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# State transitions, cyclic and linear electron transport and photophosphorylation in *Chlamydomonas reinhardtii*

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**Abstract**

The relationship between state transitions and the kinetic properties of the electron transfer chain has been studied in *Chlamydomonas reinhardtii*. The same turnover rate of cytochrome *f* was found in state 1 and 2. However, while DBMIB was inhibitory in both states, DCMU was effective only in state 1. These observations suggest that linear electron transport was active only in state 1, while a cyclic pathway around photosystem (PS) I operated in state 2. The reversible shift from linear to cyclic electron transport was modulated by changes of PSII antenna size, which inactivated the linear pathway, and by oxygen, which inhibited the cyclic one. Attainment of state 2, under anaerobiosis in the dark, was associated with the decline of the ATP/ADP ratio in the cells and the dark reduction of the intersystem carriers. Upon illumination of the cells, the ATP/ADP ratio increased in a few seconds to the aerobic level. Then, several minutes later, the $F_m$ returned to the state 1 level, and $O_2$ evolution was reactivated. This suggests that ATP, though required for photosynthesis, is not the rate-limiting factor in the reactivation of photosynthetic $O_2$ evolution, which is rather controlled by the redox state of the electron carriers. 

*Keywords:* State transition; Electron transport pathway; Cytochrome *f*; Energy charge; *Chlamydomonas reinhardtii*

## 1. Introduction

Highly efficient photosynthesis requires a balanced excitation of the two photosystems and a constant supply of ATP and reducing power (NADPH) in the ratio of not less than 3:2, as required for the assimilation of $CO_2$ in the Calvin cycle. The balanced modulation of excitation energy flux to the two photosystems is achieved by the state transitions discovered by Bonaventura and Myers [1]. This phenomenon occurs via the reversible transfer of a fraction of the light harvesting complex II (LHCII) from photosystem (PS) II to PSI (state 1 to state 2 transition), as the consequence of its phosphorylation [2–4]. Dephosphorylation of LHCII restores state 1. Both the protein kinase and the phosphatase involved are thylakoid-bound. The kinase is activated when the intersystem redox carriers are over-reduced [5–7], i.e. when PSII activity exceeds that of PSI.

Abbreviations: Chl, chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonylcyanide *p*-(trifluoromethoxy)phenylhydrazone; $F_m$, maximal value of fluorescence emission; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonate); LHC, light harvesting complex; PQ, plastoquinone; PS, photosystem; MV, methylviologen

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In higher plants, only 15–20% of the light harvesting complexes are transferred during state transitions. The excitation rates of PSII and PSI are modulated accordingly, resulting in a balanced excitation of the two photosystems [3]. In the unicellular green alga *Chlamydomonas reinhardtii*, however, the change in the relative size of the two antennae during the state transitions is much larger: ca 80% of the PSII antenna is translocated to PSI in state 2 [8,9]. Moreover, it has been demonstrated that the cytochrome $b_6f$ complex accumulates in the stroma lamellae in state 2 [10]. In *Chlamydomonas*, therefore, state 2 represents a condition where most of the excitation energy is utilised by PSI photochemistry, implying that cyclic electron transport and/or PSI-dependent photooxidation of cellular metabolites must be prevailing over linear electron transport.

Investigating state transitions in *Chlamydomonas*, Bulté and colleagues [11] reported a number of experimental conditions, all causing the transition to state 2, which were associated with a decrease of the cellular ATP content and the concomitant reduction of the intersystem electron carriers. They found that recovery of ATP levels promoted the transition to state 1. These authors therefore suggested that state transitions were regulated by the cellular level of ATP. This hypothesis is still a subject under investigation.

We have studied the changes in the pathways and rates of electron transport and of ATP level in *C. reinhardtii* as related to state transitions. We show here the transition from linear to cyclic electron flow upon attainment of state 2, as demonstrated by inhibition of cytochrome $b_6f$ complex turnover by 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), but not by 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU). This transition is modulated by the large changes in the relative antenna size of the two photosystems, which are likely to be responsible for the inhibition of linear electron transport. However, the cyclic electron flow is probably regulated by the PSII-dependent generation of oxygen, which inhibits the cyclic electron flow. The cyclic electron flow is coupled to the synthesis of ATP, which occurs immediately upon illumination.

The transition to state 1 and the recovery of photosynthetic oxygen evolution is a rather slow phenomenon, having a half-time of several minutes. This time can be significantly reduced by an addition of small amounts of $O_2$ in the dark. Thus, we conclude that the limiting factor for the restoration of photosynthesis in state 2 cells is the reoxidation of the intersystem electron carriers, which triggers the transition to state 1.

## 2. Materials and methods

### 2.1. Strains and culture conditions

*C. reinhardtii* cells (from strain 137C) were kindly provided by the Laboratoire de Physiologie Membranaire et Moléculaire du Chloroplaste at the Institut de Biologie Physico-Chimique of Paris (France). Cells were grown at 24°C in acetate-supplemented medium [12] under $\sim$60 μE $m^{-2}$ $s^{-1}$ of continuous white light. They were harvested during exponential growth and resuspended at the required concentration in HS minimal medium [13]. Chlorophyll (Chl) was measured as the absorbance at 680 nm of the cell cultures in a spectrophotometer equipped with a scatter attachment, on the basis of a calibration curve constructed after extraction of the chlorophyll with 80% acetone [14].

### 2.2. Oxygen evolution and uptake

Photosynthesis and respiration were measured as the $O_2$ exchanges by means of a Clark-type electrode (Radiometer, Denmark) in a home-made cell at 25°C. Illumination was provided by a halogen lamp. The light was filtered through a heat filter, and its intensity was defined, as needed, by neutral density filters.

