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Inhibition of a respiratory activity by short saturating flashes in *Chlamydomonas*: evidence for a chlororespiration

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Excitation with short actinic flashes (2 μs) of oxygenated dark-adapted *Chlamydomonas* cells deposited on a bare O_2 platinum electrode induces an increase of the amperometric signal after the first two flashes. Mass spectrometer experiments performed in the presence of $^{18}O_2$ showed that this signal was not due to the photolysis of water ($H_2{}^{16}O$). The insensitivity of this signal to 10 μM DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), its stimulation by acetate or high O_2 concentration as well as its inhibition by cyanide indicate that these flash-induced changes in O_2 concentration were related to the inhibition of a respiratory process. Because this rather fast inhibition of respiration is insensitive to antimycin A and to salicyl hydroxamic acid, inhibitors of mitochondrial respiration, and because it occurs on a single flash illumination, we conclude that the related respiratory activity takes place inside the chloroplast (chlororespiration) and not in the mitochondria. This interpretation is confirmed by the quite high $K_m(O_2)$ of this process (about 23 μM) compared to those measured for the mitochondrial reactions (0.2 μM for the cytochrome oxidase pathway and 5.5 μM for the alternative pathway). In a mutant lacking Photosystem I activity, no photoinhibition of respiration was observed. We conclude from the above results that the light-induced inhibition of chlororespiration is due to the oxidation by Photosystem I activity of electron carriers common to both photosynthetic and chlororespiratory chains.

Introduction

It is generally agreed that photosynthetic and respiratory activities of eukaryotic cells are located in two different cellular organelles, respectively chloroplasts and mitochondria. However, different lines of evidence indicate that, in unicellular green algae, molecular oxygen could interact in the dark with photosynthetic electron carriers, suggesting the presence of a respiratory-like activity inside the chloroplast. In the early sixties, Goedheer [1] concluded from luminescence transient studies on *Chlorella* that a dark oxidation of the acceptors of PS II could occur and was the first to postulate the existence of a chloroplastic respiratory system. In the same algal species, Diner and Mauzerall [2] proposed that the redox state of the PQ pool in the dark should result from an equilibrium between reduction and oxidation involving O_2. More recently, Bennoun [3,4] has studied using cells of *Chlamydomonas* and *Chlorella,* the effects of O_2 and of some respiratory inhibitors on the fluorescence induction kinetics and suggested that chlo-

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PQ, plastoquinone; PS I, Photosystem I; PS II, Photosystem II.

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roplasts may contain a respiratory chain (referred as chlororespiration) located between the two photosystems [3]. Furthermore, a respiratory-like NADH-plastoquinone-oxidoreductase bound to the membrane was discovered in the green alga *Chlamydomonas* [5,6]. Therefore, a respiratory chain, oxidizing NADH at the expense of O_2 and sharing at least one carrier of the photosynthetic electron-transport chain, the PQ pool [3], seems to be present inside the chloroplasts of eukaryotic microalgae.

In the present work, we have studied flash-induced oxygen exchanges in dark-adapted *Chlamydomonas* cells by amperometric and mass-spectrometric techniques. We show the existence of an O_2 signal unrelated to O_2 evolution after one or two flashes. The properties of this signal allowed us to ascribe it to a flash-induced inhibition of chlororespiration. We propose that PS I activity inhibits the chloroplastic respiratory activity by oxidizing an electron carrier common to chlororespiration and photosynthesis.

Materials and Methods

Chlamydomonas reinhardtii wild-type 137c was grown phototrophically as previously described [7]. The mutant lacking PS I activity (F15/4$^+$) was grown in TAP medium [3] under continuous illumination (25 $\mu E \cdot m^{-2} \cdot s^{-1}$). Algae were harvested by low speed centrifugation (1500 $\times$ g) and resuspended in a 0.05 M Tris buffer (pH 7.2) containing 0.2 M KCl. Flash-induced oxygen-exchange measurements were performed using a bare platinum electrode system similar to that described in Ref. 8. The cell suspension was deposited on the platinum electrode and was separated from the circulating buffer (the same as the resuspending buffer) by a dialysis membrane. Different O_2 concentrations at the sample level were obtained by bubbling either air or O_2 in the circulating buffer. The cells were allowed to settle for at least 15 min before measurement.

