*Biochimica et Biophysica Acta*, 1020 (1990) 72–80
Elsevier

BBABIO 43285

# ATP control on state transitions in vivo in *Chlamydomonas reinhardtii*

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(Received 20 April 1990)

Key words: State transition; ATP depressing treatment; Photophosphorylation, cyclic; Starch breakdown; (C. *reinhardtii*)

**We have attempted to depress the ATP content in dark adapted cells of the unicellular green algae, *C. reinhardtii*, by inhibiting ATP synthesis coupled to mitochondrial electron flow. Whether we used uncouplers, ATP synthase inhibitors, inhibitors of mitochondrial electron transport or a mutant altered in mitochondrial cytochrome *b*, we observed a fluorescence quenching at room temperature associated with an increased reduction of the intersystem electron carriers of the photosynthetic apparatus. This fluorescence quenching reflected a genuine transition to state II, since: (i) it was associated with an increase in light-harvesting complex phosphorylation and (ii) it did not occur in mutants lacking the $b_6/f$ complex which are blocked in State I. The complete reversion to state I occurred only into experimental conditions which allowed both ATP synthesis and reoxidation of intersystem electron carriers. We conclude that the intracellular demand for ATP controls state transitions in vivo. The interplay between intracellular ATP concentrations and the redox state of the kinase activator at the thylakoid membrane level is discussed.**

## Introduction

State transitions were first described as a short-term chromatic adaptation in green algae and red algae [1,2]. Considerable efforts have been made in recent years to further elucidate the molecular basis of these transitions in algae and higher plants (Ref. 3 and reviewed in Refs. 4 and 5). Depending on their state of phosphorylation, antenna proteins were shown to migrate reversibly between PS I and PS II, thus providing variations in the proportion of light excitation energy distributed to each photosystem.

The physiological significance of this extensive reorganization of the light-harvesting apparatus in the thylakoid membranes nevertheless remains obscure. It has been mainly understood as an adaptation mechanism by which plants and algae restore optimal photosynthesis in situation where the two photosystems would otherwise receive unbalanced light excitation: this would be the case of bottom leaves in a canopy, or at the inner surface of a leaf, exposed mainly to far red light due to screening effects of blue and red light.

It has also been suggested that the decrease in PS II antenna size due to LHC phosphorylation could play a role in the protection of PS II against photoinhibition [6]. However, the inactivation of the LHC-kinase during photoinhibition [7] argues against this possibility.

An alternative view has been developed by P. Horton and colleagues (Ref. 8 and also discussed in Ref. 9): state transitions would regulate the linear to PS I-mediated cyclic electron flow. This would occur in response to variations both in the redox state of some IEC and in the 'energy state' of the membrane [10] which may reflect cellular variations in ATP levels. Recently, some of us have observed that blocking mitochondrial ATP production in intact cells of *C. reinhardtii*, led to a decrease in the ratio of 685/717 fluorescence emissions at 77 K [11,12], as is the case upon transition to state II in this organism [13,14]. Here, we demonstrate that inhibition of ATP production in intact cells of *C. reinhardtii* leads to a genuine transition to state II, whereas reversion to state I requires ATP synthesis. This argues

Abbreviations: IEC, intersystem electron carriers; LHC, light harvesting complex; PS, Photosystem; DCMU, dichlorophenyldimethylurea; DCHC, dicyclohexyl-*crown*; AA, antimycin A; SHAM, salicylhydroxamic acid; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; DCCD, dicyclohexylcarbodiimide; TBT, tri-*n*-butyltin; MV, methylviologen; PMSF, phenylmethylsulfonyl fluoride; p-BQ, *para*-benzoquinone; TAP, Tris-acetate-phosphate; WT, wild type.

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for the view that state transitions in vivo are primarily aimed at responding to variations in cellular ATP demand.

## Material and Methods

The various strains of *C. reinhardtii* used in this study were grown in TAP medium, except when otherwise indicated. They were kept under continuous illumination at 300 lx except for the DUM-1 mutant which was grown at 1000 lx. The FUD6 mutant strain was previously characterized as a mutant lacking the $b_6/f$ complex owing to the absence of synthesis of subunit IV [15]. The FUD50 and ac46 mutants were characterized as chloroplast ATP synthase mutants [16,17]. The Dum-1 mutant was isolated and characterized by Matagne [18] as a mutant defective in mitochondrial electron transport.

Cells were harvested at mid-exponential phase ((3–4) $\cdot 10^6$ cells/ml) and resuspended at a concentration of $2 \cdot 10^7$ cells/ml in TAP medium for fluorescence induction experiments, or in the same buffer lacking phosphate (TA) for $^{32}P$ labeling. In the latter case cells were incubated for 90 min in the presence of 75 MBq/ml [$^{32}P$]$P_i$ then washed in TA medium and placed in the various states under investigation. Starved cells were obtained after growth in minimum medium (lacking acetate) under 1000 lx illumination followed by an incubation in darkness for several hours.