### 2.3. Determination of ATP and ADP

The determination of these metabolites was made in trichloroacetic acid (TCA) extracts of the cells. After thawing, the extract was centrifuged in a microfuge to obtain a clear acid-soluble fraction. ATP and ADP were measured enzymatically after two extractions of the TCA by ethyl ether. The TCA-soluble fractions, after extraction with ether, have a pH of 5–5.5. They do not show any adenylate kinase

activity (which may be found in neutralised perchloric acid extracts) and are suitable for enzymatic measurements. The extracts were added to a spectrophotometer cuvette together with a Tris buffer at a final concentration of 0.2 M, pH 8.0, and 0.5 mM NADP. The absorbance at 340 nm was read, then glucose 6-phosphate dehydrogenase (6 units) was added, and the glucose 6-P present was measured as the increase of absorbance. ATP was measured as the further increase of absorbance upon addition of hexokinase (10 units) and glucose (5 mM, final concentration). After the reaction was completed (ca 30 s), adenylate kinase was added (10 units) to measure ATP+ADP. ADP in the extract was measured upon subtraction of the amount of ATP. This method was found to be more reliable, though less sensitive, than the luciferin-luciferase method, which gave essentially the same results (not shown), but the firefly enzyme luciferase was more sensitive to the small amounts of trichloroacetate ion remaining in the extract, which may severely inhibit its activity.

### 2.4. Spectroscopic measurements

For spectroscopic measurements, algae were harvested during exponential phase ($\sim 2\times10^6$ cells $ml^{-1}$), and resuspended in a HEPES-KOH 20 mM buffer, pH 7.2, with the addition of 20% Ficoll (w/v) to avoid cell sedimentation during measurements. The cytochrome *f* redox changes upon illumination were measured in intact cells as the difference between the absorption at 554 nm and a baseline drawn between 545 and 573 nm. All experiments were performed at room temperature, using a home-made spectrophotometer similar to that described by Joliot and Joliot [15]. Continuous red illumination was provided by a LED array placed on both sides of the measuring cuvette. Heat-absorbing filters were placed between the LED arrays and the cuvette.

Fluorescence was measured using the same optical apparatus. Emission was excited at 480 nm, and measured in the near infrared region. Actinic illumination was provided by the same LED array as in cytochrome *f* measurements. Alternatively, fluorescence was measured in the same chamber used for oxygen measurements, by means of a PAM fluorimeter (Walz, Germany).

## 3. Results

### 3.1. Electron transfer properties of C. reinhardtii cells under state 1 and state 2 conditions

We have measured the kinetics of cytochrome *f* redox changes in *C. reinhardtii* under continuous illumination. In dark-adapted aerobic algae, switching the light on generated an oxidation signal (absorption decrease), which rapidly attained a plateau level (Fig. 1A). After the light was switched off a reduction could be observed, which brought the absorption signal to its initial value. This profile is typical of the redox changes of cytochrome *f* under continuous illumination [16]. The signal was sensitive to DCMU, a well known inhibitor of plastoquinone reduction by PSII [17]. Increasing the concentration of DCMU increased the extent of the oxidation, demonstrating that the latter is sensitive to the amount of photogenerated plastoquinol, which is known to be the electron donor for cytochrome *f* reduction. Moreover, the oxidation signal was sensitive to DBMIB, an inhibitor of cytochrome *f* reduction by plastoquinol [18]. The spectrum of the signals is reported in Fig. 1B: in all cases (without additions, plus DCMU and/or DBMIB), it corresponded to that of cytochrome *f* [19], confirming that the observed kinetics represent genuine cytochrome *f* redox changes.

Fig. 2 reports the kinetics of cytochrome *f* in algae under different experimental conditions, suitable to establish either state 1 or state 2. To allow a better comparison of the results, all the measurements of Fig. 2 were performed on algae of the same culture. Thus, panel A substantially reproduced the same condition as in Fig. 1A, i.e. aerobic condition (state 1). In panels B and C, the cells were placed in state 2, by two different treatments: aerobic conditions in the presence of an uncoupler (carbonylcyanide *p*-(trifluoromethoxy)phenylhydrazone, FCCP 5 μM, panel B) or anaerobiosis (panel C). Both treatments promote the transition to state 2 in the dark, through the reduction of the plastoquinone (PQ) pool [11,20]. The fluorescence measurements reported in panel D confirmed the attainment of state 2: both in anaerobiosis (dotted line) and in aerobiosis in the presence of FCCP (dashed line); the maximal level of fluorescence yield ($F_m$) decreased about 40% (compared to