Measurements of oxygen exchanges following flash excitation were also performed by using a magnetic sector mass spectrometer (type 14-80, VG Instruments). 4 ml of the algal suspension were deposited at the bottom of a close vessel (total volume of about 40 ml) on a gas-permeable membrane which allowed dissolved gases to diffuse toward the mass spectrometer for analysis. After bubbling the sample with N_2 to remove the $^{16}O_2$ present, $^{18}O_2$ (98.1% ^{18}O) was injected in the gas phase. The concentrations of gases dissolved in the algal suspension were analyzed by continuously and simultaneously recording masses 32 ($^{16}O_2$) and 36 ($^{18}O_2$).

For both amperometric and mass spectrometric experiments, flash excitation was provided by a xenon flash (EG and G, PS302). The duration of the flash was 2 μs at its half peak height.

Respiration was measured with a Clark-type electrode (Rank Brothers). Chlorophyll content was determined after extraction with 90% (v/v) methanol as previously described [7].

Antimycin A and salicyl hydroxamic acid were used dissolved in ethanol and were purchased from Sigma. At the concentration used, ethanol had no effect on respiratory and photosynthetic activities.

Results

When dark-adapted *Chlamydomonas* cells were deposited on a bare O_2 platinum electrode and illuminated with a series of short (2 μs) saturating flashes, a typical damped oscillation of oxygen yields with period 4 was observed (Fig. 1A). This characteristic pattern first observed by Joliot et al. [9] has been interpreted [10] as being due to the accumulation of four oxidizing equivalents on the donor side of PS II necessary to bring about the evolution of one O_2 molecule. In the general pattern, almost no O_2 is produced on the first two flashes (Fig. 1A). When the basal concentration of oxygen at the level of the electrode was raised by oxygenating the circulating buffer, we observed small signals but of significant amplitude following the first two flashes (Fig. 1B). At the same time, the stationary oxygen yield was increased by about 20%. The polarogram of the signal observed on the first two flashes was identical to the one observed on following flashes (data not shown), indicating that this signal was due to the increase in the O_2 concentration. The oxygen-exchange signal occurring on the first flash was much longer ($t_{1/2}$ = 350 ms – see Fig. 2A) than the one observed on the third flash ($t_{1/2}$ = 5 ms – see Fig.

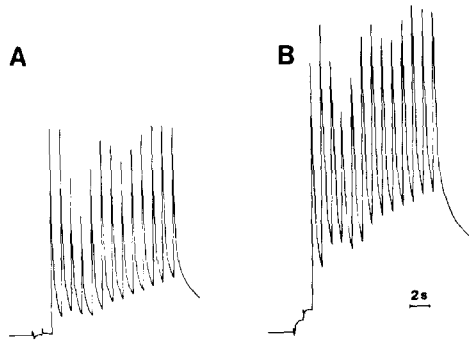

Fig. 1. Amperometric signal measured following the flash illumination of *Chlamydomonas* cells deposited on a bare platinum electrode. (A) Control; air was bubbled in the circulating buffer. (B) In the presence of an increased oxygen concentration obtained by bubbling O_2 in the circulating buffer. Flashes were spaced by 1 s.

2B) and corresponding to O_2 evolution. These differences in the amperometric signal pattern indicate that a process different from O_2 evolution may occur at least after the first two flashes.

