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Oxygen-exchange studies in *Chlamydomonas* mutants deficient in photosynthetic electron transport: evidence for a Photosystem II-dependent oxygen uptake in vivo

Gilles Peltier and Pierre Thibault

Service de Radioagronomie, C.E.N. de Cadarache, Saint-Paul-lez-Durance (France)

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Photosynthetic oxygen exchange has been measured using $^{18}O_2$ and the mass-spectrometric technique in two mutant strains of *Chlamydomonas reinhardtii* deficient in electron transport. In the F15 mutant, deficient in PS I, O_2 was evolved in the light at a constant rate of about 145 nmol O_2/min per mg chlorophyll. At the same time, O_2 uptake was increased in the light by about 28%. O_2 evolution and the light-stimulation of O_2 uptake were inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea. Antimycin A and salicylhydroxamic acid, both inhibitors of mitochondrial respiration, when added together, inhibited dark respiration and also the light-dependent O_2 evolution by about 80%. Similar properties were observed in a mutant strain of *Chlamydomonas* (F18) lacking the cytochrome b_6-f complex. We conclude from these results that in the absence of active Photosystem I, a permanent electron flow can occur in the light from Photosystem II to molecular O_2. This electron transfer pathway would involve the plastoquinone pool and the mitochondrial electron transport chain. Because O_2 evolution measured in the F15 mutant was severely inhibited by the uncoupler cyanide *m*-chlorophenylhydrazone, we propose that an energy-dependent reverse electron transfer similar to that of *Rhodospirillaceae* might occur in the chloroplast of *Chlamydomonas*.

Introduction

During chloroplastic photosynthesis, the main flow of electrons proceeds from water to the PQ pool via PS II and from the PQ pool to NADP via PS I. However, alternative electron transfer pathways have been identified. Cyclic electron flow connected to PS I can occur under certain conditions [1] and recently, the operation of a light-induced cyclic pathway around PS II has been described [2]. Oxygen has been reported to act as a natural electron acceptor during light reactions [3–7] and its reduction site is generally thought to be located at the level of the PS I electron acceptors such as ferredoxin [4–7]. However, interaction of O_2 with light-generated PS II reductants has been evoked to explain the existence of constant O_2 production under flash illumination in a mutant of *Scenedesmus* lacking PS I [8]. PS II has also been reported to catalyze hydrogen peroxide production in vitro in isolated chloroplast lamellae [9], but this electron pathway is generally considered as a minor route of O_2 consumption in vivo [5,7,10].

Abbreviations: CCCP, *m*-chlorophenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea); PS, Photosystem; PQ, plastoquinone.

Correspondence: G. Peltier, Service de Radioagronomie, C.E.N. de Cadarache, 13108 Saint-Paul-lez-Durance, France.

In the present work, we study in vivo oxygen exchange in two mutant strains of *Chlamydomonas*, one (F15) lacking PS I and the other (F18) lacking the cytochrome b_6-f complex, using $^{18}O_2$ and the mass spectrometry. When illuminated, these mutants exhibit a permanent electron flow from water to O_2 involving the PQ pool and probably requiring the cooperation of the mitochondrial electron transport chain.

Materials and Methods

The two mutant strains of *C. reinhardtii*, F15/4$^+$ lacking PS I and F18/1$^+$ lacking the cytochrome b_6-f complex were generously provided by Dr. Girard-Bascou and were isolated as described in Ref. 11. Algae were grown in a Tris-acetate-phosphate medium at a light intensity of 20 μE/m^2 per s. During exponential growth, algae were harvested by low-speed centrifugation (1500 $\times g$) and resuspended in a 10 mM phosphate buffer (pH 7.0).