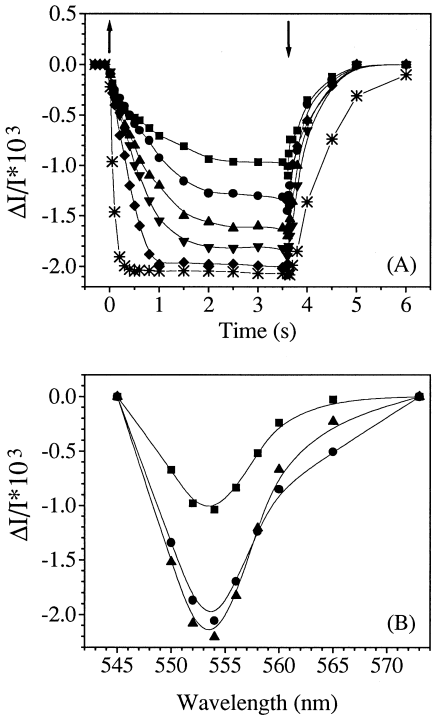


Fig. 1. Light-induced redox changes in cytochrome $b_6f$ complex of *C. reinhardtii* cells under continuous illumination. Algae were collected during the exponential growth phase and resuspended in HEPES 20 mM, pH 7.2, with the addition of 20% (w/v) Ficoll to avoid sedimentation. Chlorophyll concentration was 30 μg $ml^{-1}$. Red actinic light was provided by a LED array placed on both sides of the cuvette. Light intensity was 223 μE $m^{-2}$ $s^{-1}$ (A) Effects of DCMU on the redox changes of cytochrome *f* upon continuous illumination. DCMU concentrations were 0 (squares), 20 nM (circles), 50 nM (upward triangles), 100 nM (downward triangles) and 2 μM (diamonds). DBMIB-treated samples (2 μM, crosses) are also shown for comparison. After attainment of steady-state conditions, the actinic light was switched off (arrows: up, light on; down, light off), and the relaxation of absorption changes was followed for ~3 s. (B) Light-induced absorption spectra. Spectra were recorded after attainment of steady-state absorption changes, and were normalised at 545 and 573 nm for comparison. Squares: no addition; circles: DCMU 10 μM; triangles: DCMU 10 μM plus DBMIB 2 μM.

control, continuous line), in agreement with previous reports in *Chlamydomonas* [11,21,22].

No matter how state 2 was reached, a signal reflecting the electron transfer through cytochrome *f* could be observed: the plateau level was essentially the same as in state 1, indicating very similar rates of turnover. However, while DBMIB inhibited the reduction of cytochrome *f* both in state 1 and in state 2, increasing the extent of the oxidation signal, DCMU inhibited only when the cells were in state 1. This was not due to an incomplete block by DCMU of the photochemical activity of PSII in state 2, as indicated by the observation that the rise of fluorescence to the maximal value was even faster in state 2 than in state 1, in agreement with previous observations [22]. Rather, these data suggest that a shift from linear to cyclic electron transfer (DCMU-insensitive) was induced upon transition from state 1 to state 2. Upon switching the light off, the signals relaxed to the initial dark level. The rate of this recovery was markedly inhibited by the addition of DBMIB in both state 1 and state 2, while it was

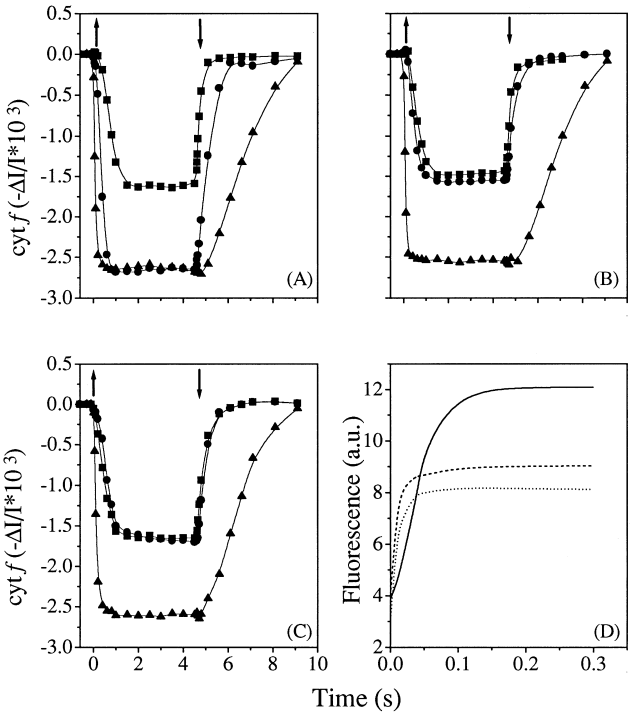


Fig. 2. Light-induced cytochrome *f* redox changes in state 1 and state 2 conditions. (A) State 1, algae were kept in darkness under continuous vigorous agitation in air to prevent reduction of the PQ pool. (B) State 2, obtained through dark addition of 5 μM of FCCP to the same algae of panel A. (C) State 2, obtained through dark incubation of algae in argon. Squares, no addition; circles, DCMU 10 μM; triangles, DCMU 10 μM plus DBMIB 2 μM. (D) Fluorescence emission measurements. Continuous line: same algae as in panel A; dashed line: same algae as in panel B; dotted line: same algae as in panel C. Fluorescence measurements were recorded on dark-adapted algae, placed in the same conditions used for electron transport measurement, as described in Section 2. Other conditions as in Fig. 1.

affected by DCMU only in state 1. In addition, a lag in the reduction of cytochrome *f* was observed in the presence of DBMIB (Fig. 2). Addition of methylviologen (MV), at the concentration of 100 μM, completely restored the DCMU sensitivity of cytochrome *f* reduction in state 2 (not shown).

We observed that FCCP treatment induced a less defined state 2, as shown by the smaller quenching of fluorescence (Fig. 2D). This was probably due to the very strong oxygenation of the algae necessary to keep them in the aerobic state, which partially prevented the reduction of the PQ pool – as required to induce state 1 to state 2 transition [20] – even in the presence of uncouplers. The less efficient attainment of state 2 resulted also in a slightly more pronounced effect of DCMU on the electron transport through cytochrome *f* (Fig. 2C). Therefore, we decided to induce the transition to state 2 through anaerobic incubation.