For nucleotides determination, cells were resuspended in minimum medium and stirred for 1 h in the dark. For the determination in the presence of methylviologen, catalase was added in the medium at a concentration of 120 $\mu$g/ml in order to eliminate hydrogen peroxide. Pyridine and adenine nucleotides were determined as described in Ref. 11. Starch content was estimated according to Ref. 19.

Cells were placed in state I either by an incubation for 20 min under continuous illumination (2700 lx) in the presence of DCMU or in darkness under strong aeration in the case of the FUD6 mutant. Conventional state II was achieved by anaerobic incubation in darkness under nitrogen atmosphere for 30 min.

For determination of antenna polypeptide phosphorylation, $^{32}P$-labeled cells were fixed in their final state by p-BQ incubation as described in Ref. 20. Thylakoid membrane preparations were then performed as previously described [13] in the presence of 10 mM EDTA, 200 $\mu$M PMSF and 10 mM NaF.

Fluorescence inductions, gel electrophoresis and autoradiography were performed as in Ref. 13.

## Results

### *State I to state II transition upon ATP-depressing treatments*

Shown on Table I are the maximal fluorescence levels, at room temperature, of WT cells of *C. reinhardtii* placed in state I (DCMU + light), state II (anaerobiosis) or after various treatments aimed at inhibiting the synthesis of ATP in the dark, which then originates from oxidative phosphorylation in the mitochondria. To this end we incubated the WT cells with uncouplers, 5 $\mu$M FCCP or 1 mM DCHC, ATP synthase inhibitors, 2 $\mu$M TBT or 5 $\mu$M DCCD, or with 1 $\mu$M AA + 1 mM SHAM, which block respectively the cytochrome oxidase and the alternative oxidase in the mitochondria. The latter situation could be readily obtained by incubating in darkness the Dum-1 mutant with SHAM only, since it has a block in mitochondrial electron transport at the level of cytochrone *b*. Most of these treatments induced a drop in total content in intracellular ATP, as shown in Table I.

As previously reported [13], the $F_{max}$ level was almost 2-times higher in state I than in state II, due to a decreased PS II sensitization in the latter case. Cells in which ATP production was blocked by any of the treatments listed above, had a low fluorescence yield

TABLE I

*Changes in metabolites and fluorescence yields upon inhibition of ATP synthesis of mitochondrial origin*

Measurements are given relative to the control (State I or no ad.).

| | WT | | | | | | | | | Dum-1 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | State I [a] | State II [b] | FCCP | DCHC | TBT | DCCD | AA/SHAM | SHAM | no ad. | no ad. | SHAM | State I |
| ATP | 100 | 65 | 40 | 65 | 75 | 90 | 45 | 100 | 100 | 80 | 40 | 100 |
| ATP/ADP [c] | 7 | 2 | 0.8 | 1.3 | 3 | 7 | 1 | 7 | 7 | 2.5 | 1 | 5 |
| NADPH [d] | – | – | 158 | – | – | – | 200 | 100 | 100 | – | – | – |
| $F_{max}$ | 100 | 61 | 56 | 63 | 50 | 59 | 54 | 85 | 85 | 55 | 45 | 100 |

[a] DCMU + light (2700 lx).

[b] Anaerobiosis.

[c] ATP + ADP = 70–130 nmol/mg Chl; ratios are given ±25%.

[d] NADPH + NADPH = 9–16 nmol/mg Chl, with NADPH about 35% of the total in the control (no ad.). All values are an average of at least three experiments.

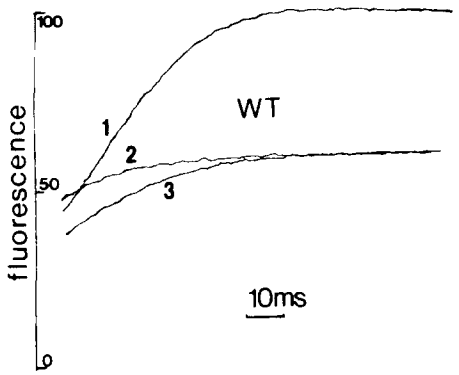


Fig. 1. Fluorescence induction curves in the presence of $10^{-5}$ M DCMU using WT cells of *C. reinhardtii*. Curve 1: 2700 lx illumination in the presence of DCMU for 20 min (state I conditions). Curve 2: dark incubation with 5 μM FCCP for 30 min. Curve 3: dark incubation with 1 μM AA + 1 mM SHAM for 30 min.