Oxygen exchange was measured using $^{18}O_2$ and a magnetic sector mass-spectrometer (VG Instruments, type 14-80). The algal suspension (2 ml) was introduced in a thermostated (25°C) glass reaction vessel and stirred with a rotating magnetic bar. A polyethylene membrane at the bottom of the reaction vessel allowed dissolved gases to be introduced into the mass spectrometer. After bubbling, the algal suspension with N_2, $^{18}O_2$ (98.1% ^{18}O from C.E.A. Saclay, France) was injected to obtain an initial O_2 concentration of about 25% O_2. After closing the reaction vessel, m/e 32 ($^{16}O_2$) and 36 ($^{18}O_2$) were simultaneously and continuously recorded. Oxygen uptake, true O_2 evolution and net O_2 evolution were determined as described in Ref. 12. During some experiments, $^{13}CO_2$ was injected and CO_2 exchange was followed by recording m/e 44 ($^{12}CO_2$) and 45 ($^{13}CO_2$).

Illumination was provided by a projector (Schott, type KL 1500) equipped with a heat-filter. Neutral density filters were used to obtain the light intensities required.

Results

Oxygen exchange has been measured using $^{18}O_2$ and the mass-spectrometric technique in the dark and in the light in the mutant strain of *Chlamydomonas* F15 deficient in PS I (Fig. 1). Following illumination, O_2 was evolved from te photolysis of water at a constant rate of 117 nmol O_2/min per mg chlorophyll and the rate of O_2 uptake increased from 207 to 281 nmol O_2/min per mg chlorophyll. In this case, net O_2 exchange, which is the difference between O_2 evolution and O_2 uptake, corresponded to consumption and was inhibited by about 21% in the light. We then studied the effect of various light intensities on O_2 exchange in the F15 mutant (Fig. 2). O_2 evolution increased progressively as a function of light intensity and was almost completely saturated at a value of 825 μE/m^2 per s. The maximum rate of O_2 evolution was about 7% of the maximal CO_2-supported O_2 evolution rate measured in the wild type (see Ref. 12). O_2 uptake was stimulated below 100 μE/m^2 per s and then remained virtually constant up to 825 μE/m^2 per s. Conversely, net O_2 consumption was progressively inhibited as light intensity was increased.

A first important result is that molecular O_2 is evolved in the light at a constant rate in a mutant lacking PS I. A similar conclusion was reached by Radmer and Kok [8] from the observation that the *Scenedesmus* mutant No. 8 deficient in PS I activity, was evolving O_2 under flash illumination in the steady state. The authors suggested the existence of a leak path via the PS II and probably

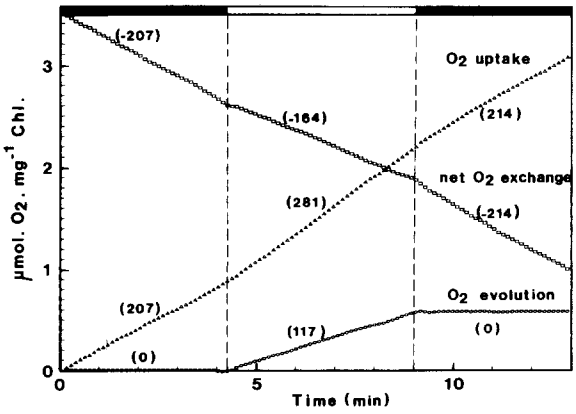

Fig. 1. O_2 evolution, O_2 uptake and net O_2 exchange measured in the dark and in the light in the mutant strain of *Chlamydomonas* F15 lacking PS I. Numbers in brackets indicate the O_2 exchange rates in nmol O_2/min per mg chlorophyll. Light intensity was 640 μE/m^2 per s.