In both states 1 and 2, the steady-state level of cytochrome *f* oxidation remained unchanged when the light intensity was increased, while the oxidation rate measured in the presence of DBMIB increased significantly (not shown). This indicates that the reduction rate of cytochrome *f* increased as the light intensity became stronger. We have calculated the rates of electron injection into cytochrome *f* as a function of light intensity. This rate is governed by several factors, and should therefore be expressed as $-df^{+}/dt = k_{red}*[f.FeS.b_l.b_h]^{+}*[PQH_2]$, where $k_{red}$ is the second-order rate constant for PQ oxidation at the Qo site of the cytochrome $b_6f$ complex, while *f*, FeS, $b_l$ and $b_h$ represent, respectively, the cytochrome *f*, the Rieske protein and the low and high potential haems of the cytochrome *b* moiety. The concentration of most of these components, however, is not

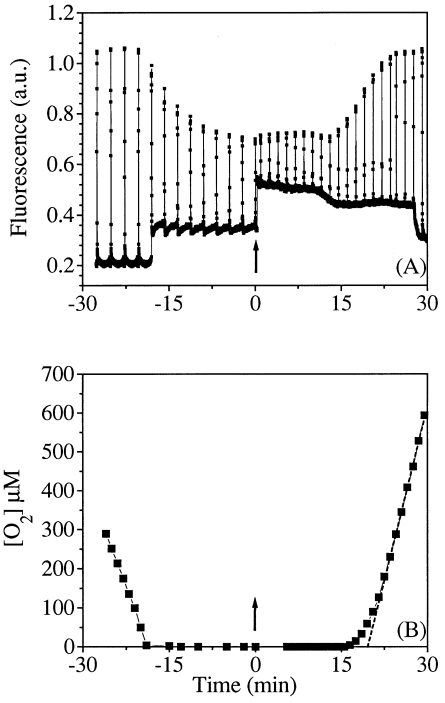


Fig. 3. Fluorescence emission, oxygen consumption and evolution in *C. reinhardtii* during state transitions. (A) Fluorescence emission measured continuously with a PAM fluorimeter. $F_m$ level was measured upon illumination of the algae with a 2 s saturating pulse of white light (2200 μE $m^{-2}$ $s^{-1}$). [Chl] = 200 μg $ml^{-1}$. (B) Oxygen concentration changes. Dark-adapted algae were placed in the oxygen electrode chamber (at time −25 min), and oxygen consumption by respiration was monitored continuously by a Clark-type electrode, simultaneously to fluorescence emission. At time ca −18 min, anaerobiosis was reached (see B), and fluorescence rise was concomitantly observed. Actinic light (850 μE $m^{-2}$ $s^{-1}$) was switched on at time 0 (arrows).

Table 1
Rates of electron transfer through cytochrome $b_6f$ complex in state 1 and state 2 as a function of light intensity

| Light intensity (μE $m^{-2}$ $s^{-1}$) | State 1 | | State 2 | |
|---|---|---|---|---|
| | no addition | DCMU | no addition | DCMU |
| 221 | 10.7 | ~0 | 12.1 | 12.5 |
| 578 | 23.2 | ~0 | 25.1 | 24.8 |
| 986 | 29.5 | ~0 | 50.3 | 50.0 |
| 1190 | 34.1 | ~0 | 43.6 | 45.0 |
| 1700 | 71.2 | ~0 | 65.4 | 70.0 |

The rates of cytochrome *f* reduction were calculated from the traces of Figs. 2 and 3 as explained in the text. Rates are expressed as mol $e^{-}$ $s^{-1}$.

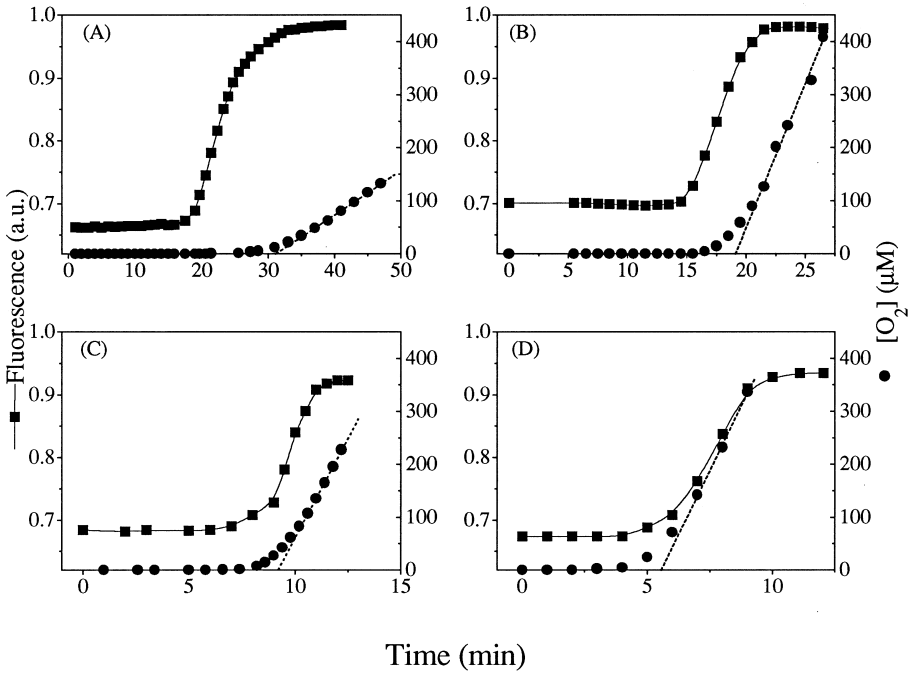


Fig. 4. Light intensity dependence of maximal fluorescence emission and $O_2$ evolution in *C. reinhardtii* during state 2 to state 1 transition. Light intensities were 300 μE $m^{-2}$ $s^{-1}$ in panel A, 850 μE $m^{-2}$ $s^{-1}$ in panel B, 1400 μE $m^{-2}$ $s^{-1}$ in panel C and 3000 μE $m^{-2}$ $s^{-1}$ in panel D. Squares represent the $F_m$ values measured as in Fig. 3. Circles represent oxygen concentration, expressed as nmol $ml^{-1}$. Other conditions as in Fig. 3.

known with sufficient precision to estimate the reduction rate of cytochrome *f* in our conditions.

