled (Fig. 1, right-hand side reading from right to left) and uncoupled conditions (Fig. 1, left-hand side). The F_o level was detected under pulsed light (1,6 kHz) which was confirmed non-actinic, because the enhancement of the modulation frequency (100 kHz) did not change the fluorescence level. The F_{max} level was depicted by the application of saturating light pulses with a duration of 300 ms. The sequence of several flashes has no influence on the F_o or the F_{max} level. As shown in Fig. 1 the addition of 5 mM NH_4Cl (arrow) does not affect F_o but the F_{max} level. Therefore, it can be concluded that the lack of q_E during a dark–light transient is due to the presence of a pH gradient, even in the dark.

If the photochemical quenching is set to zero by the addition of 20 μM DCMU under saturating light intensities, a pronounced decrease of fluorescence became obvious in a few seconds when the strong light is replaced by modulated light of low intensity. This drop of the fluorescence level will be recovered after some minutes to the initial F_{max} level (Fig. 2). This quenching after strong light and its subsequent recovery under low light are shown to be fully reversible in Fig. 3. In the following it will be called q_{PQ}.

As given in Fig. 4 the fluorescence decay after saturating illumination (q_{PQ}) is not related to an increase of the membrane energization, because uncouplers abolish neither the recovery of fluorescence under low light nor its rise. The only effect of ammonium is the enhancement of the fluorescence level, because q_E is cancelled.

DCCD, an inhibitor of the ATP synthase and the cytochrome-*c* oxidase [15], stimulates the rate of fluorescence recovery leading to a F_{max} level higher than before, under conditions where q_Q und q_E are kept at

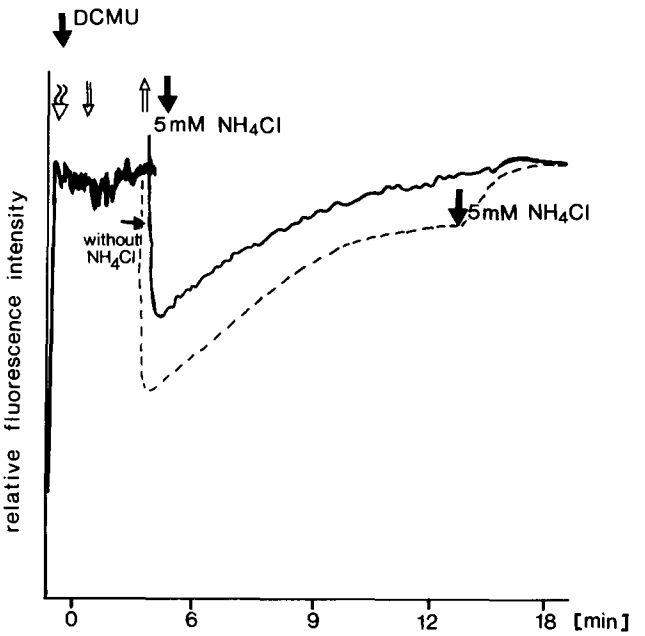

Fig. 4. The modulated fluorescence response of dark-adapted, DCMU-treated cells from *M. squamata* under the influence of NH_4Cl. Symbols, see Fig. 2.

