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Chlororespiration and the process of carotenoid biosynthesis

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Abstract

The plastoquinone pool during dark adaptation is reduced by endogenous reductants and oxidized at the expense of molecular oxygen. We report here on the redox state of plastoquinone in darkness, using as an indicator the chlorophyll fluorescence kinetics of whole cells of a *Chlamydomonas reinhardtii* mutant strain lacking the cytochrome b_6f complex. When algae were equilibrated with a mixture of air and argon at 1.45% air, plastoquinol oxidation was inhibited whereas mitochondrial respiration was not. Consequently, mitochondrial oxidases cannot be responsible for the oxygen consumption linked to plastoquinol oxidation. Plastoquinol oxidation in darkness turned out to be sensitive to *n*-propyl gallate (PG) and insensitive to salicylhydroxamic acid (SHAM), whereas mitochondrial respiration was sensitive to SHAM and PG. Thus, both PG treatment and partial anaerobiosis allow to draw a distinction between an inhibition of plastoquinol oxidation and an inhibition of mitochondrial respiration, indicating the presence of a plastoquinol:oxygen oxidoreductase. The possible identification of this oxidase with an oxidase involved in carotenoid biosynthesis is discussed in view of various experimental data.

Keywords: Chlorophyll fluorescence kinetics; Chlororespiration; Plastoquinol:oxygen oxidoreductase; *n*-Propyl gallate; Carotenoid biosynthesis; *Chlamydomonas reinhardtii*

1. Introduction

The model of 'chlororespiration' was proposed originally to account for two sets of observations by a single model [1]. The first one referred to the changes of the redox state of the plastoquinone pool that can be observed in darkness, the second one to the transmembrane potential of thylakoids detected in darkness in the absence of the chloroplast F_0F_1 ATP synthase (referred to below as the permanent membrane potential). In this model, plastoquinone was supposed to be reduced by an NAD(P)H dehydrogenase, and reoxidized by molecular oxygen through a chloroplast oxidase. Electron transfer through this respiratory chain from NAD(P)H to oxygen was supposed to be electrogenic and thus responsible for the observed permanent membrane potential. A careful examination of mitochondrial–chloroplast interactions led us to conclude afterwards that the permanent membrane potential resulted mostly from a new ATP-dependent electrogenic pump present in the thylakoid membranes rather

Abbreviations: SHAM, salicylhydroxamic acid; PG, *n*-propyl gallate; NAD(P)H, nicotinamide adenine dinucleotide (phosphate); DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ATP, adenosine 5′-triphosphate

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than from chlororespiration [2,3]. On this basis, the actual model of chlororespiration concerns mainly the processes of reduction and oxidation of plastoquinone in darkness. The reduction of plastoquinone was assigned to the presence of an NAD(P)H:plastoquinone oxidoreductase. In line with this prediction, low amounts of a chloroplast homologue of the mitochondrial NADH dehydrogenase were found in thylakoid membranes of higher plants [4–6]. In algae, however, there are few molecular evidences for the existence of such a protein, although physiological evidence shows that plastoquinone reduction in darkness is catalyzed in some way. For instance in *Chlamydomonas reinhardtii*, some reduction of plastoquinone was observed in darkness in response to an inhibition of the mitochondrial electron transport [2]. The identification of the algal complex responsible for the dark reduction of plastoquinone remains to be achieved.