In fact, the increase in the O_2 concentration measured with the O_2 electrode system used can be due to either a production of O_2 (from the photolysis of water) or the inhibition of a respiratory process. The latter phenomenon has been observed for photosynthetic bacteria (which do not evolve oxygen) when illuminated by flashes [11]. To choose between these two hypotheses we performed oxygen exchange experiments in the presence of $^{18}O_2$ using a mass spectrometer. The experiments are based on the rationalization that, in the presence of a large excess of $^{18}O_2$, changes in respiratory activity will only significantly affect the $^{18}O_2$ concentration, while photolysis of water ($H_2{}^{16}O$) will cause an increase in the $^{16}O_2$ concentration. Illumination of algae with one or two flashes in the presence of $^{18}O_2$, modified only the $^{18}O_2$ concentration (Fig. 3A and B). On the other hand, variations in both $^{18}O_2$ and $^{16}O_2$ concentrations were induced by the third flash (Fig. 3C). This experiment clearly demonstrates that a respiratory process was inhibited after the three first flashes at least. A further confirmation of this interpretation is found in the experiment performed in the presence of DCMU. Fig. 4 shows

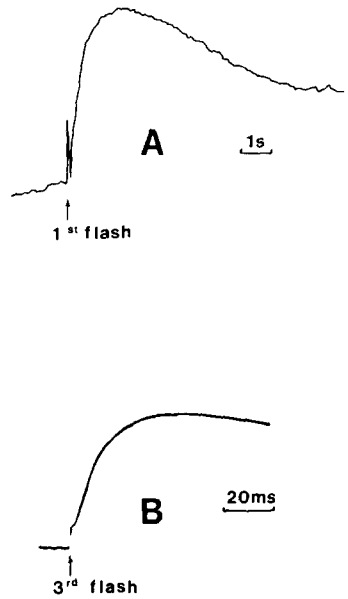

Fig. 2. Kinetics of the amperometric signal measured following the flash illumination of *Chlamydomonas* cells deposited on a bare platinum electrode. (A) Measured after the first flash. (B) Measured after the third flash. Note the different time scale between traces A and B.

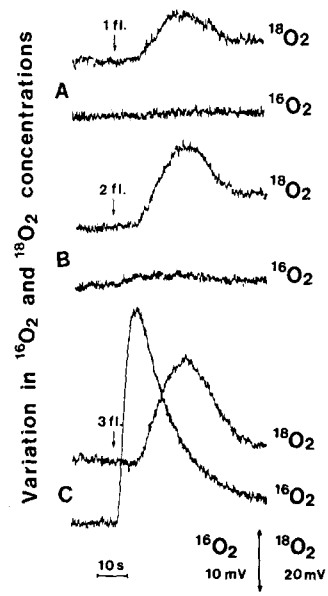

Fig. 3. Mass spectrometer measurements of masses 32 and 36 following the illumination of dark-adapted *Chlamydomonas* by one (A), two (B) or three (C) flashes (fl.) spaced by 0.1 s. Background signal values (volts) before the flashes were fired: mass 32, 255 mV; mass 36, 6.09 V.

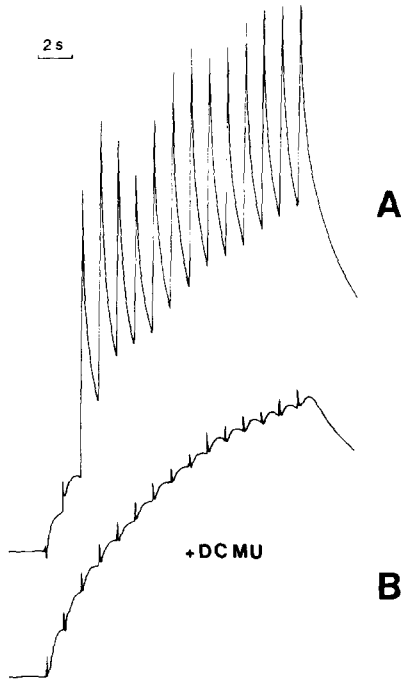

Fig. 4. Same as Fig. 1B. (A) Control in the presence of 1 mM acetate; (B) +10 μM DCMU to (A).

that, at a DCMU concentration of 10 μM, which is known to inhibit O_2 evolution completely, the light inhibition of respiration induced by the first two flashes is unaffected and still occurs after subsequent flashes. The excitation flashes induced some artefactual signals of small amplitude clearly discernible after each flash in the presence of DCMU. On the other hand, we observed that addition of 1 mM acetate increased the O_2 signal of the first flash by a factor 3 (compare with Fig. 1B and Fig. 4A).

