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Control in the dark of the plastoquinone redox state by mitochondrial activity in *Chlamydomonas reinhardtii*

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In *Chlamydomonas reinhardtii*, addition of mitochondrial oxidases inhibitors or uncouplers in the dark resulted in an increase of the variable fluorescence level, a decrease of the maximum fluorescence level and a rise of the slow electrochromic phase in flash-induced 520 nm absorption changes. Fluorescence emission spectra recorded at 77 K presented a marked decrease in the F_{686nm}/F_{715nm} ratio which was suggestive of a state I–state II transition. Biochemical analysis indicated that the ATP and glucose 6-phosphate pools decreased markedly under these conditions, whereas the NADPH concentration increased. The NAD pool remained totally oxidised. These results strongly suggest that alterations of the mitochondrial oxidative phosphorylations in the dark could induce an increase of the reduction state of the plastoquinone pool and a redistribution of the antennae between the two photosystems. It is proposed that these variations were linked to an acceleration of the NADPH production in the chloroplast through the glycolytic activity.

Introduction

In photosynthetic eukaryotic cells, interaction between chloroplasts and mitochondria is still an open question. The problem has been studied mainly with respect to the light-induced variations of the pyridine and adenine nucleotides concentrations, effectors known to control mitochondrial respiration [1–6]. In a previous paper, we presented evidence indicating that in a strain of *Chlamydomonas reinhardtii* devoid of active Rubisco, interactions between chloroplast and mitochondria electron transport chains could also occur in the dark [7]. Indeed, addition in the dark of inhibitors of the mitochondrial oxidative phosphorylations resulted in a marked increase of the fluorescence level which was presumably representative of changes in the redox state of some components of the chloroplastic electron transport chain. Because the presence in *C. reinhardtii* of a chloroplastic respiratory pathway initiated by a NAD(P)H plastoquinone oxidoreductase has been reported by several authors [8–11], it was suggested that in the dark these conditions favour the operation of this pathway.

In the present paper, we attempt to determine further the relationship between the mitochondrial activity and the redox state of the PQ pool in the wild type of *C. reinhardtii*. The possible role of glycolysis in this interaction is also discussed.

Material and Methods

C. reinhardtii wild type 137 c strain was grown photoautotrophically as previously described [12]. For each experiment, algae in the mid-exponential phase of growth were harvested by low-speed centrifugation ($1500 \times g$, 15 °C) and resuspended in a fresh culture medium at the desired cell concentration.

Biochemical analysis

Chlorophyll content was determined after extraction with 90% (v/v) methanol as previously described [13].

For nucleotides and glucose 6-phosphate determination, algae (3 ml suspension) were quickly dropped into liquid nitrogen and the frozen suspensions were ho-

Abbreviations: PQ, plastoquinone; Rubisco, ribulose-1,5 bisphosphate carboxylase/oxygenase; SHAM, salicylhydroxamic acid; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; G-6-*P*, glucose 6-phosphate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; LHC II, light harvesting complex of Photosystem II; Q_A, primary acceptor of Photosystem II; TNBT, tri-*n*-butyltin.

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mogenized in the presence of perchloric acid (5% final) as previously described [14]. After thawing, the solution was centrifuged (10000 × *g*, 15 min, 0 °C) and then neutralised with ice-cold saturated potassium hydrogenocarbonate. Pyridine nucleotides were determined using an enzymatic cylic method [15] as described in Ref. 14. Adenine nucleotides were determined as described by Strehler [16]. Glucose 6-phosphate was measured according to Lang and Michal [17].

Fluorescence induction

Chlorophyll fluorescence induction was measured with a pulse modulation technique (basic system PAM 101 and saturation pulse unit 103, H. WALZ, Effeltrich, F.R.G.) [18], as previously described [7]. The fluorescence level was measured with a weak modulated light (averaged intensity 0.8 $\mu E \cdot m^{-2} \cdot s^{-1}$) and the maximal fluorescence yield was determined with 500 ms pulses (light intensity 1500 $\mu E \cdot m^{-2} \cdot s^{-1}$) applied every minute. The variable fluorescence F_v, the maximal variable fluorescence $(F_v)_s$, the photochemical quenching q_Q and the non-photochemical quenching q_{NP} were determined as in Ref. 18.