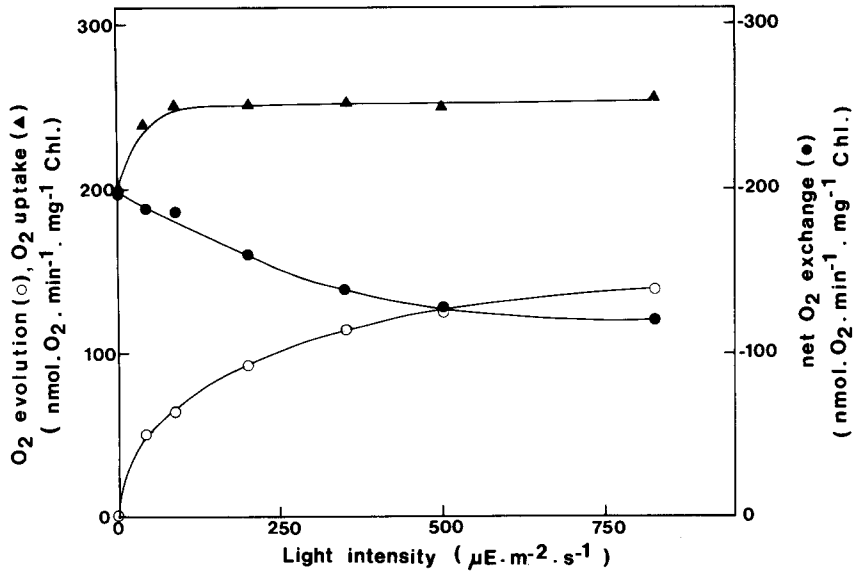

Fig. 2. O_2 evolution (○), O_2 uptake (▲) and net O_2 exchange (●) rates measured as a function of light intensity in the mutant strain of *Chlamydomonas* F15 lacking PS I.

to O_2, but further attempts to measure this leak by using isotopes were unsuccessful [7].

A second result which should be noticed is that net O_2 consumption is inhibited by light. To study this phenomenon further, we have simultaneously measured net CO_2 and net O_2 exchange (Fig. 3). We observed that net CO_2 production (measured as the *m/e* 44 increase) was inhibited by light in the same proportion as O_2 consumption (measured as the *m/e* 32 decrease). We checked using

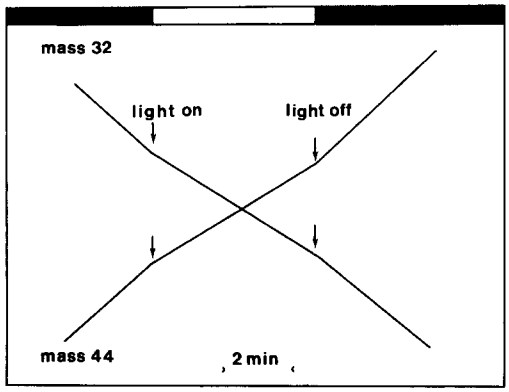

Fig. 3. Effect of illumination on the relative rates of net O_2 uptake (measured as the *m/e* 32 signal decrease) and of net CO_2 production (measured as the *m/e* 44 signal increase) in the mutant strain of *Chlamydomonas* F15 lacking PS I. Light intensity was 640 $\mu E/m^2$ per s.

$^{13}CO_2$ that the apparent inhibition of the CO_2 production rate was not due to a residual photosynthetic CO_2 assimilation (data not shown). Note that such an inhibition of respiration by light was observed in a *Chlamydomonas* mutant lacking functional Rubisco [13].

TABLE I

DARK AND LIGHT O_2 EXCHANGE IN THE F15 MUTANT (LACKING PS I) AND IN THE F18 MUTANT (LACKING THE CYTOCHROME b_6-*f* COMPLEX) OF *CHLAMYDOMONAS*

Effect of some inhibitors on O_2 exchange measured in the F15 strain. Light intensity was 640 $\mu E/m^2$ per s. Abbreviation: SHAM, salicylhydroxamic acid. Values represent nmol O_2/min per mg chlorophyll.