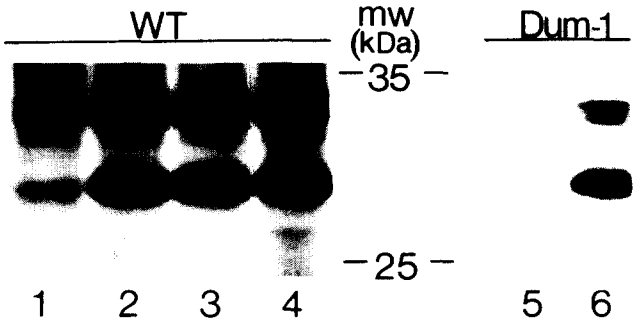


Fig. 2. Antenna phosphopolypeptides viewed after autoradiography of the 25–35 kDa region on an electrophoretogram loaded with SDS-solubilized thylakoid membrane proteins from $^{32}$P-labeled cells. Left: WT cells were placed (1) in state I by 2700 lx illumination in the presence of $10^{-5}$ M DCMU; (2) in the dark with $5 \cdot 10^{-6}$ M FCCP; (3) in the dark with $10^{-6}$ M AA + $10^{-3}$ M SHAM; (4) in state II by incubation in the dark under $N_2$ atmosphere. Right: Dum-1 mutant cells were placed (5) in state I by 2700 lx illumination in the presence of $10^{-5}$ M DCMU; (6) in the dark with no addition.

with $F_{max}$ levels similar to that in state II (Table I). In particular, cells from the DUM-1 mutant when aerated in darkness displayed a low fluorescence yield, whereas WT cells when similarly treated had a fluorescence yield close to that of state I.

For further comparison are shown on Fig. 1 the fluorescence induction curves in state I (curve 1) and after FCCP treatment (curve 2) or AA + SHAM treatment (curve 3). We note that the extensive fluorescence quenching at $F_{max}$ observed in the latter cases (curves 2 and 3) was not accompanied by a similar quenching at $F_0$. A similar observation has been previously reported for cells placed in state II by an anaerobic incubation [20]. Because the $F_0$ is in part controlled by the proportion of PS II traps which are closed upon addition of DCMU – due to the conversion of $Q_a/Q_b^-$ centers to $Q_a^-$/DCMU centers (21,22) – p-BQ treatment, which oxidizes the PS II acceptors but preserves state II [20], restored a quenching both at $F_0$ and $F_{max}$ as compared to cells in state I (results not shown). Therefore the high $F_0$ in curves 2 and 3 was indicative of an increased reduction of the IEC upon incubation in the dark with FCCP or AA + SHAM.

Shown in Fig. 2 is the autoradiography of the 25–35 kDa region, where apoproteins of Chl *a/b* protein complexes migrate, in an electrophoretogram of SDS-solubilized thylakoid membranes. Cells were labelled with $^{32}$P and placed either in state I or in state II and compared with cells treated with AA. + SHAM or FCCP. In the latter cases, the algae displayed the same increased phosphorylation on antenna polypeptides as after the anaerobic incubation leading to state II. Antennae polypeptides from algae aerated in darkness were extensively dephosphorylated as previously reported [23]. A similar increase in polypeptide phosphorylation was observed after a TBT, DCCD or DCHC treatments (results not shown). The mere incubation in darkness of the DUM-1 mutant also led to an extensive phosphorylation of LHC subunits (right of Fig. 2).

In contrast with the WT, the FUD6 mutant, lacking $b_6/f$ complexes, showed no changes at the $F_{max}$ level upon treatments which inhibited mitochondrial ATP production (Table II). Such mutants were previously shown to be blocked in state I due to the absence, or to the lack of activation, of the LHC-kinase [24,23].

These treatments, however, induced a major change in the fluorescence rise in the absence of DCMU. This is easily observed on Fig. 3 where fluorescence inductions from cells aerated in darkness (curve 1) or treated with FCCP (curve 2) or AA + SHAM (curve 3) are compared. Owing to the absence of $b_6/f$ complexes (which results in a block in photosynthetic electron transfer after the PQ pool), the fluorescence kinetics in the absence of DCMU rise up to the $F_{max}$ level. The

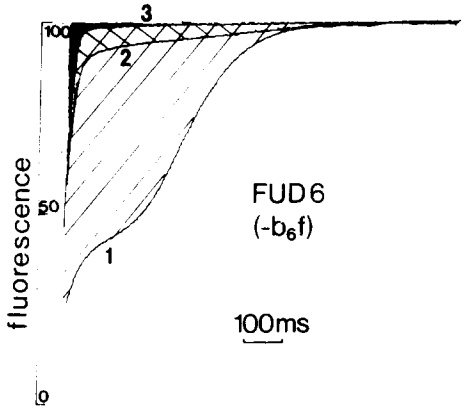


Fig. 3. Fluorescence induction curves in the absence of DCMU, using cells from the FUD6 mutant, lacking the $b_6/f$ complex. Curve 1: cells aerated in darkness. Curve 2: dark incubation with 5 μM FCCP. Curve 3: dark incubation with 1 μM AA + 1 mM SHAM. The hatched area are a measure of the size of the PQ pool. Note that the time-scale is 10-times longer than in Fig. 1.