The 77 K fluorescence emission spectra were recorded with a Jobin-Yvon (France) spectrophotometer after a rapid freezing in liquid nitrogen of an aliquot of the cell suspension kept well-aerated in the dark. The device used in this procedure was similar to that described in Ref. 19. Phycoerythrin, a generous gift from Dr. D. Dermoun, was used as internal standard.

Absorption changes

Spectrophotometric measurements at 520 nm were performed with an apparatus similar to that described in Ref. 20 and improved according to Ref. 21. Actinic excitation was provided by Xenon flashes (3 μs half-time duration), filtered through an RG 680 filter. Algae (in the presence of 7% Ficoll to avoid sedimentation) were kept well aerated and in darkness for 5–10 min before the measurement.

Results

Fluorescence kinetics

Simultaneous measurements of O_2 uptake rates and chlorophyll induction fluorescence are presented in Fig. 1. Under our experimental conditions, the weak modulated light needed for the fluorescence measurement did not significantly affect the rate of dark respiration (result not shown). Addition of antimycin a (1 μM) (Fig. 1A), an inhibitor of the mitochondrial cytochrome oxidase pathway [1], had no major effect on the respiratory rate as already reported [10], suggesting the operation of an alternative oxidase pathway under this condition. As is also shown in this figure, the fluorescence yield was not modified. Likewise, SHAM (1 mM), an inhibitor of the mitochondrial alternative oxidase pathway [1], had no effect on the O_2 uptake rate nor on the fluorescence (result not shown). As expected, the combination of these two inhibitors induced a marked inhibition (60–70%) of the respiratory rate. However, large changes of the fluorescence induction kinetics, i.e., an increase of the variable fluorescence F_v and a decrease of the saturated signal $(F_v)_s$, slowly appeared under this condition. These changes corresponded respectively to a decrease of the photochemical quenching, q_Q, and an increase of the non-photochemcial quenching q_{NP} (Fig. 1C). The variation of the photochemical quenching was indicative of a reduction of the primary acceptor Q_A [18], whereas the increase of the non-photochemical quenching was related to either a rise of the intrathylakoid pH or thylakoid membrane phosphorylations [18,26]. These fluorescence changes were observed only if the concentrations of both inhibitors were sufficient to inhibit the respiration.

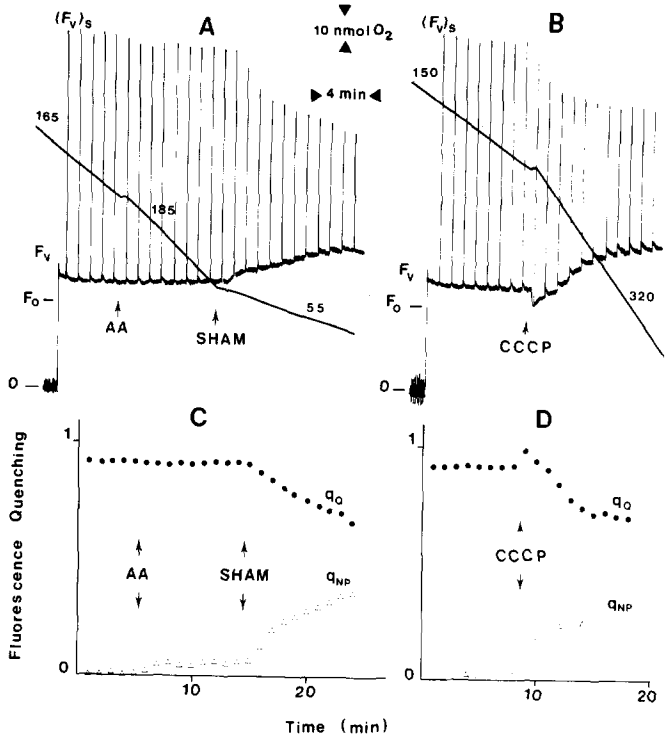

Fig. 1. O_2 electrode traces and fluorescence kinetics in *C. reinhardtii* (A) Effect of antimycin a (1 μM) and SHAM (1 mM). (B) Effect of CCCP (2 μM). (C) Variations of the photochemical quenching (q_Q) and the non-photochemical quenching (q_{NP}) obtained from (A). (D) Variations of the photochemical quenching (q_Q) and the non-photochemical quenching (q_{NP}) obtained from (B). Numbers along the lines are nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ Chl. q_Q and q_{NP} were calculated from F_0, F_V and $(F_V)_S$ as described in Ref. 18.