It was shown that plastoquinol oxidation proceeded in the dark at the expense of molecular oxygen [7]. In the model of chlororespiration, this oxidation was assigned to the presence of a plastoquinol:oxygen oxidoreductase. However, the occurrence of interactions between chloroplasts and mitochondria made the mechanism of this oxidation difficult to resolve [2,8]. A stimulation of oxygen uptake was observed upon illumination of *C. reinhardtii* mutants devoid of photosystem I reaction centers. The additional oxygen uptake was attributed to the presence of a plastoquinol:oxygen oxidoreductase sensitive to *n*-propyl gallate (PG) [9], an inhibitor of the plant mitochondrial alternative oxidases [10]. A nuclear gene product IM was recently reported in *Arabidopsis thaliana*, showing amino acid similarity to the plant mitochondrial alternative oxidase known as a quinol:oxygen oxidoreductase [11,12]. Upon expression in *Escherichia coli*, the IM protein was shown to confer a cyanide-resistant electron transport to isolated membranes, thus acting as an alternative terminal oxidase. Interestingly enough, this activity appeared to be sensitive to PG. The IM protein was shown to be located in the thylakoid membrane and to contain an iron-binding motif similar to that of the plant mitochondrial alternative oxidase. The *im* mutants of *A. thaliana* show a light-dependent variegated phenotype. Their albino sectors contain reduced amounts of carotenoids and increased levels of the carotenoid precursor phytoene. It was proposed that phytoene desaturation would be coupled with the reduction of plastoquinone, and plastoquinol oxidation carried out by the IM protein acting as a plastoquinol:oxygen oxidoreductase. In addition to its role during the early chloroplast differentiation, the IM protein was shown to be essential to carotenoid accumulation in petal and fruit chromoplasts, a process also linked to a respiratory redox pathway (see [13] for a review). It was thus of interest to further investigate the process of plastoquinol oxidation in darkness in view of these new sets of data.

2. Materials and methods

2.1. Strain

The *C. reinhardtii* strain FUD6 was used throughout this work. This strain is derived from wild type 137-C following 5-fluorodeoxyuridine mutagenesis and fluorescence screening [14], and lacks the cytochrome b_6f complex [15]. This strain was backcrossed several times to wild type in order to select offspring showing a high growth rate in darkness indicative of a high rate of mitochondrial respiration. This is important since spontaneous mutations leading to slow growers are extremely frequent in *C. reinhardtii*.

2.2. Cultures

Algae were inoculated in liquid Tris–acetate–phosphate medium [16] at 25°C at a light intensity of 250 lux and allowed to grow on a rotatory shaker. Cells were harvested in exponential phase (around 2–4×10^6 cells/ml) and resuspended in liquid minimal HS medium [17] at the same density. The culture was further shaken in the same conditions for 1–2 h before use. This procedure allows to fully oxidize the plastoquinone pool in darkness.

2.3. Measurements of the amounts of electron acceptors to photosystem II by fluorescence

Plastoquinone is an electron carrier of the photosynthetic electron transport chain located between

the two photosystems, that is reduced by photosystem II reaction centers and reoxidized by cytochrome b_6f complexes and photosystem I reaction centers. The size of the plastoquinone pool is conveniently measured in the absence of either cytochrome b_6f complex or photosystem I reaction centers because under these conditions photosystem II can fully reduce this pool, leading to a fluorescence rise. In the course of this rise, a complementary relation is observed between the fluorescence yield and the photochemical rate of photosystem II reaction centers. The area delimited by the fluorescence rise and its asymptote is thus proportional to the amount of electron acceptors available to photosystem II. In the presence of the herbicide diuron (DCMU), only one charge separation can be achieved per photosystem II reaction center and this is reflected by a fast fluorescence rise. Consequently, the ratio of the area over the fluorescence rise observed in the absence and in the presence of diuron allows the estimation of the amount of electron acceptors available per active photosystem II reaction center. In our experiments, a ratio of 10 is currently found for dark-adapted algae. The electron acceptors available to photosystem II in strains lacking the cytochrome b_6f complex are plastoquinone molecules each accepting two electrons. The observed ratio of 10 therefore indicates the presence, on the average, of five plastoquinone molecules per photosystem II reaction center. As plastoquinol is reoxidized in darkness at the expense of oxygen [7], the rate of regeneration of the pool of electron acceptors in darkness as deduced from fluorescence measurements provides a specific measurement of the oxygen uptake related to plastoquinone oxidation, that is to chlororespiration. The measurements of chlorophyll fluorescence kinetics in vivo were achieved on whole cells as previously described [2]. The Hamilton syringe of our setup was replaced by disposable cuvettes of 1.4 ml. Continuous illumination was delivered at 590 nm at the intensity of 60 μE m^{-2} s^{-1}. In the experiment shown in Fig. 4, the algae were incubated with a mixture of argon and air, their ratio regulated by a digital mass flow controller (Bronkhorst Hi-Tec). Algae were kept in a special tank under moderate overpressure and transferred to the measuring cell through an airtight pipe. The measuring cell was made of a glass cylinder with an internal diameter of 1.4 mm.