TABLE II

*Changes in metabolites and fluorescence yields in cytochrone $b_6/f$ mutants, upon inhibition of ATP synthesis*

Measurements are given relative to the control (no ad.).

| | No ad. | FCCP | DCHC | TBT | DCCD | AA/SHAM |
|---|---|---|---|---|---|---|
| ATP | 100 | 50 | 50 | 78 | 73 | 55 |
| ATP/ADP [a] | 7 | 0.8 | 1.2 | 2.5 | 3 | 1.2 |
| NADPH [b] | 100 | 200 | – | – | 265 | |
| $F_{max}$ | 100 | 98 | 96 | 90 | 94 | 100 |
| Area [c] | 100 | 19 | 8 | 34 | 13 | 5 |

[a] ATP + ADP = 150–200 nmol/mg Chl; ratios are given ±25%.
[b] NADPH + NADP = 13 nmol/mg Chl with NADPH about 15% of the total, in the control (no ad.).
[c] Area bound by the fluorescence induction curve and its $F_{max}$ asymptote, indicative of the size of the plastoquinone pool.

pool size of PS II acceptors may then be evaluated through the area, hatched on Fig. 3, bound by the fluorescence rise and its $F_{max}$ asymptote [25]. The various treatments used to inhibit ATP production resulted in a large decrease in this area (Fig. 3 and Table II). This indicated an extensive reduction of the PQ pool upon inhibition of ATP synthesis in darkness. From the comparison of the two sets of experiments with the WT and FUD6 mutant, it turns out that ATP-depressing treatments induced a transition to state II in vivo which was associated with an increased reduction of the IEC. The mechanism by which this transition occurs in vivo may then involve the same redox control on kinase activity that has been previously characterized in a number of in vitro studies [3–5].

*Reversion from State II to State I*

We then investigated whether the reversion from state II to state I required a restoration of high intracellular ATP levels or the mere reoxidation of some IEC. We attempted to reoxidize the IEC through a 20 min illumination (2700 lx) in the presence of DCMU (condition 1 in Table III). These experiments were performed with algae previously placed in state II by several treatments which blocked ATP synthesis in darkness, but were distinguishable by their ability to synthesize or not ATP upon illumination.

The effect of such reversion experiments on the phosphorylation of the LHC subunits is shown in Fig. 4 in three cases. In case 1, state II was reached by incubating WT cells in the dark with the uncoupler FCCP. This treatment prevented mitochondrial ATP production as well as cyclic photophosphorylation. Consequently, we detected no increase in intracellular ATP content upon transfer of DCMU-poisoned algae from darkness (35

TABLE III

*ATP sources allowing state II to state I transition*

| Sample in state II (darkness) | Condition of illumination (1–4) [a] | putative ATP sources [b] | $F_{max}$ increase [c] (%) | Final state [d] |
|---|---|---|---|---|
| WT( N2) | 1, 2 | M, Pc | 100 | I |
| | 3, 4 | M, Pl | 79 | I |
| WT (FCCP) | 1–4 | none | 20 | II |
| WT, Dum-1 (AA + SHAM) (unstarved) | 1 | Pc | 100 | I |
| | 2 | Pseudo Pl | 97 | I |
| | 3 | $Pl^-$ | 10 | II |
| | 4 | $Pl^+$ | 95 → 59 | I |
| WT, Dum-1 (AA + SHAM) (starved) | 1 | Pc | 100 | I |
| | 2 | none | 20 | II |
| | 3 | $Pl^-$ | 29 | II |
| | 4 | $Pl^+$ | 63 → 17 | I → II |
| ac46, FUD50 (AA + SHAM) | 1 | none | 22 | II |
| | 2 | Starch | 74 | I |
| | 3 | none | 29 | II |