As shown in Fig. 1B, addition of the uncoupler CCCP (2 μM) stimulated the O_2 uptake rate more than 2-fold, but had a similar effect on the fluorescence induction curves as did antimycin a plus SHAM indicating a reduction of Q_A. Taking into account the known effect of an uncoupler on the transmembrane

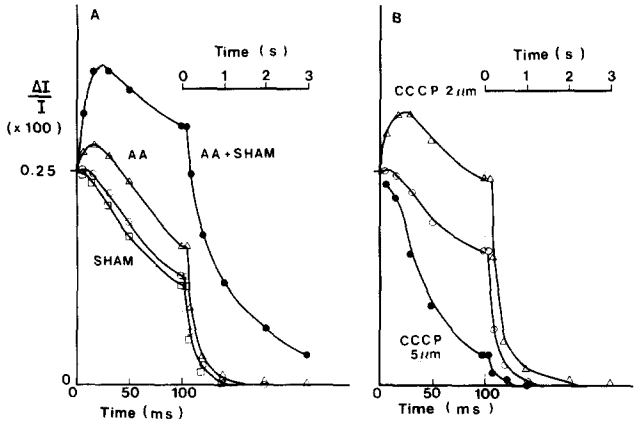

Fig. 2. Time course of the 520 nm absorption change after a saturating flash in *C. reinhardtii*. To avoid anaerobiosis, the cell suspension was kept well agitated in the dark during the experiments. The 520 nm absorption change was recorded 5–10 min after addition of the inhibitors. Chlorophyll concentration was in the range 10–20 μg Chl·ml^{-1}. (A) Effect of mitochondrial oxidase inhibitors. (○), dark-adapted cells; (□), +1 mM SHAM; (△), +1 μM antimycin a; (●), +1 μM antimycin a and 1 mM SHAM. (B) Effect of CCCP. (○), dark-adapted cells; (△), +2 μM CCCP; (●), +5 μM CCCP.

pH gradient, the increase of q_{NP} (Fig. 1D) can probably not be ascribed to an increase of the intrathylakoid pH gradient. As for antimycin a and SHAM, fluorescence changes were observed with CCCP only if the concentration was sufficient to uncouple the dark O_2 uptake. In addition, the effect of these inhibitors cannot be attributed to an alteration of PS II, since the maximal fluorescence yield could be totally recovered in the presence of light and DCMU [36].

The higher reduction state of Q_A was possibly related to a reduction of the plastoquinone pool. This was investigated by measuring the carotenoid spectral shift after a short saturating flash.

Flash-induced 520 nm absorption change

The flash-induced 520 nm absorption increase in the 1–10 ms range (phase b of the electrochromic response [22]) has been attributed to an electrogenic electron transfer, depending on the presence of a reduced bound plastoquinone, in rapid equilibrium with the pool [23,24]. As shown in Fig. 2A, the slow electrochromic phase (phase b) was not seen under standard conditions, which suggest that the PQ pool was largely oxidised in these algae [35]. In the presence of SHAM alone, no effect on the 520 nm absorption change was detected. On the other hand, addition of antimycin a resulted in a slight increase of phase b, largely stimulated when SHAM was added together with antimycin a. This electrochromic rise was very similar to that obtained with algae placed in the anaerobic state (result not shown), a situation known to reduce the PQ pool in *C. reinhardtii* [26]. Note also that the electrical membrane potential decreased more slowly in the presence of antimycin a plus SHAM. It could, therefore, be possible that this slower decrease uncovered a phase b previously hidden in the control by a rapid decrease of the electrical field. We verified, by analysis of the signal into different exponential components (see Ref. 22), that the appearance of phase b in the presence of antimycin a plus SHAM was due to a real increase of this phase (result not shown). The effect of CCCP on the electrochromic response was more complex and depended on the concentration used (Fig. 2B). At 2 μM, the membrane potential decrease was not accelerated compared to the control, and a phase b was observed although its amplitude was lower than in the presence of antimycin a plus SHAM. This result is in agreement with earlier studies [12] indicating that below 2 μM, CCCP was a weak uncoupler of the chloroplast in *C. reinhardtii*. However, at a concentration higher than 4 μM, the decrease of the membrane potential was strongly accelerated (Fig. 2B), as expected after addition of an uncoupler.