We therefore conclude from these experiments that the increase in the amperometric signal induced by the first two flashes is not related to O_2 evolution but rather to the inhibition of a respiratory process. Note that the experiments shown in Fig. 3 and the following experiments were performed in the presence of 1 mM acetate. Under these conditions, the signal observed on the first two flashes had its maximal intensity.

We further characterized this respiratory process by investigating the effect of different respiratory inhibitors. We found that cyanide (0.5 mM) inhibited the slow amperometric signal by about 75% (Fig. 5 A and B). At a cyanide concentration of 1 mM, inhibition was almost complete. A similar cyanide sensitivity was observed for the flash-induced variation in the $^{18}O_2$ concentration recorded in the mass spectrometer experiment (data not shown). At the same time, the oscillations of oxygen evolution were strongly affected and the stationary evolution, measured after 15 flashes, was inhibited by about 22% (Fig. 5B). However, when the experiment was performed after a preillumination with low intensity far-red, we recorded a stationary oxygen production similar to that of the control (Fig. 5C). These experiments show that cyanide drastically inhibited the phenomenon oc-

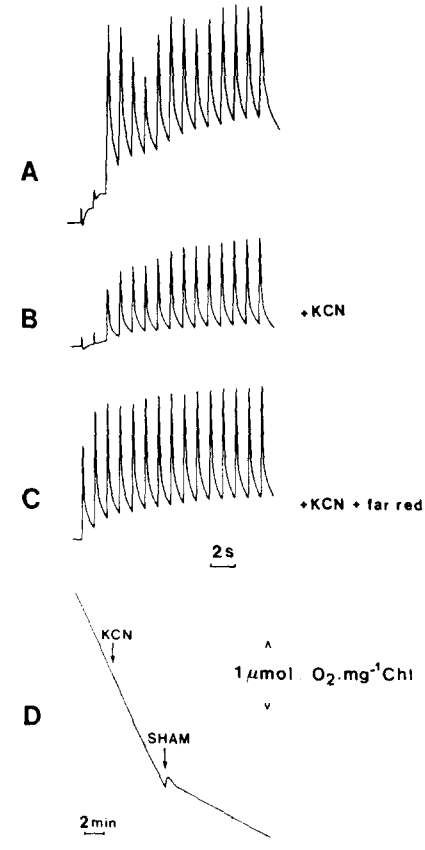

Fig. 5. Effect of 0.5 mM cyanide on flash-induced O_2 exchange and on dark respiration rate. (A) Amperomeric signal recorded after a series of 15 flashes in the presence of 1 mM acetate. (B) +0.5 mM cyanide. (C) +0.5 mM cyanide after a low intensity far-red preillumination (3 min). (D) Effect of 0.5 mM cyanide and of 1 mM salicyl hydroxamic (SHAM) acid on the respiration rate of *Chlamydomonas* cells measured with a Clark-type O_2 electrode.

curring on the first two flashes without affecting the activity of PS II. Cyanide is known to block mitochondrial cytochrome oxidase activity completely at this concentration. However, in our experiments it inhibited the algal respiration rate by only 6% (Fig. 5D). This apparent ineffectiveness of cyanide addition is probably due to the participation of an important alternative, cyanide-insensitive pathway which operates when the cytochrome oxidase is inhibited. This interpretation is supported by the effect of salicyl hydroxamic acid, a well-known inhibitor of the alternative oxidase [12]. Indeed, respiration was insensitive to the addition of salicyl hydroxamic acid only (data not shown), but was strongly inhibited after addition of both cyanide and salicyl hydroxamic acid (Fig. 5D).

When algae were treated by 1 μM antimycin A and 1 mM salicyl hydroxamic acid, inhibitors which would be expected to block completely the mitochondrial respiratory activity, the respiration rate was inhibited by about 84% (Fig. 6A). However, these compounds did not affect the oxygen pattern recorded on the platinum electrode (Fig. 6B).