Nevertheless, it is possible to derive a simpler equation that describes the phenomenon, starting from the consideration that, in steady state, the rates of reduction and oxidation of cytochrome *f* are the same. The latter process can be expressed simply as $df/dt = k_{ox}*[f]*[PC^+]$, where $k_{ox}$ is the second-order rate constant for cytochrome *f* oxidation by plastocyanin, which is indicated here as PC. Thus, the rates of cytochrome *f* reduction can be computed as the product of the fraction of reduced cytochrome *f* – which is indicated by the difference between the plateau levels in the absence and the presence of DBMIB (Figs. 2 and 3) – and the factor $k_2*[PC^+]$, which can be calculated from the initial rate of cytochrome *f* oxidation when its reduction is inhibited, i.e. in the presence of DBMIB. The latter inhibitor, indeed, does not affect the electron transfer from reduced cytochrome *f* to PSI.

The results are presented in Table 1. There, it is shown that the rates of electron injection into the cytochrome $b_6f$ complex were very similar in state 1 and in state 2 and increased similarly as a function of light intensity, to values that were comparable to previous estimations from thylakoids of higher plants [23]. Very small differences, if any, were found between the rates calculated in the absence and in the presence of DCMU in state 2, indicating that the rate of cyclic electron transfer did not decrease when light intensity approached saturation.

### 3.2. Oxygen evolution and fluorescence changes under state 1 and state 2

We have sought further evidence for the hypothesis that in state 2 most, if not all, of the photosynthetic activity involves only cyclic electron flow around PSI. To this end, we measured oxygen evolution by the algae, as a probe for the activity of PSII. The results are presented in Fig. 3, where the changes in oxygen consumption and evolution are presented, together with the simultaneous measurement of fluorescence emission. Upon incubation of *C. reinhardtii* in the dark, $O_2$ was consumed and the cells became anaerobic. When the $O_2$ concentration

approached zero (Fig. 3B), the fluorescence rose abruptly, and $F_m$ started to decrease (Fig. 3A), as a consequence of state 1 to state 2 transition. The decrease of $F_m$ started immediately after attaining anaerobiosis, then proceeded with a half-time of 5–7 min, observed in different cultures of cells, to a constant low value (see example in Fig. 3A). This observation is in agreement with previous reports [24]. The attainment of anaerobiosis was an absolute requirement to observe the onset of state 1 to state 2 transition, in agreement with the notion that transition to state 2 requires the reduction of the PQ pool [5].

In state 2, no oxygen evolution was observed upon switching the light on (Fig. 3B), in spite of the fact that electron transfer through the cytochrome $b_6f$ complex occurred at approximately the same rate as in state 1 (see Fig. 2 and Table 1). This, again, indicated that only PSI-dependent cyclic electron transport was active in state 2. The onset of oxygen evolution required several minutes of illumination, and was preceded by the recovery of fluorescence to the state 1 level (Fig. 3A,B). Apparently the two phenomena (state 2 to state 1 transition and the restoration of oxygen evolution) are strictly connected.

We further analysed the correlation between fluorescence changes and oxygen evolution in state 2 cells at different light intensities (Fig. 4). At low light intensity (panel A), the state 2 to state 1 transition largely preceded the onset of oxygen evolution (see also Fig. 3). At increasing light intensities the two phenomena became closer in time (panels B and C), and under strong illumination they occurred simultaneously (panel D). From the traces in Figs. 3 and 4, we quantified the 'lag' of photosynthesis, i.e. the time required to observe a constant rate of oxygen evolution upon illumination of dark-anaerobic cells. This was done by tracing the intercept (dashed lines, Figs. 3 and 4) between the steady-state rate of oxygen evolution eventually reached and the abscissa axis.

Table 2
Estimation of the lag of photosynthesis upon illumination of anaerobic *C. reinhardtii* cells

| Additions | 'Lag' (min) | Steady-state $O_2$ evolution (μmol $O_2$ mg $Chl^{-1}$ $h^{-1}$) |
|---|---|---|
| None | 17.0 | 17.1 |
| $O_2$ 10 μM | 7.5 | 16.0 |
| Nigericin 2 μM+ nonactin 4 μM | 33.5 | 17.0 |

Algae (200 μg Chl $ml^{-1}$) were dark-incubated until oxygen was consumed by respiration and state 2 was consequently reached. Light was switched on after attainment of a steady state of maximal fluorescence quenching (12–15 min). The 'lag' of photosynthesis was estimated as described in the text (see also Fig. 3). When oxygen was added, the light was switched on 30 s after the addition, to allow the consumption of most of it by respiration. Nigericin and nonactin were added initially to the aerobic algae. Other conditions as in Fig. 4. Light intensity was 850 μE $s^{-1}$ $m^{-2}$.