	O_2 uptake dark	O_2 uptake light	O_2 evolution light
F15 Control	195	250	128
DCMU 50 μM	205	210	0
Antimycin A 2 μM	174	214	75
KCN 1 mM	171	226	72
Antimycin A 2 μM + SHAM 0.4 mM	45	71	22
SHAM 0.4 mM	212	262	129
CCCP 2 μM	360	390	15
F18 Control	194	310	232

At this point, we can ask ourselves about the nature of the electron transport carriers involved in the PS-II-induced O_2 uptake. To answer this question, we have first studied light-induced O_2 exchange in the F15 mutant treated with DCMU and then in a *Chlamydomonas* mutant (F18) lacking the cytochrome b_6-f complex. When treated with DCMU, the F15 mutant did not evolve O_2 in the light and O_2 uptake was not stimulated by light (Table I), thus showing the involvement of the components directly located after the blocking site of DCMU (i.e., at least the PQ pool) in the electron pathway from PS-II to O_2. In the *Chlamydomonas* mutant (F18) lacking the cytochrome b_6-f complex, O_2 was continuously evolved in the light and O_2 uptake was stimulated by light as in the PS-I-deficient strain (Table I). This result clearly demonstrates that the cytochrome b_6-f complex and probably also the following electron carrier plastocyanin do not participate to the electron pathway from PS II to O_2.

To investigate further the nature of the electron pathway leading to the reduction of O_2, we then studied the effect of antimycin A, cyanide and salicylhydroxamic acid, inhibitors of the mitochondrial electron transport chain, and of an uncoupler CCCP on the rates of O_2 exchange measured in the F15 strain (Table I). Antimycin A (2 μM) or cyanide (1 mM), both inhibitors of the cytochrome oxidase pathway, when added alone inhibited O_2 evolution by about 40% and slightly inhibited dark respiration (about 10%). Simultaneous addition of salicylhydroxamic acid (inhibitor of the alternative pathway) was required to strongly inhibit (77%) the rate of respiration. This was due to the presence in *Chlamydomonas* of a very efficient alternative pathway [12,14]. In these conditions, O_2 evolution was inhibited by 83%. Note that salicylhydroxamic acid, when added alone, had no effect on the O_2 exchange rates. Similar effects of antimycin A, cyanide and salicyl hydroxamic acid were observed in the F18 strain (data not shown). Addition of CCCP (2 μM) inhibited O_2 evolution by about 90% and stimulated dark respiration by a factor of 1.8.

Discussion

Results presented in this paper clearly show that a mutant of *Chlamydomonas* lacking PS I activity is able to establish in the light a permanent electron flow from water to O_2 involving PS II activity and the PQ pool. Because similar O_2 exchange were observed in a mutant lacking the cytochrome b_6-f complex any participation of this complex and also probably of the following carrier plastocyanin to this electron pathway can be ruled out. On the basis of recent reports [15,16] concerning the existence of a respiratory activity (chlororespiration) in the chloroplast of the green alga *Chlamydomonas*, it is tempting to assume that electrons originating from PS II are driven towards this respiratory chain. However, the compared effects of antimycin A and cyanide do not agree with such an interpretation. This comes from the fact that antimycin A had the same effect as cyanide on the O_2 exchange rate (Table I), whereas cyanide but not antimycin A inhibits chlororespiration [16]. On the other hand, cyanide, like antimycin A, is a well-known inhibitor of the mitochondrial cytochrome oxidase pathway, indicating that the observed inhibition of O_2 evolution is more likely due to an effect on mitochondrial respiration than to an effect on chlororespiration. This interpretation is supported by the fact that salicylhydroxamic acid, when added to antimycin-A-treated cells, strongly inhibited both O_2 uptake and evolution, whereas when added alone, this compound did not inhibit O_2 exchange. This conditional effect of salicylhydroxamic acid is generally considered as a specific effect related to the inhibition of the mitochondrial alternative pathway which is operative in *Chlamydomonas* when the cytochrome oxidase pathway is blocked. Moreover, salicylhydroxamic acid alone or antimycin A plus salicylhydroxamic acid have been reported to not affect chlororespiration [16,17]. We conclude from the effects of the mitochondrial electron transport inhibitors that the permanent electron flow from water to O_2 via the PQ pool occurs only if the mitochondrial electron transport is operating. It is therefore probable that final interaction of electrons with molecular O_2 occurs at the level of mitochondrial oxidases.