As previously shown in Fig. 1, the flash-induced inhibition of respiration was found to be highly sensitive to O_2 concentration. The apparent $K_m(O_2)$ of this phenomenon was found to be about 23 μM (Fig. 7A). This value is much higher than the apparent $K_m(O_2)$ of total dark respiration measured in the same alga (about 0.2 μM – see Fig. 7B) and also higher than the $K_m(O_2)$ for the mitochondrial alternative pathway measured in the presence of 1 mM KCN (about 5.5 μM – see Fig. 7A).

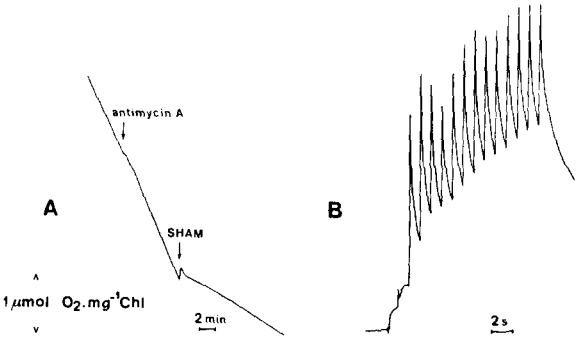

Fig. 6. (A) Effect of 1 μM antimycin A and 1 mM salicyl hydroxamic acid on the respiration rate of *Chlamydomonas* cells measured with a Clark-type O_2 electrode. (B) Amperometric signal recorded after a series of 15 flashes in the presence of 1 mM acetate, 1 μM antimycin A and 1 mM salicyl hydroxamic (SHAM) acid.

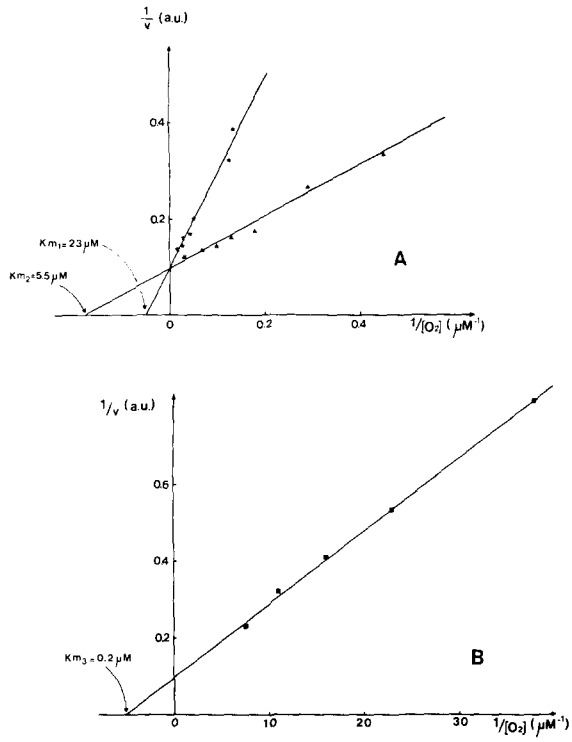

Fig. 7. Effect of O_2 concentration on the flash-induced inhibition of respiration (★), on the total dark respiration (■) and on the alternative pathway (▲) in *Chlamydomonas*. Maximum rates were arbitrarily normalized to 10. The flash-induced inhibition of respiration (★) was measured by mass spectrometry following a three flashes illumination at different $^{18}O_2$ concentrations. The total dark respiration (■) and the alternative pathway activity (▲) were measured using a Clark-type O_2 electrode. The latter experiment was performed in the presence of 1 mM cyanide to inhibit the cytochrome oxidase.

We inferred from the experiment performed in the presence of DCMU (Fig. 4) that the photochemical reactions responsible for the inhibition of respiration observed on the first two flashes are mediated by PS I. This hypothesis is further confirmed by the experiment carried out with a mutant of *Chlamydomonas* (F15/4^+) lacking PS I activity (Fig. 8). In this mutant, the slow increase of the amperometric signal occurring in the wild type in response to the first two flashes was absent.