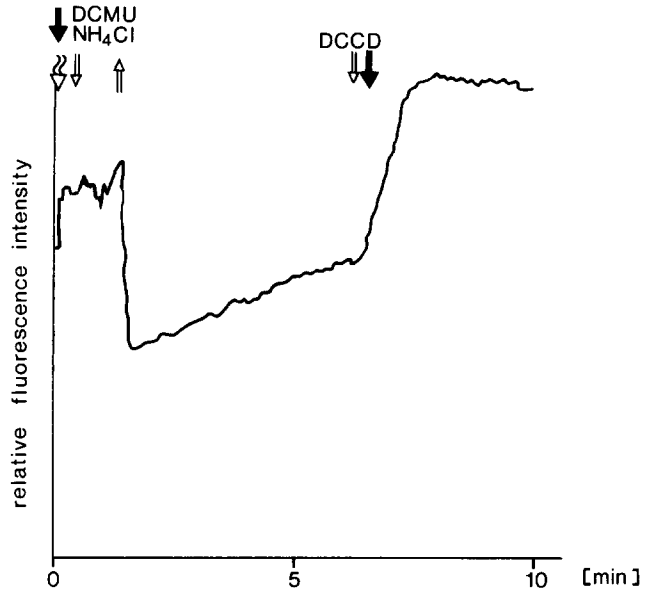

Fig. 5. The influence of DCCD on the fluorescence of DCMU-treated cells of *M. squamata*. Symbols, see Fig. 2.

zero level by DCMU and NH_4Cl (Fig. 5). Fig. 6 shows that the fluorescence rise due to the action of DCCD can not be due to q_E because uncouplers induce and increase of fluorescence level. The action of DCCD eliminates the fluorescence drop after the application of saturating light intensity as well as the subsequent recovery under low actinic light (Fig. 7). There is slight gradual decrease of fluorescence with time which may be caused by the stepwise enhancement of the pH gradient during the light phases. This gradient is thought to persist because under DCCD the protons cannot leave the thylakoid lumen. Therefore, the variable, non-

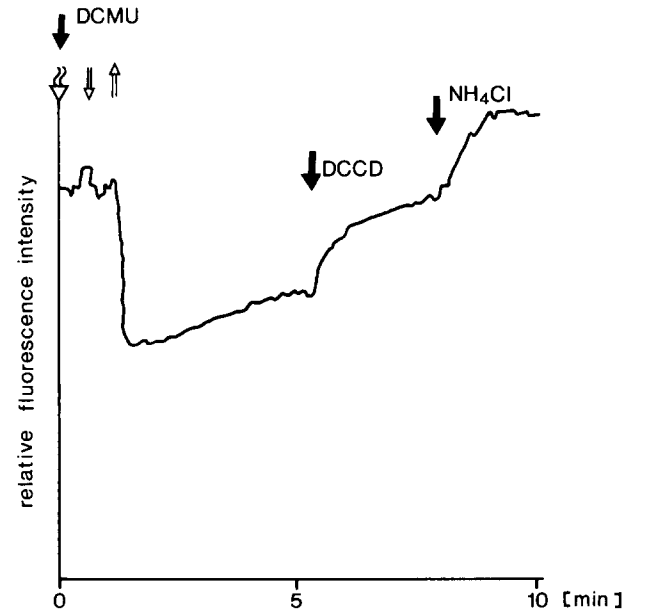

Fig. 6. The influence of DCCD on the fluorescence of DCMU-treated cells of *M. squamata* under uncoupled conditions. Symbols see Fig. 2.

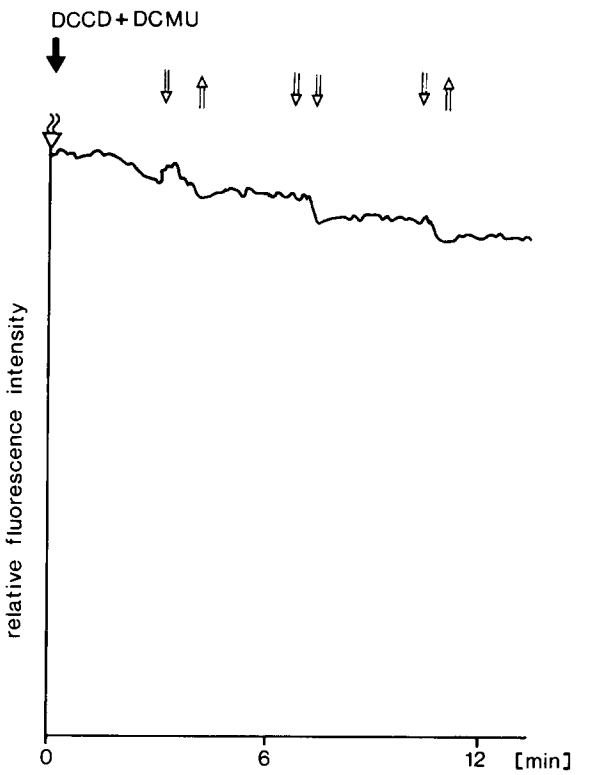

Fig. 7. The fluorescence induction kinetic of *M. squamata* after the addition of DCMU and DCCD under saturating and low (actinic) light. Symbols see Fig. 2.