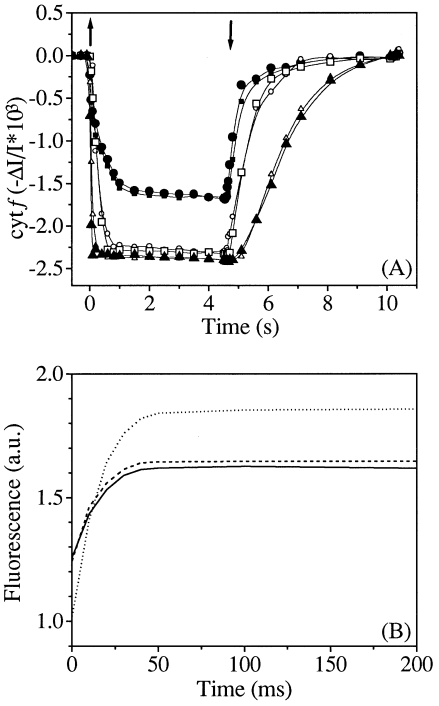


Fig. 5. Effect of oxygen on cyclic electron transport in state 2 *C. reinhardtii* cells. Algae were collected as in Fig. 1, and resuspended in the same medium, with the addition of 5000 U $ml^{-1}$ of catalase. State 2 was induced by anaerobic incubation in argon. Oxygen was generated by injection of calibrated amounts of $H_2O_2$ into the cell suspension, and the measurement of cytochrome *f* turnover was initiated after 2 s. The oxygen concentration (~200 μM) decreased during the measurement because of respiration (not shown). Its concentration at the end of the measurement was ~50 μM. Other conditions as in Fig. 1. (A) Light-induced cytochrome *f* redox changes. Circles: control; squares: DCMU 10 μM; triangles: DCMU 10 μM plus DBMIB 2 μM. Closed symbols: anaerobiosis; open symbols: oxygen added, ~200 μM. (B) Fluorescence emission. Continuous line: no oxygen added; dashed line: oxygen added, ~200 μM; dotted line: aerobiosis ($[O_2]$ = 257 μM).

The results are reported in Table 2, where the effects of the addition of uncouplers or of oxygen on the 'lag' of photosynthesis are also shown. Uncouplers greatly increased the lag, even at concentrations that did not inhibit the steady-state rate of photosynthesis. When added at higher concentrations, however, they completely inhibited photosynthesis (not shown).

We observed that oxygen had a dual effect on the photosynthetic electron transport activity of *Chlamydomonas*: on the one hand, it reduced the lag of $O_2$ evolution when added to the dark-anaerobic cells, even at the rather low concentration of 10 μM (Table 2). This effect was probably related to the oxidation of the PQ pool, as indicated by the decrease of the steady-state fluorescence emission upon its addition (not shown).

On the other hand, the addition of oxygen during illumination blocked the cyclic electron flow, as revealed by its strong inhibitory effect on the kinetics of cytochrome *f* in anaerobic state 2 cells (Fig. 5A). The cells remained in state 2 during the short time required to measure electron transport, as shown by the fluorescence levels (Fig. 5B).

### 3.3. ATP synthesis under state 2 conditions

In a previous work, Bulté et al. [11] analysed the changes of the ATP/ADP ratio during the transition from state 2 to state 1, and suggested a regulatory role of ATP in this process. We therefore monitored the changes of ATP and ADP under the same conditions used above to measure the changes of fluorescence and $O_2$ concentration. The contents of ATP and ADP in dark-aerobic *C. reinhardtii* cells, during the transition to anaerobiosis, and upon illumination with low intensity are presented in Table 3. The measurements obviously concern all cellular nucleotides, and do not distinguish the different compartments (chloroplast, cytosol, mitochondria). This does not affect our conclusions, inasmuch the illumination affects first the chloroplast compartment.

The ATP/ADP ratios were very high in aerobic *Chlamydomonas* in darkness, and decreased strongly upon reaching anaerobiosis, without changes in the ATP+ADP sum, in agreement with previous reports [11,25]. During prolonged anaerobic incubation in the dark, the ATP content slowly increased (Table 3), probably because of the increased rate of glycolysis, due to the reversal of the Pasteur effect [26]. A subsequent illumination of the cells raised their ATP concentration in a few seconds (Table 3) to its maximal value, which was similar to that observed in aerobic conditions or under saturating light intensity (not shown). The synthesis of ATP upon illumination was slightly slowed down in the presence of uncouplers, when they were added at the same concentration at which they increased the lag of $O_2$ evolution (Table 3).

Table 3
Effect of uncouplers on changes of ATP and ADP upon transition to anaerobiosis and illumination in anaerobic conditions

| | Time | | Control | | | Nigericin+Nonactin | | |
|---|---|---|---|---|---|---|---|---|
| Conditions | Dark | Light | ATP | ADP | ATP/ADP | ATP | ADP | ATP/ADP |
| Aerobiosis | – | off | 114 | 12.0 | 9.6 | 131 | 10.3 | 12.7 |
| Anaerobiosis | 1–2 s | off | 83.0 | 26.0 | 3.2 | 74.0 | 27.7 | 2.7 |
| Anaerobiosis | 90 s | off | 82.0 | 26.0 | 3.2 | 81.0 | 29.0 | 2.8 |
| Anaerobiosis | 180 s | off | 95.0 | 23.0 | 4.1 | - | - | - |
| Anaerobiosis | 180 s | on 5 s | 106 | 9.0 | 11.8 | 104 | 19.9 | 5.2 |
| Anaerobiosis | 180 s | on 60 s | 110 | 11.0 | 10.0 | 120 | 9.2 | 13.0 |
| Anaerobiosis | 180 s | on 600 s | 103 | 8.5 | 12.0 | 111 | 8.6 | 12.9 |

Measurements are reported as nmol mg $Chl^{-1}$. Light (400–700 nm) intensity was 850 μE $m^{-2}$ $s^{-1}$. Aerobiosis corresponds to an oxygen concentration of 257 μM. The concentrations of nigericin and nonactin were, respectively, 2 and 4 μM. *Chlamydomonas* was incubated in the $O_2$ electrode cell at a concentration of 200 μg Chl $ml^{-1}$; samples were taken for ATP and ADP analysis initially (aerobic condition), then after anaerobic incubation for the times indicated in column 2. The other samples were fixed in the light after the indicated time of anaerobiosis followed by illumination as indicated in column 3. Figures refer to a single experiment measurement. However, the amount of ATP found in 14 measurements was 114 ± 5.9 and ADP was 11.9 ± 1.3.