[a] Illumination conditions 1–4 are for 20 min, except for two cases in condition 4 where the first value is after 5 min and the second after 20 min.
(1) 2700 lx illumination in the presence of DCMU.
(2) same as 1 with $10^{-3}$ M MV.
(3) 2700 lx illumination.
(4) same as 3 with $10^{-3}$ M MV.
[b] Putative ATP source. M, coupled electron flow in the mitochondria; Pc, cyclic photophosphorylation Pl, linear photophosphorylation; $^-$ATP consumption in Calvin cycle; $^+$ no ATP. consumption in Calvin cycle.
[c] The $F_{max}$ increase is given as % of $\Delta$ $F_{max}$ between that in state I (2700 lx illumination in the presence of DCMU) and that in state II in each set of experiments (i.e., in the dark, either under $N_2$ or with FCCP or AA + SHAM).
[d] Final state considered as state I when $F_{max}$ increase is above 60% and considered as state II when $F_{max}$ increase is below 30%.

nmol/mg Chl) to light in the presence of DCMU (25 nmol/mg Chl). Their fluorescence yield remained low (Table III) and the light-harvesting complex subunits remained highly phosphorylated (Fig. 4). Thus FCCP prevented transition from state II to state I, although the illumination conditions were suitable for a reoxidation of the IEC. In case 2, state II was reached by incubating WT cells in the presence of inhibitors of mitochondrial electron transport (AA + SHAM). This treatment prevented mitochondrial ATP production, but cyclic photophosphorylation was preserved. Owing to the latter property, a rise in ATP was observed upon transfer of the algae from darkness to light in the presence of DCMU (from 50 to 90 nmol/mg). This was accompanied by a reversion from state II to state I as indicated by the increase in maximal fluorescence yield (Table III), the restoration of a high $F_{685}/F_{717}$ fluorescence emission ratio at 77 K (result not shown) and the extensive dephosphorylation of the LHC subunits (Fig. 4). However, in case 3, when a similar experiment was performed using mutants lacking the chloroplast ATP synthase (FUD50 or ac46 mutants) and therefore unable to perform cyclic photophosphorylation, we observed no significant light-induced reversion to state I (Table III and Fig. 4) and no rise in intracellular ATP content (50 vs. 53 nmol/mg Chl). We interpret these data as an evidence for the requirement of cyclic photophosphorylation to promote transition to state I in DCMU-poisoned cells lacking ATP synthesis of mitochondrial origin.

In order to assess whether any ATP source or specifically cyclic photophosphorylation were required to promote transition to state I, we analyzed the increases in $F_{max}$ observed with algae placed originally in state II in darkness by various treatments and subsequently illuminated at 2700 lx in four different conditions: in the presence or absence of DCMU after being incubated without MV (conditions 1, 3 in Table III) and with MV (conditions 2, 4). The addition of MV aimed at preventing cyclic electron flow around PS I. It also prevented

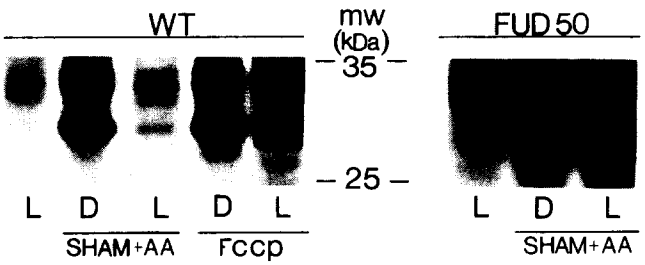


Fig. 4. Phosphorylation pattern of antenna polypeptides upon changes from state II conditions (D) to state I conditions (L: 2700 lx illumination in the presence of DCMU) in three cases. Case 1: state II in WT cells due to uncoupling of mitochondrial electron transport (FCCP); case 2: state II in WT cells due to inhibition of mitochondrial electron transport (AA + SHAM); case 3 same as case 1 using FUD50 cells lacking the chloroplast ATP synthase.

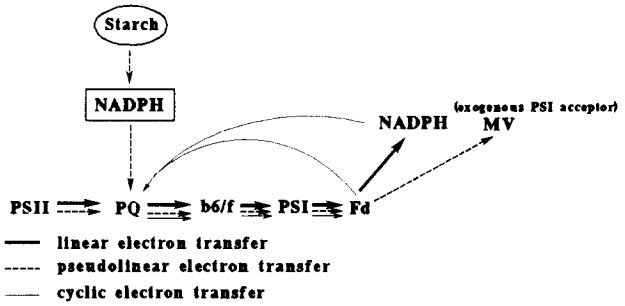


Fig. 5. Various electron flows coupled to ATP production.

ATP consumption in the Calvin cycle. The various pathways of photosynthetic electron flows operating in these experiments are schematically represented in Fig. 5. The fluorescence increases after an illumination are given in Table III relative to the amplitude ($\Delta F = 100$) of a current state II to state I transition, i.e., from a dark anaerobic state to an aerobic state in the presence of light and DCMU.