photochemical fluorescence quenching, q_{PQ}, in *Mantoniella* strictly depends on the activity of the ATP synthase and/or cytochrome-*c* oxidase.

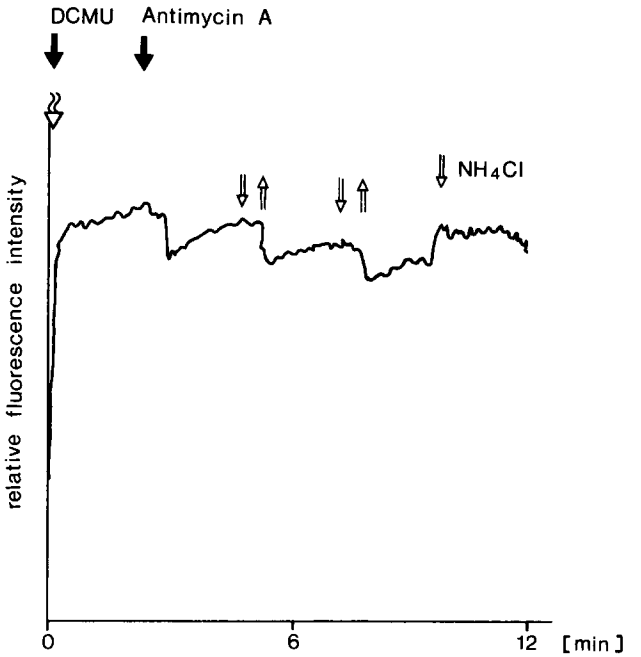

Fig. 8. The influence of antimycin A on the fluorescence of DCMU-treated cells of *M. squamata* under coupled and uncoupled conditions. Symbols, see Fig. 2.

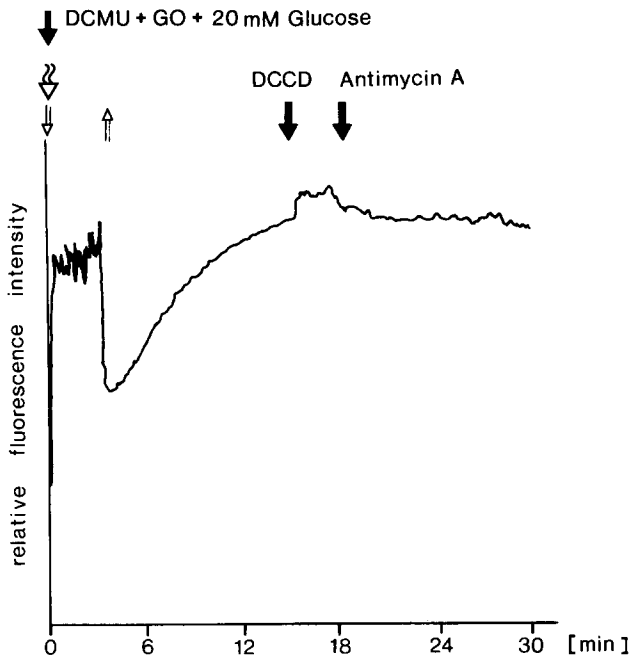

Fig. 9. The influence of anaerobic incubation (glucose oxidase with catalase) on the effect of DCCD and antimycin A on the fluorescence of DCMU-treated cells of *M. squamata*. Symbols, see Fig. 2.