## 4. Discussion

### 4.1. Properties of the electron transfer system under state 1 and state 2 conditions

Our results show that in *C. reinhardtii* cells placed in state 2, the turnover of cytochrome *f* occurs at a rate very close to that observed in state 1 (ca 70 $e^-$ $s^{-1}$, see Fig. 2 and Table 1), though no oxygen evolution by PSII can be observed (see Figs. 3 and 4). This demonstrates that only PSI-dependent photochemistry is responsible for cytochrome *f* reduction under these conditions. This is further confirmed by the finding that the reduction of cytochrome *f* is sensitive to DCMU only in state 1, but is substantially insensitive to this inhibitor in state 2 (Fig. 2 and Table 1). In contrast, DBMIB, a well known inhibitor of cytochrome $b_6f$ reduction by PQ [18], is effective in both state 1 and state 2, indicating that the kinetics of cytochrome *f* observed by us depend upon the normal functioning of the cytochrome $b_6f$ complex. The latter, however, is functionally connected to PSII only in state 1.

In principle, in anaerobic state 2 cells another source of electrons to cytochrome *f* reduction could be envisaged, i.e. the non-photochemical, dark reduction of the PQ pool by the cytoplasmic metabolism previously observed in green algae [15,27–29]. This process reduces the PQ, allowing cytochrome *f* reduction through a DBMIB-sensitive reaction, which is coupled to the generation of the $\Delta\tilde{\mu}_{H^+}$ necessary for ATP synthesis. We consider this possibility rather unlikely, since the rate of glycolysis of *C. reinhardtii* has been estimated to be in the range of 10–30 μmol of carbon mg $Chl^{-1}$ $h^{-1}$ [26,30]. This rate is at least two orders of magnitude slower than the maximum rate of cytochrome *f* turnover ($\sim$70 $e^-$ $s^{-1}$) observed under the same conditions. As to the rate of ATP synthesis, the glycolytic activity mentioned above could account for less than 3 nmol of ATP formed mg $Chl^{-1}$ $s^{-1}$, assuming a stoichiometry of 2 ATP/glucose fermented. We conclude, therefore, that PSI-dependent cyclic photophosphorylation is by far the major source of ATP for anaerobic *Chlamydomonas* cells in state 2, and it accounts for the observed rapid formation of ATP upon illumination (Table 3).

It remained to be established whether cyclic electron transport around PSI is also active in state 1, as the process supplying ATP in the stoichiometry of 3:2 NADPH, as needed by the assimilation of $CO_2$. This seems to be ruled out by our experiments, as we found that the same extent of cytochrome *f* oxidation is reached in the presence of DCMU and of DBMIB. This indicates that in state 1, under steady-state conditions, no electrons are injected into the cytochrome $b_6f$ complex in the presence of DCMU, i.e. no cyclic electron flow from PSI is operative.

In state 1, however, some differences exist at the level of the pre-steady-state kinetics of cytochrome *f*: the oxidation rate is faster in the presence of DBMIB than DCMU (Fig. 2). This is due to the fact that the PQ pool is partially reduced in the dark, even in aerated *C. reinhardtii* cells [27], and is therefore able to re-reduce cytochrome *f* when DCMU is added. In contrast, in the presence of DBMIB, which binds directly to the cytochrome $b_6f$ complex, electron transfer from the PQ pool is prevented and the oxidation kinetics are consequently faster.

Also the rates of cytochrome *f* reduction – measured after the light is switched off – are different in the presence of DBMIB and DCMU: they are much slower in the presence of the former. This difference is probably related to the properties of these inhibitors. As DBMIB binds directly to the cytochrome *f* reducing site, it blocks the reduction of the *f* haem not only in the light, but also in the dark, until it unbinds from the complex. Therefore, the differences existing between the rates of cytochrome *f* reduction in the dark observed in the presence of DCMU and DBMIB are probably due to the very slow unbinding rate of the latter [31], rather than to differences in the steady-state kinetics of cytochrome *f* turnover during illumination.

The prevailing, if not exclusive, operation of cyclic electron transport in state 2 is a major functional difference between *C. reinhardtii* and other organisms, such as higher plants. In the latter, state transitions involve the migration of only 15–20% of the antenna from PSII to PSI and vice versa (reviewed in [2,3]), and are considered to serve the purpose of balancing the excitation energy distribution between the two photosystems. In contrast, in *Chlamydomonas* state transitions displace most of the PSII pigments that became connected to PSI in state 2 [8,9]. Also, it is known that cyclic photophosphorylation,

as observed in isolated thylakoids from higher plants, is a rather slow process catalysed by ferredoxin [32], and can occur at high rates only in completely artificial systems, with the addition of non-physiological electron carriers (reviewed in [33]).

In isolated, intact chloroplasts from spinach, Heber and colleagues (see e.g. [34]) have shown that the quenching of Δ-aminoacridine fluorescence (a measure of ΔpH) is inhibited by antimycin A only when an efficient electron acceptor is absent. They concluded that cyclic phosphorylation occurs in intact thylakoids, on the basis of the notion that antimycin A inhibits cyclic electron transport, but does not affect the linear one. The existence of cyclic photophosphorylation in vivo, however, has been demonstrated only under non-physiological conditions established upon addition of inhibitors which prevented photosynthesis, such as in the presence of DCMU in higher plants [35], and in *Chlorella* and *Scenedesmus* [36]. Moreover, Myers [37] did not find any evidence in favour of cyclic electron transport in intact cells of several cyanobacteria.

At the moment, the physiological relevance of the shift between linear and cyclic electron transport in *C. reinhardtii* upon state transitions is unclear, but it probably represents a way to utilise light energy for ATP synthesis when PSII is inactive, i.e. in conditions of anaerobic stress, which can be experienced by these cells in natural conditions.

### 4.2. Regulation of linear and cyclic electron transport during state transitions

The switch from linear to cyclic electron flow observed upon transition to state 2 (and vice versa) strongly suggests that both electron transfer pathways are modulated and can be switched on or off depending on the conditions of the cells.