77 K fluorescence emission spectra

Thylakoid membrane polypeptides, mainly the light harvesting complexes, can be phosphorylated and their phosphorylation level is likely to be controlled by the redox state of the PQ pool [25,26]. Therefore, the decrease of the maximal variable fluorescence signal observed in Fig. 1 could be due, in part, to phosphorylation of light harvesting complexes of PS II and migration of these complexes toward PS I. In order to test this hypothesis, we measured the 77 K fluorescence emission spectra of cells incubated under the same

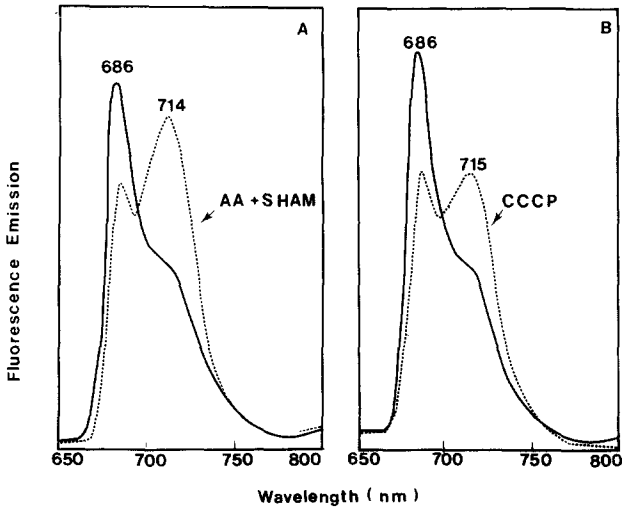

Fig. 3. Effect of mitochondrial oxidase inhibitors and uncoupler on 77 K fluorescence emission spectra of *C. reinhardtii*. (A) solid line, cells kept well aerated in the dark; dashed line, cells incubated 15 min in the dark in the presence of antimycin a (1 μM) and SHAM (1 mM). (B) solid line, cells kept well aerated in the dark; dashed line, cells incubated 15 min in the dark in the presence of CCCP (2 μM). Excitation wavelength 440 nm. Phycoerythrin in a capillary tube was added to the sample and used as internal standard.

conditions as above. As shown in Fig. 3, the F_{686nm}/F_{715nm} ratio (respectively, PS II and PS I fluorescence) decreased markedly after addition of either antimycin a plus SHAM (Fig. 3A) or CCCP (Fig. 3B). These changes were similar to that observed in anaerobiosis [26] and were suggestive of a state I to state II transition.

Biochemical analysis

At this point, it may be asked why the plastoquinone pool is more reduced in the dark, after addition of antimycin a and SHAM or CCCP. One may suspect that the above observations were the result of a direct interaction of these chemicals with the chloroplastic electron transport chain. However, it has been previously established that neither 1 mM SHAM nor 1 μM antimycin a, added separately or in combination, significantly affected the maximal rate of gross photosynthesis (Refs. 12, 27, and results not shown). Likewise, as pointed out above, CCCP (2 μM) was a weak uncoupler of the chloroplastic chain [12]. In addition, the possibility that inhibition of a chlororespiration would be responsible for the above results can be ruled out, since it has been shown that these products, at least antimycin a plus SHAM, have no effect on this respiratory pathway [10]. Therefore, it is likely that the modifications of the PQ redox state in the dark was the indirect consequence of alterations of the mitochondrial respiratory activity. This idea is supported by the much slower effect of these products on the fluorescence than on the respiratory rate (see Fig. 1). In order to gain better insight into this process, we measured the effect of these mitochondrial inhibitors upon the concentration of different metabolic pools.