The addition of antimycin A, which inhibits cyclic electron flow [16], results also in a strong reduction of q_{PQ} (Fig. 8). Therefore, it can be concluded that the variable fluorescence under DCMU depends strongly on the activity of the ferredoxin-quinone reductase in the cyclic electron flow around PS I. The inhibition of this electron pathway does not abolish the pH gradient, as previously noted for DCCD.

In order to test the sensitivity of q_{PQ} to the redox state of the plastoquinone pool, the action of DCCD and antimycin A were analyzed in the absence of oxygen. The result is presented in Fig. 9. The cells were incubated 5 min in DCMU and glucose oxidase with 20 mM glucose and catalase. Obviously, the q_{PQ} quench occurs after switching off the saturating light intensity, but the subsequent increase of fluorescence is slightly faster. The effect of DCCD is strongly reduced and that of antimycin A is completely eliminated.

Conclusion and proposed model

The present results confirm our previous assumption that in *Mantoniella* a pH gradient persists also in the dark [11]. It is likely that this membrane energization is generated by the activity of NAD(P)H-dependent

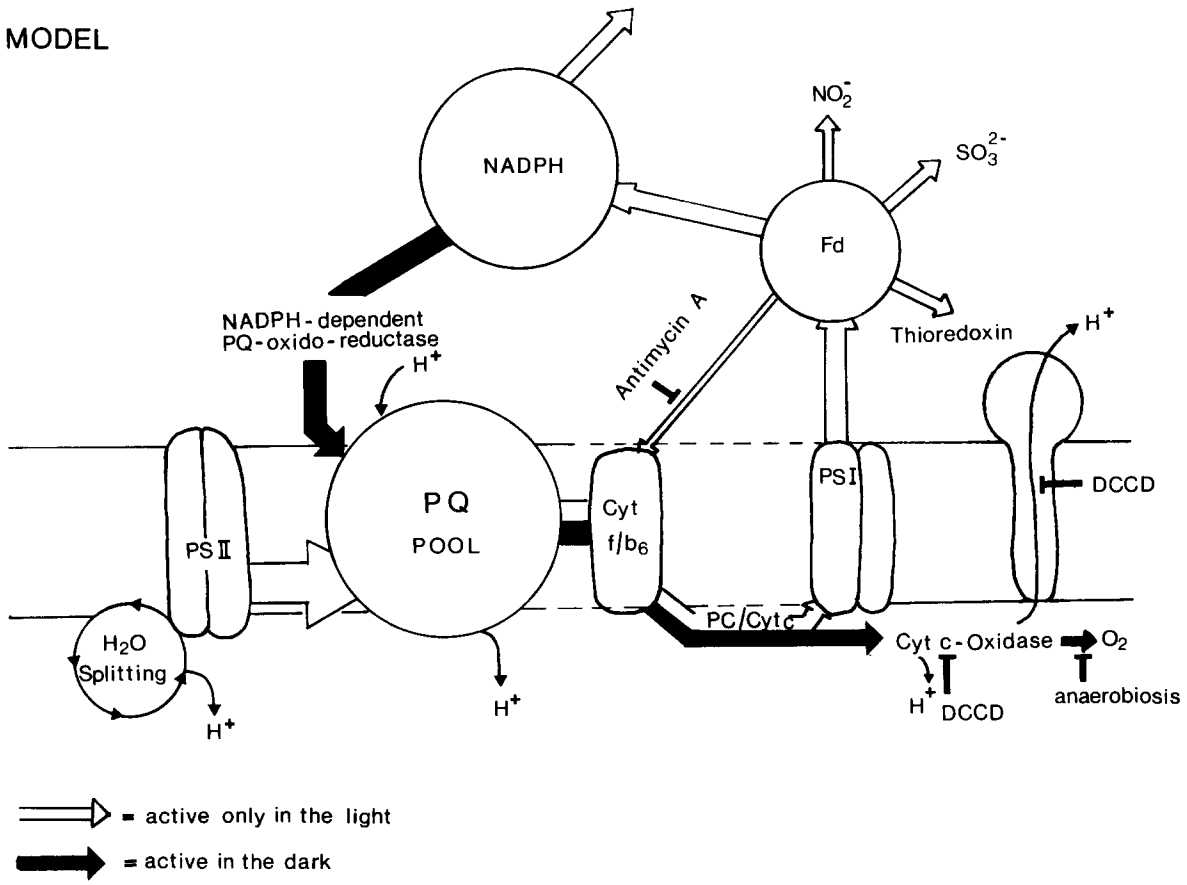

Fig. 10. Proposed model for the regulation of electron pathways in the light and the dark in *M. squamata*. For explanation see text. Symbols see Fig. 2.