It should first be noted that an aerobic illumination, in any of the conditions 1–4, of the WT (as well as of mutants lacking the chloroplast ATP synthase) previously adapted to state II by an anaerobic incubation, led to state I. This observation is consistent with the results shown in Table I where aerated WT cells placed in darkness were close to state I. In all cases, there was a possible ATP supply coupled to mitochondrial electron flow. In contrast, the mere illumination of either the DUM-1 mutant or the WT treated with AA + SHAM (no ATP of mitochondrial origin) was unable to induce a transition to state I (condition 3). Such a reversion to state I was restored in the light when the same algae were preincubated in darkness with MV (illumination condition 4). Prolonged illumination, however, caused a subsequent quenching of variable amplitude which may be attributed to a re-establishment of state II for some reason as yet unknown.

Possibly, the presence of MV allowed a state II to state I transition because this chemical prevented ATP consumption in the Calvin cycle. Fractionation experiments of subcellular compartments of protoplasts of higher plants have shown the importance of the activity of the Calvin cycle in establishing the ATP/ADP ratio in vegetative cells in the light [26,27]: at high $CO_2$ concentrations, when ATP consumption in Calvin cycle is optimum, there is a limited increase in ATP/ADP ratio in the chloroplast, upon illumination, and it is even lowered in the cytosol. In contrast, under limiting $CO_2$ concentrations, a significant light-induced increase of the ATP/ADP ratio is observed in all subcellular compartments. When mitochondrial ATP production is impaired, ATP consumption in Calvin cycle could thus account for the absence of reversion from state II to state I upon illumination.

TABLE IV

*Starch and ATP control on the reversion to state I in the presence of MV*

Measurements were performed on various WT (No. 1–3) and DUM-1 (No. 4) samples.

| No. experiment | Starch [a] | ATP increase (%) [b] | $F_{max}$ increase (%) [c] |
|---|---|---|---|
| 1 | 2.2 | 0 | 15 |
| 2 | 4.5 | 10 | 25 |
| 3 | 11 | 60 | 85 |
| 4 | 18 | 70 | 100 |

[a] Starch levels were estimated through determination of glucose content ($\mu$M/mg Chl).

[b] ATP (light + DCMU + MV) minus ATP (state II)/ATP(state I) minus ATP (state II).

[c] $F_{max}$ (light + DCMU + MV) minus $F_{max}$ (stateII)/$F_{max}$ (state I minus $F_{max}$ (state II), measured after 6–8 min illumination.

Alternatively, the MV effect could be attributed to an increased oxidation of the IEC through an increase in the rate of PS I photochemistry. This would account for kinase deactivation through an increased oxidation of the IEC with, as a result, a rise in $F_{max}$. Although this hypothesis could also explain the increase in $F_{max}$ observed with the DUM-1 mutant and the WT (AA + SHAM-treated) when illuminated in the presence of DCMU and MV (condition 2 in Table III), it should be considered that a pseudo-linear electron flow, operating between chlororespiration [29,30], supplemented in NADPH by starch breakdown and the operation of a NADPH plastoquinone-oxidoreductase, may elicit ATP synthesis. In order to discriminate between the two hypotheses we starved WT or DUM-1 cells in darkness, in the absence of acetate, for several hours. This pretreatment aimed at decreasing the storage of reducing power by starch accumulation, then inhibiting the coupled pseudo-linear electron flow. In these conditions, most of our attempts to revert from state II (obtained by inhibition of mitochondrial electron transport) to state I by an illumination of DCMU-poisoned cells in the presence of MV proved unsuccessful (condition 2 in Table III). Table III shows that reversion to state I was, however, still observed in the absence of MV, when cyclic photophosphorylation was operating (condition 1). In Table IV are shown the results of four experiments in condition 2, using algae containing various amounts of starch. We measured simultaneously the ATP and $F_{max}$ increases upon illumination. These experiments show a close correlation between the amount of starch, the extent of light-induced increase in ATP and the ability to recover a fluorescence yield characteristic of state I.

## Discussion

In the present study, we have investigated the possible control of in vivo state transitions by the cellular demand for ATP. The ATP supply in plant cells and green algae originates mainly from three interacting sources: from coupled electron transfer processes in the mitochondria and in the chloroplast and from the breakdown of carbohydrate sources, sucrose and starch in plant cells but starch only in *C. reinhardtii* [31]. This latter contribution is expected to occur mainly upon ATP shortage, resulting in an acceleration of the glycolytic pathway (Pasteur effect) and in ATP synthesis through phosphoglycerate and phosphoenolpyruvate kinases activities [32]. Moreover, this event should lead to a rise in the stromal NAD(P)H concentration through the operation of glyceraldehyde-phosphate-dehydrogenase. Studies based on fluorescence [30] and biochemical [33] data have pointed to the existence of an input of electrons in the plastoquinone pool, presumably via a NADPH/plastoquinone oxidoreductase [33] or a succinodehydrogenase [34]. Such a pathway has also been postulated in higher plants on the basis of fluorescence [35] and gene sequencing [36,37] data. Therefore, the deregulation of the glycolytic pathway should induce an increased reduction of some intermediates in the photosynthetic electron transport chain. Within this framework, previous observations that green algae treated with uncouplers or inhibitors of mitochondrial electron transport [12,28,38] displayed a low fluorescence yield could be understood as a transition to state II upon NADPH-mediated reduction of the IEC in the dark.