As shown in Fig. 4A, the ATP pool in the dark was not significantly affected by antimycin a alone, nor by

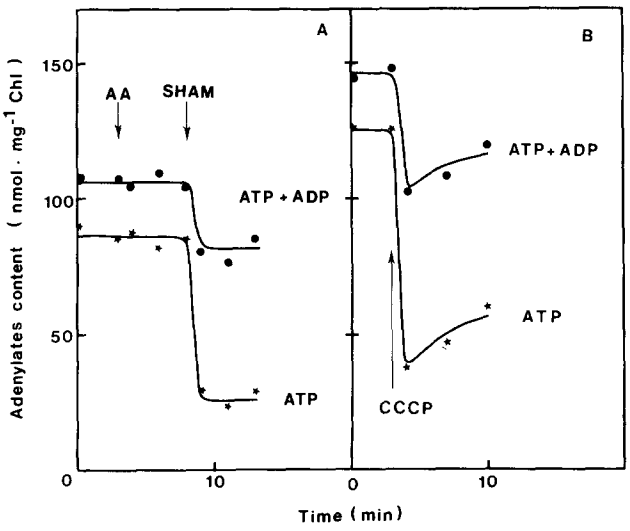

Fig. 4. Effect of mitochondrial oxidase inhibitors and uncoupler on the cellular ATP and ATP+ADP content in *C. reinhardtii*. (A) 1 μM antimycin a, 1 mM SHAM. (B) 2 μM CCCP.

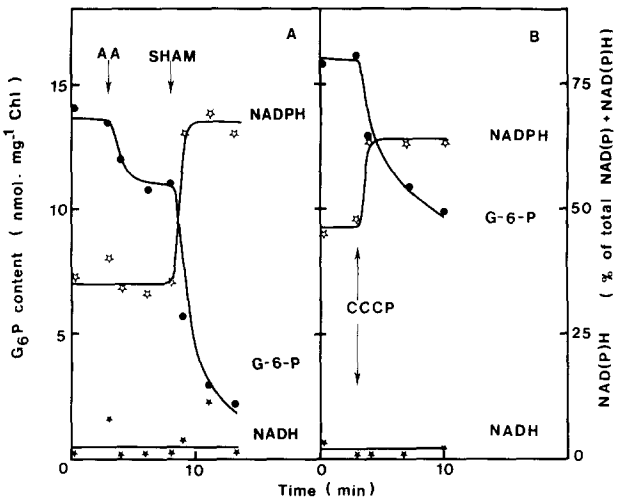

Fig. 5. Effect of mitochondrial oxidase inhibitors and uncoupler on the cellular NADPH, NADH and G-6-*P* content in *C. reinhardtii*. (A) 1 μM antimycin a, 1 mM SHAM; the total amount of NADP+NADPH and NAD+NADH were, respectively, 8 and 28 nmol·mg^{-1} Chl. (B) 2 μM CCCP; the total amount of NADP+NADPH and NAD+NADH were, respectively, 15 and 35 nmol·mg^{-1} Chl.

SHAM alone (result not shown), but in the presence of both inhibitors, the ATP concentration dropped markedly during the first minute. The effect of the uncoupler CCCP (Fig. 4B) was similar to that observed with antimycin a plus SHAM. Under all the conditions tested, the decrease of the ATP pool was associated with both, a diminution of the G-6-*P* pool and an increase of the NADPH concentration (Fig. 5). In the presence of CCCP, the G-6-*P* and NADPH levels varied in a lower extent, however, than in presence of antimycin a plus SHAM (compare parts A and B of Fig. 5). No variation of the NADH concentration was observed (Fig. 5).

Discussion

The results presented here confirm and extend earlier studies [7] and indicate that alteration in the dark of the mitochondrial oxidative phosphorylations in *C. reinhardtii* induces the reduction of some components of the chloroplastic chain, most probably at the PQ pool level. The concomitant rise of NADPH strongly suggests that this metabolite was responsible for the higher reduction state of the PQ pool. Indeed, it has been shown that, in open-cell preparation of *C. reinhardtii*, the PQ pool can be reduced by addition of NADPH [8]. Possibly, NADPH could be reoxidised via a NAD(P)H plastoquinone oxidoreductase [11], an enzyme bound to the thylakoid membrane and reported to initiate the chlororespiratory pathway [8]. This point is currently under investigation.