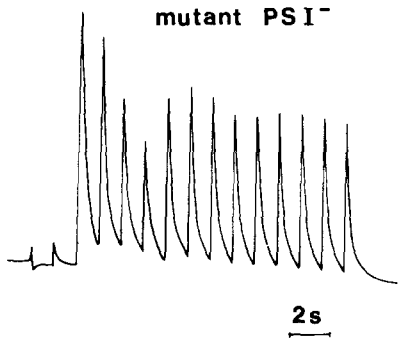

Fig. 8. Same as Fig. 1B with a mutant strain (F15/4$^+$) of *Chlamydomonas* lacking PS I activity (in the presence of 1 mM acetate).

Discussion

As demonstrated by the mass-spectrometer experiments and the experiment performed in the presence of DCMU, the rising O_2 signal observed following single or double flashes illumination of dark-adapted *Chlamydomonas* cells is not due to the photolysis of water but to the inhibition of a respiratory process. This O_2 signal is sensitive to O_2 concentration, increased in the presence of acetate and inhibited by cyanide. When mitochondrial respiration is inhibited by the addition of antimycin A and salicyl hydroxamic acid, the flash-induced inhibition of respiration is unaffected (Fig. 6). This experiment shows that mitochondrial respiration is not involved in this process. This interpretation is further confirmed by the occurrence of the light-inhibition after a single flash illumination. This event does not induce significant changes in ATP and in NADPH concentrations (probable mediators of interactions between chloroplasts and mitochondria). For all these reasons, we suggest that chlororespiration rather than mitochondrial respiration is inhibited under the conditions employed. The involvement of a respiratory process different from mitochondrial respiration is also supported by the $K_m(O_2)$ measurements. Indeed, the apparent $K_m(O_2)$ measured for the flash-induced inhibition of respiration (about 23 μM) is quite different from the apparent $K_m(O_2)$ of the total dark respiration (about 0.2 μM) mediated primarily by the mitochondrial cytochrome oxidase and also from the apparent $K_m(O_2)$ of the cyanide-insensitive pathway (about 5.5 μM) mediated primarily by the mitochondrial alternative oxidase. Therefore, the chloroplastic respiratory chain may contain a cyanide-sensitive oxidation pathway with an affinity for O_2 different from the affinities of the oxidases involved in the mitochondrial respiratory chain.

The inhibition of chlororespiration by flash illumination can be easily interpreted in a scheme where PS I activity diverts the electrons from a chloroplastic respiratory chain into the photosynthetic chain by oxidizing an electron carrier which is common to both chains. The identity of these common electron carriers is difficult to determine from the experiments reported in the present paper. By studying the rate of oxidation of the PQ pool in different mutants of *Chlamydomonas* with deficient electron transport between the PQ pool and PS I, Bennoun [4] concluded that the chlororespiratory and photosynthetic electron-transport chains share the PQ pool but not the cytochrome b_6f complex. The chlororespiratory chain would therefore resemble an alternative respiratory electron-transport chain with an oxidase branched directly on the PQ pool. In such a scheme, the light-induced inhibition of respiratory activity we observed will be induced by the oxidation of the quinone pool. In photosynthetic bacteria [11,13,14] and in cyanobacteria [15–17] light has been shown to inhibit respiratory activity. This inhibition has been interpreted as being due to the existence of a common segment of respiratory and photosynthetic electron-transport chains involving the ubiquinone pool, the cytochrome *bc* complex and the soluble cytochrome c_2 in the case of photosynthetic bacteria [18,19] and the plastoquinone pool, the cytochrome b_6f complex and the soluble plastocyanin/cytochrome *c*-553 in the case of cyanobacteria [20]. The extension of this type of interaction to the chloroplast of microalgae will therefore implicate the presence of both a cytochrome oxidase-like pathway (involving the PQ pool, the cytochrome *bc* complex and the plastocyanin) and an alternative oxidase-like pathway (involving the only PQ pool). Additional experiments are required to elucidate, unequivocally, the

nature of the oxidases and the photosynthetic electron carriers implicated in the respiratory chain located in the chloroplast.