In particular, linear electron transport efficiency is mainly modulated by the changes in the antenna size of PSII which occur during state transitions. The migration of most of the PSII antenna to PSI drastically decreases PSII activity, consequently strongly inhibiting the efficiency of linear electron flow. Upon state 2 to state 1 transition, recovery of its antenna restores PSII activity, and therefore reactivates linear electron transfer. In particular, at low light intensity, where absorption by PSII limits its overall activity, a nearly complete transition to state 1 would be required before $O_2$ evolution activity is recovered. This requirement should be less stringent as light intensity increases. This is shown in Fig. 4, where it appears that the antenna migration from PSI to PSII largely precedes $O_2$ evolution at low light intensity, while the two phenomena tend to become simultaneous at higher intensities.

However, it is known that a fraction of PSII antenna is still connected to it even in state 2 [9]. Thus, one would expect to observe some linear flow even in this condition, especially at high light intensity, where antenna deprivation is less restrictive for PSII activity. Apparently, this is not the case (Fig. 2). We interpret this apparent contradiction as the consequence of the decreased availability of cytochrome *f* to electrons coming from PSII, because of the concentration of the former in the stroma thylakoids [10]. The increased distance between PSII and the cytochrome complex, indeed, would increase the time required for plastoquinone shuttling beyond a limit compatible with the persistence of linear electron flow (see e.g. the discussion in [38]). PSII would nevertheless be photochemically active, as indicated by the persistence of a fluorescence level below $F_m$ (Fig. 3), but would not contribute electrons to cytochrome *f*. Instead, its electrons would probably recombine with the donor side, as previously suggested in the case of *Chlamydomonas* cells where electron transfer from PSII to cytochrome *f* is prevented by chemical treatments [29].

In contrast, cyclic electron flow modulation cannot be explained only in terms of antenna size. Upon transition to state 1, cyclic electron flow is switched off, in spite of the fact that PSI is active and its antenna size is still large [9]. Therefore, the existence of a different regulation, which should be able to switch off cyclic electron transport upon transition to state 1, i.e. when PSII is active, must be postulated.

We believe that this factor is PSII-dependent $O_2$ evolution, as we have demonstrated that the presence of oxygen is able to switch off completely cyclic electron transport in state 2 cells (Fig. 5), provided that the electrochemical proton gradient is present (Fig. 2C). We consider this observation physiologically very relevant. However, the mechanism of the inhibition by $O_2$ is not known. It seems probable that it

competes for electrons with the cyclic pathway at the reducing side of PSI. It is well known that this is the case in the presence of MV, and indirect evidence has been provided that the Mehler peroxidase reaction may act as a competitor for the cyclic process, in the presence of a $\Delta\tilde{\mu}_{H^+}$ in intact chloroplasts (e.g. [39]). The activity of the ascorbate-monodehydroascorbate linear system is high [40–42] and it has been demonstrated that this reaction represents a significant percentage of electron transfer activity during photosynthetic carbon assimilation in intact spinach chloroplasts [43]. Therefore, we propose that the mechanism responsible for switching off cyclic electron flow in state 2 when oxygen is added (Fig. 5) is the Mehler peroxidase reaction, i.e. the reduction of molecular oxygen by photosynthesis.

### 4.3. Modulation of state transitions through cellular energy metabolism

We have shown that, upon illumination of anaerobic state 2 cells, the ATP/ADP ratio strongly increased in less than 5 s even at rather low light intensity (see Table 3). The level attained is the same as that observed in aerobic conditions and in saturating light intensity, nor could ATP be further increased appreciably, because ADP is almost undetectable. After this rapid increase, the transition to state 1 and the recovery of $O_2$ evolution occur, but these latter processes require several minutes. Thus, we conclude that photophosphorylation, though an absolute requirement for photosynthetic $O_2$ evolution, is not the kinetically limiting factor for the reactivation of photosynthesis in dark-anaerobic *C. reinhardtii*.

Instead, the light dependence of both the recovery of fluorescence and oxygen evolution is indicative of the influence of electron transfer rate on the reversal to state 1, i.e. on the deactivation of the LHCII kinase. This enzyme is, indeed, activated by the presence of a $PQH_2$ molecule in the Qo site of cytochrome $b_6f$ [7,21], and is deactivated by its oxidation [44]. The phosphatase, in contrast, can be considered constitutively active in our experimental conditions [21]. The effect of light intensities on the transition to state 1 can be explained, therefore, in terms of the slower turnover rate of cytochrome $b_6f$ in low light, which increases the permanence of $PQH_2$ in the Qo site and consequently the deactivation time of the kinase. In agreement with this concept, we found that only at saturating light the recovery time of fluorescence and oxygen evolution is similar to that required by the antenna to back-migrate from PSI to PSII [24].

Therefore, the redox state of quinols in the Qo site of cytochrome $b_6f$ complexes seems to be the main regulator of state transitions: this is also shown by the complementary effect of oxygen and uncouplers on the 'lag' of photosynthesis (Table 2). Oxygen induced a transient oxidation of the pool (probably via a respiratory oxidation of the electron carriers [28,29]) in the dark, and then inactivates PQ reduction by cyclic electron transport when the light is switched on. The two effects of $O_2$, therefore, induce a strong oxidation of the PQ pool, which is a prerequisite to re-establish state 1 [20]. Conversely, the uncouplers induce the reduction of the PQ pool (in agreement with [11]) which is probably not overcome by the oxidising activity of cytochrome $b_6f$ complex. For this reason, they dramatically increase the lag of photosynthesis, in spite of the fact that, at the concentrations employed, the ATP content rose to its maximum in a time that is still very short as compared to that required to resume photosynthetic activity (Table 2).

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