These conclusions imply that direct reduction of molecular O_2 by reduced plastoquinones, which is probably linked to hydrogen peroxide production, if it occurs in vivo, represents a very minor route of O_2 consumption. Indeed, the maximal

rate of this reaction is given by the light-induced O_2 exchange, which is insensitive to antimycin A plus salicylhydroxamic acid, and is less than 22 nmol O_2/min per mg chlorophyll (see Table I).

Transfer of reducing power from chloroplasts to mitochondria has already been shown to occur in the light [12,18,19]. Such a transfer, coupled to mitochondrial oxidative phosphorylations, has been considered to explain photosynthetic CO_2 fixation in a mutant strain of *Chlamydomonas* lacking chloroplastic ATPase [19] and also to supply extra ATP during photosynthesis of barley protoplasts [18]. Moreover, chloroplasts were shown to have a high capacity for transferring redox equivalents from the stroma to the cytosol via the malate-oxaloacetate shuttle [20]. Generally, NADPH produced by PS I is recognized as the reductant involved in such a process. Clearly, this cannot be the case in mutant strains lacking either PS I or the cytochrome b_6-f complex, except if reducing power (NADH or NADPH) can be produced in the light by PS II activity.

Several authors have noticed that NADH [21] and NADPH [22] may reduce the PQ pool of the green alga *Chlamydomonas*. In the same algal species, Godde discovered an NADH-PQ oxidoreductase activity associated to the thylakoid membranes [23]. One may speculate that, in conditions where the PQ pool is fully reduced (which is generally the case in the light), this enzyme might catalyze the photoreduction of NAD^+. Such a reduction, which is thermodynamically unfavourable, may proceed, as it is the case in *Rhodospirillaceae*, via an energy-dependent reverse electron transfer [24]. This assumption is well-supported by the high sensitivity of O_2 evolution to the uncoupler CCCP observed in the F15 mutant (Table I). Indeed, uncouplers have been shown to inhibit succinate-linked NAD^+ photoreduction in *Rhodopseudomonas viridis* [25], whereas they are known to not inhibit chloroplastic electron transfer. We therefore suggest that in *Chlamydomonas*, as in *Rhodospirillaceae* [25], a $\Delta\tilde{\mu}_{H^+}$-driven QH_2 to NAD^+ electron transfer reaction could occur. In *Rhodospirillaceae*, this reaction is catalyzed by an enzymatic complex, analogous to the complex I of mitochondria, which is inhibited by rotenone [24,25]. In *Chlamydomonas*, the NADH-PQ oxidoreductase has been also reported to be sensitive to rotenone [21,23]. In these conditions, if the NADH-PQ oxidoreductase is involved in the reverse electron transfer reaction, one should expect a rotenone sensitivity of the PS-II-driven electron flow which occurs in the two mutants of *Chlamydomonas* studied. Recent experiments in our laboratory have shown that O_2 evolution in the F18 mutant was sensitive to rotenone (unpublished data). These results will be reported more in detail in a subsequent paper. However, direct measurements of PS-I-independent NAD^+ reduction will be necessary to confirm such an assumption concerning the occurrence of a reverse electron transfer pathway in the chloroplast of *Chlamydomonas*.

It has been recently established that the chloroplast DNA of a liverwort and of tobacco contained some genes showing important homologies to genes encoding for the NADH dehydrogenase of human mitochondria [26]. This suggests that a NADH dehydrogenase could be present in the chloroplasts of plants, as it is the case in the chloroplast of the green alga *Chlamydomonas*. In these conditions, the possible existence of an energy-dependent reverse electron flow catalyzed by PS II in the chloroplasts of higher plants has to be questioned in the future.

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