It appears likely from our experiments that under certain conditions the PS II activity may be affected by the activity of chlororespiration. Indeed, it is probable that the decrease of the stationary O_2 yield induced by cyanide and relieved by far-red preillumination (Fig. 5) as well as the decrease of the stationary O_2 yield observed in the presence of a low O_2 concentration (Fig. 1) are due to an over-reduction of the PS II acceptors (principally the PQ pool) consecutive to the inhibition of the chloroplastic oxidase either by cyanide or by a low O_2 concentration. A similar interpretation has been proposed by Bennoun [3], who investigated the effect of chlororespiratory activity on the redox state of the PQ pool using a fluorescence method.

It has been postulated [17,21] that the inhibition of respiration by light is responsible for the nonlinearity in the curve of net O_2 exchange as a function of light intensity (Kok effect). This effect is not suppressed by DCMU and is more apparent in far red than in red light [22]. Two different hypotheses have been proposed to explain this light-induced inhibition of respiration. In the first, it is supposed that an increase in the ATP/ADP ratio causes the inhibition of the respiratory electron flow [17]. In the second, a diversion of reductant away from respiratory transport and into PS I would cause the inhibition of respiration [22]. The first hypothesis is inconsistent with the insensitivity of the Kok effect to the uncoupler CCCP observed in *Chlamydomonas* cells [22]. In the present work, we found that PS I activity is responsible for the inhibition of a respiratory process located inside the chloroplast. Therefore, it seems likely that the PS I-mediated inhibition of respiration responsible for the Kok effect observed in *Chlamydomonas* may be due, at least in part, to the diversion of electrons from the chlororespiratory to the photosynthetic electron transport chain.

The effect of the different respiratory inhibitors permits the differentiation between two types of respiratory process. Chlororespiration is inhibited by flashes, sensitive to cyanide but insensitive to antimycin A and salicyl hydroxamic acid. Conversely, mitochondrial respiration is inhibited by a combination of antimycin A and salicyl hydroxamic acid, but is insensitive to cyanide alone. These different effects of the respiratory inhibitors on chlororespiration and on mitochondrial respiration allow us to make a quantitative estimation of the chlororespiration rate. Indeed, one may consider that the cyanide-sensitive oxygen uptake component remaining when algae were treated with both antimycin A and salicyl hydroxamic acid (condition where the mitochondrial respiration is inhibited) is an estimation of the maximum oxygen uptake rate involved in chlororespiration. The basal respiration rate (330 nmol O_2/min per mg Chl) was reduced to 108 nmol O_2/min per mg Chl by simultaneous addition of 1 μM antimycin A and 1 mM salicyl hydroxamic acid. The remaining activity was inhibited by about 40 nmol O_2/min per mg Chl following subsequent addition of 1 mM cyanide. Therefore, we can estimate the maximal rate of chlororespiration to about 40 nmol O_2/min per mg Chl. That is 12% of the respiration rate measured in the control. From the kinetics of reoxidation of the pool of electron acceptors of PS II, Bennoun [3] calculated that the chlororespiration rate was about 20% of total respiration. These calculations are in agreement with our estimations.

Chlororespiration could be regarded as the residual respiratory activity of the primitive prokaryote (presumably a *Prochloron*-like organism) which is thought to be at the origin of the chloroplasts of green algae [23]. We can ask ourselves about the physiological role of this chloroplast respiratory pathway. Since the electron flow rate involved in chlororespiration represents only 12% of that involved in the mitochondrial respiration, the contribution of this process to the respiratory function of the green cell seems to be marginal. As chlororespiration is inhibited by light, its participation to the accomplishment of the photosynthetic function appears unlikely. However, chlororespiration may contribute efficiently to the metabolism of acetate inside the chloroplast, especially under dark conditions. One may also speculate that chlororespiration, by maintaining the acceptors of PS II (principally the PQ pool) in an appropriate redox state in the dark, or by producing a sufficient pH gradient to maintain the

ATPases in a functional form [24], allows an efficient initiation of photosynthesis during a dark-to-light transition.

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