The observed intracellular NADPH accumulation could result from the inhibition of mitochondrial NADH

oxidation and transfer of reducing equivalents towards the chloroplast. However, in this case, an increase of the NADH concentration would be expected. This was not observed. Furthermore, the fact that the uncoupler CCCP, which favours the NADH oxidation through the mitochondrial electron transport chain, had a similar effect as antimycin a plus SHAM is not in favour of this first hypothesis. On the other hand, it is now well established that ATP exerts a tight control on key enzymes of the glycolytic pathway (pyruvate kinase and phosphofructokinase [1]). It might, therefore, be speculated that the observed drop of the cellular ATP content led to an increase of the glycolytic rate with, as a consequence, a rise of NAD(P)H production. Such an hypothesis is well supported by the observed decrease of G-6-*P* (Fig. 5) and the reported acceleration of the starch breakdown in the wild type after addition of CCCP [28] and in the F-60 mutant strain (devoid of ribulose kinase activity) after addition of FCCP or cyanide plus SHAM [29]. In *C. reinhardtii,* starch is the only source of carbohydrates and the initial part of the glycolysis to 3-phosphoglyceric acid and the oxidative pentose phosphate pathway are located in the chloroplast [30]. Any increase of the glycolytic rate is therefore expected to induce an increase of the NADPH production in this compartment through the NADP glyceraldehydephosphate dehydrogenase activity. Alternatively, the variations of NADPH and G-6-*P* could be due to a higher engagement of the pentose phosphate pathway, although the high NADPH/NADP ratio would tend rather to decrease the glucose-6-phosphate dehydrogenase activity [31].

In the presence of antimycin a, the alternative oxidase pathway was engaged and was able to sustain NADH oxidation at a rate similar to that of cytochrome oxidase. Under this condition, a decrease of the mitochondrial ATP synthesis would be expected, since only one site of phosphorylation remained associated with NADH oxidation [1]. As a drop of the intracellular ATP concentration was not observed in this situation, it is most probable that the glycolytic rate increased in order to maintain the cellular ATP level. This may account for the small decrease of G-6-*P* and rise of the slow electrochromic phase observed after addition of antimycin a.

The results obtained with room temperature fluorescence and 77 K fluorescence emission spectra suggested that the changes of the redox state of the thylakoid membrane were associated with a state I state II transition. Such a transition may reflect phosphorylations of the LHC II and its migration from the photosystem II to the photosystem I, as it has been shown under anaerobiotic conditions [26]. In *Chlorella,* uncoupler-induced changes of fluorescence, similar to state I–state II transitions, have been reported earlier in DCMU-poisoned algae [32], while other authors have proposed that the effect of CCCP on the maximum fluorescence yield was due to cation concentration changes [33]. Although this last hypothesis cannot be ruled out, phosphorylations of the LHC II were observed in *C. reinhardtii* in the presence of either FCCP or antimycin a plus SHAM [36].

The results described here support the view that ATP might be the prime controller in the dark of the reduction state of the PQ pool and consequently of the antennae repartition around the photosystems. From this point of view, it must be noted that the use of mitochondrial oxidase inhibitors, uncouplers or ATP-synthase inhibitors might have indirect effects on the chloroplastic chain via a modification of the cellular ATP level. In this connection we observed that TNBT, an ATP-synthase inhibitor, or acetate, which is often considered as an 'uncoupler' [34], induced similar changes of the fluorescence kinetics, a decrease of the F_{686nm}/F_{715nm} ratio in the 77 K fluorescence reemission spectra and an apparition of a slow electrochromic phase. Although the physiological implications of a state I–state II transition in the dark remain to be determined, one may suspect, as was suggested by Horton [37], that in a situation where the ATP level is low, the operation of a cyclic electron transport around PS I would be favoured during the first steps of illumination and thus ATP synthesis versus NADPH production.

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