In contrast with *Chlorella* cells [38], cells from *C. reinhardtii* when aerated in darkness are close to state I [23]. Here we have demonstrated that intact cells of the WT strain of *C. reinhardtii*, when treated in darkness with uncouplers (FCCP, DCHC), ATP synthase inhibitors (TBT, DCCD) or inhibitors of mitochondrial electron transport (AA + SHAM) were in a genuine state II: they displayed both a low fluorescence yield and an extensive phosphorylation of the LHC subunits. The kinetics of the fluorescence quenching upon FCCP and SHAM + AA treatments displayed half-times in the 115–130 s range [28], which compared well with those previously reported, 120 s, for a transition to state II in *C. reinhardtii* [14]. Furthermore $b_6/f$ mutants, which are blocked in state I [24,23], did not develop this low fluorescence state when subjected to the same treatments.

The decrease in cellular ATP varied from one treatment to another. It decreased extensively upon FCCP treatment, as previously reported by Stitt et al. [26], but was barely detectable with ATP synthase inhibitors. These differences probably arise from the fact that, in the latter cases, ATP production by starch breakdown is preserved from hydrolysis by membrane-bound ATP synthases, whereas, in the presence of uncouplers, hydrolysis will be stimulated. However, as expected from an onset of starch breakdown upon ATP deprivation, we observed an increased reduction of the IEC as

indicated both by the high $F_0$ level in WT cells treated with DCMU, and by the large decrease in the area bound by the fluorescence induction curve (with no DCMU added) and its $F_{max}$ asymptote in the FUD6 mutant lacking the $b_6/f$ complex. In most cases this increased reduction of the IEC was accompanied by an increased level of NADPH. The cascade of metabolic events most likely leading to state II transition, in the absence of mitochondrial ATP production, is schematically summarized below.

$$\underset{\text{depression}}{\text{ATP}} \rightarrow \underset{\text{degradation}}{\text{starch}} \rightarrow \underset{\text{formation}}{\text{NADPH}} \rightarrow \underset{\text{reduction}}{\text{IEC}} \rightarrow \underset{\text{transition}}{\text{state II}}$$

Thus, transition to state II upon ATP depressing treatments in the WT can be understood through a redox control on the activity of the LHC-kinase. However, it remains to be investigated whether the sole reduction of the IEC, under conditions where there would be no alteration of ATP synthesis, is able to induce a transition to state II in vivo.

It is of note that anaerobiosis, previously used to induce a transition to state II in darkness [13,14], also inhibits mitochondrial electron transport. That ATP production in mitochondria controls state transitions in the chloroplast in vivo is further demonstrated by our experiments in aerobic conditions with the DUM-1 mutant strain, defective in mitochondrial activity. In contrast with the WT strain, this mutant has state II characteristics when transfered to darkness with no further treatment.

It is at first sight paradoxical that a decrease in ATP would promote an ATP-consuming reaction as is the case of the LHC phosphorylation. One possibility is that the phosphatase activity would be severely decreased upon ATP-depleting treatments. If this is not the case, the affinity of the kinase for ATP should be very high or, the substrate for phosphorylation should originate from an ATP pool in slow equilibrium with the bulk of intracellular ATP. Chase experiments with unlabeled $P_i$ added to cells incubated with $^{32}P$ for 2 h, indeed showed that there was now detectable decrease in $^{32}P$-labeling of the LHC subunits within 1 h of chase (Wollman and Bulté, unpublished observations), which is in good agreement with the last hypothesis.

Experiments with intact chloroplasts from maize [39,40] have similarly shown that uncouplers or ATP sinks, like externally added pyruvate, caused LHC-kinase activation. We believe that these experiments could be interpreted by an increase in IEC reduction upon ATP-controled starch breakdown. Such an increased reduction of the PQ pool was indeed observed upon addition of pyruvate [39,41]. In addition, in their comparative metabolic study, Fernyhough et al. [39] observed that when NADPH consumption occurred together with ATP consumption, there was no significant activation of the kinase as compared to another situation where only ATP was consumed by pyruvate dikinase activity. Horton and colleagues have, however, favored an additional and independant control on kinase activity by the 'high energy state' quenching ($q_E$) of the membranes [8,40] with high $q_E$ favoring kinase deactivation. Mutants lacking the chloroplast ATP synthase should display a low $q_E$ in darkness and developp a particularly high $q_E$ upon illumination. They should then be close to state II in darkness but close to state I upon illumination. Our observation that such mutants, were in state I when aerated in darkness but remained in state II, when treated with mitochondrial inhibitors and subsequently illuminated, argues against a role of $q_E$ in kinase (de)activation. The ATP control on the transition to state 2 in vivo is then probably mediated by a change in the redox state of the same regulatory species that controls kinase activity in broken chloroplasts.