plastoquinone oxidoreductase, which imports electrons into the transport chain also in the dark. If this influx of electrons is used not only to maintain an energized state of the membrane but also to produce ATP, the subsequent pathway of these electrons demands an acceptor system which has a relatively high oxidation capacity in the dark. The enzyme that corresponds best with these features is a cytochrome-*c* oxidase, usually present in the inner membranes of mitochondria.

The variability of the fluorescence intensity after a short period of saturating light can not be explained by photochemical activity, because the concentrations of DCMU used completely blocks PS II. Therefore, all changes in the fluorescence yield must be attributed either to non-photochemical quenching mechanisms or to effects caused by electron flows around PS I. The so-called q_{PQ} is not affected by the pH gradient, because active uncouplers like ammoniumchloride or FCCP (not shown) changed neither the drop nor the kinetics of the fluorescence recovery under low actinic light. Because q_{PQ} is strongly reversible without any indication of photoinhibition, q_{PQ} is surely different from q_I in the sense of Oxborough and Horton [5]. The fast fluorescence rise (about 1 s) after the addition of DCCD seems to be due to a strong reduction of the plastoquinone pool, because a postulated cytochrome-*c* oxidase is unable to accept imported electrons from a NAD(P)H-dependent plastoquinone oxidoreductase [15]. Because DCCD also blocks the ATP synthase, a strong pH gradient persists which becomes obvious upon the addition of NH_4Cl (Fig. 6). The observed action of antimycin A corresponds with our hypothesis that q_{PQ} is due to the redox state of the plastoquinone pool. Assuming that antimycin A blocks the cyclic electron flow via the ferredoxin-quinone reductase [16], there is under DCMU a very strong limitation of the influx of electrons, whereas the export is high by the light-driven reactions of PS I. In this case the remaining influx of electrons via chlororespiration is not strong enough to compensate the efflux of the electrons to the different acceptors of the FNR. The consequence is a light-driven, irreversible oxidation of PQ. The most convincing argument for the assumption that q_{PQ} is due to the redox state of the plastoquinone pool is based on the effects of antimycin A and DCCD under anaerobic conditions. When the PQ pool is fully reduced under the absence of oxygen, antimycin A prevents its oxidation in the light by the activity of PS I. In addition, anaerobiosis blocks also the cytochrome-*c* oxidase and no fluorescence enhancement is observed after the addition of DCCD.

In the following model we try to summarize our ideas to explain the unusual fluorescence kinetics observed in *Mantoniella*. The PQ pool can be reduced from three different sources: in the light, the influx is determined mainly by the activity of PS II, secondly by the capacity of the cyclic electron flow (depending on the activity of the FNR) and thirdly by the activity of a membrane-bound NAD(P)H-dependent plastoquinone reductase. In the dark, the latter pathway is the only one for the membrane energization. The oxidation of the PQ pool depends on the activity of two different acceptors: in high light, PS I is the predominant electron acceptor, but in the dark the postulated cytochrome-*c* reductase is able to oxidize this pool and to induce a pH gradient. The variability of the redox state of the PQ pool depends on the capacities and the activities of the different sources and sinks in response to the incident light.

This highly regulated system exihibits a surprising similarity with the thylakoid membrane of blue-green algae, which is characterized by the coexistence of photosynthesis and respiration [17]. Further studies have to be done to prove the model by the direct measurement of the activities of the components involved in this pathway.

Acknowledgements

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