Whereas ATP shortage induced transition to state II together with an increased reduction of some IEC, subsequent reoxidation of the IEC alone did not allow reversion to state I. The reoxidizing conditions had to elicit simultaneously a restoration of higher ATP contents: when state II was established in the dark by an inhibition of mitochondrial electron flow (WT with SHAM + AA or DUM-1 mutant), a reversion to state I was observed upon illumination of DCMU-poisoned algae together with a net ATP synthesis attributed to PS-I-mediated cyclic photophosphorylation. This reversion to state I was not observed in the absence of active chloroplast ATP-synthase, i.e., when using either mutants lacking this enzyme or the WT treated with uncouplers. Contrary to what has been suggested by Satoh and Fork [42], reversion to state I did not require specifically PS I cyclic photophosphorylation. Illumination in the presence of MV also allowed transition to state I provided there was a source of electron donors to allow coupled electron flow. This was observed in the presence as well as in the absence of DCMU. In the latter case ATP synthesis was born out by linear electron flow whereas in the former we assume that it originated from a pseudo-linear electron flow from NADPH produced by starch degradation and PSI. The occurrence of such a pseudo-linear electron transport has been previously implicated in the hydrogenase activity observed in anaerobic conditions in *C. reinhardtii* [49]. Consistent with this hypothesis is our observation that starved cells when similarly treated in the presence of MV remained in state II. In this case, illumination did not allow net ATP synthesis, although the presence of DCMU and MV ensured oxidation of the IEC. Thus we conclude that the restoration of high ATP contents is a prerequisite for the occurrence of a transition to state I controlled by the deactivation of the LHC-kinase upon oxidation of the IEC.

Williams and Dominy [43] have recently reported that both uncouplers and ATP synthase inhibitors have a same effect on state transitions in cyanobacteria. In a given concentration range, they both block transition from state II to state I. In this respect, the results of our study add some similarities between the regulation mechanisms operating in vivo in green algae and cyanobacteria, although the implication of a reversible phosphorylation in the latter remains questionable.

Our study suggests that, in vivo, the photosynthetic apparatus is locked in a state II configuration in ATP depleting conditions, whereas it can adopt state I or state II configuration depending on the redox state of some IEC, in the presence of ATP. These characteristics no longer prevail in broken chloroplasts which have state I characteristics in the absence of ATP. Therefore the ATP control in vivo probably involves some additional soluble factor in the stroma of the chloroplast. This ATP control may act directly on the kinase/phosphatase activities, for instance if the phosphatase activity was ATP dependent in vivo. Alternatively, it may act through a change in the organization of the IEC responsible for the redox control on the activity of the LHC-kinase: when state II is induced by ATP depletion, the IEC would not have access to the regulation site controling kinase deactivation, even in oxidizing conditions. It is now widely accepted that $b_6/f$ complexes are implicated in the activation of the kinase [24,23,44,45]. Supercomplexes between the bc complex and the bacterial reaction center have been identified in *Rhodobacter sphaeroides* [46]. On the other hand, some of us have provided preliminary evidence for an increased association of $b_6/f$ complexes with PS I reaction centers in state II [47]. Whether an ATP-controlled dissociation of such supercomplexes is required for kinase deactivation deserves further investigation.

Our study raises the question of the physiological role of chlororespiration [30]. It has been proposed that this pathway could generate a sufficient pH gradient to maintain the chloroplastic ATP synthase in an active form [48], or serve to recycle in the dark the NADPH produced by glycolysis [30]. One may now speculate that the main physiological role for chlororespiration is to control the functional organization of the photosynthetic apparatus: under low ATP level conditions, such as anaerobiosis or prolonged starvation, a positioning in state II would favor immediate operation of cyclic electron transfer coupled to ATP synthesis upon illumination.

## Acknowledgements

We thank R. Delosme, P. Joliot, J. Lavergne and A. Vermeglio for stimulating discussions. This work was supported by the M.R.T., contract no. 89 C.066501, and the C.N.R.S. URA D1187. L. Bulté is a recipient of a Roussel-Uclaf fellowship.

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