3. Results

3.1. Influence of the growth phase on plastoquinol oxidation in darkness

The basics of the correlation between the size of the plastoquinone pool and fluorescence kinetics are

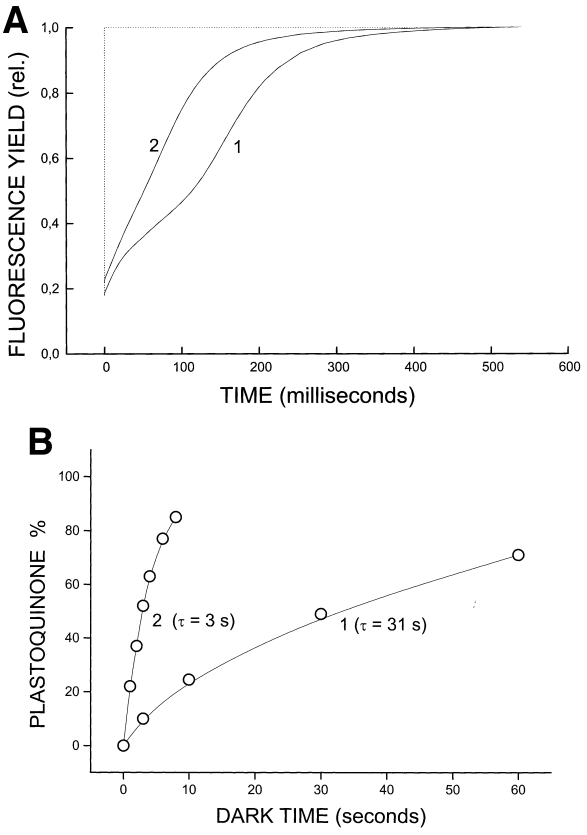


Fig. 1. Reoxidation of the plastoquinol pool in darkness following reduction by light. Fluorescence induction kinetics were achieved on whole cells of the FUD6 mutant lacking the cytochrome b_6f complex. (A) 1, dark adapted; 2, preilluminated for 600 ms to achieve full reduction of the plastoquinone pool and then incubated in darkness for 3 s to allow partial reoxidation of plastoquinol. The area over the fluorescence rise is proportional to the size of the plastoquinone pool. The comparison of kinetics 1 and 2 shows that 58% of the plastoquinol pool was reoxidized within the 3 s dark period. (B) The kinetics of reoxidation of the plastoquinol pool in darkness. Fluorescence experiments were conducted as described in A and the dark time between the two illuminations was varied. This dark time is indicated on the X axis whereas the Y axis shows the amount of oxidized plastoquinone present at the end of each dark time and revealed by the second illumination. 1, algae harvested in the early exponential phase of growth (about 10^6 cells/ml); 2, algae harvested in the stationary phase (about 10^7 cells/ml). The half-times of the kinetics (τ) are shown in the figure.

summarized in Section 2. Fig. 1A curve 1 shows the light-induced fluorescence rise obtained in dark-adapted whole cells of *C. reinhardtii* FUD6 lacking the cytochrome b_6f complex. This fluorescence rise reflects the complete reduction of the plastoquinone pool induced by the light. At the end of this illumination, algae were kept in darkness for 3 s in order to allow some plastoquinol oxidation to proceed. A second illumination was then given resulting once more in the reduction of the remaining plastoquinone pool as revealed by the fluorescence rise shown in curve 2. The comparison of the area above the two fluorescence rises indicates that 58% of the plastoquinol pool was reoxidized during this dark adaptation. As mentioned above, this reoxidation proceeds at the expense of molecular oxygen [7]. The reoxidation of the plastoquinol deduced from the above analysis reflects therefore the oxygen uptake due to chlororespiration. This estimation is more specific than the overall oxygen measurements that fail to distinguish oxygen uptakes related to various processes. The kinetics of plastoquinol oxidation in darkness can be derived from this type of experiments by varying the dark time between the two illuminations. The kinetics for cells harvested either in the early exponential phase of growth or in the stationary phase are shown on Fig. 1B. A drastic slow-down in the kinetics of plastoquinol oxidation in darkness was observed in cells reaching the stationary phase (half-time changing from 3 to 31 s). Similar observations (not shown) were made in the *Chlorella sorokiniana* S14 strain lacking the cytochrome b_6f complex [18], with the half-time changing from 1.2 to 24 s.

3.2. Sensitivity of plastoquinone oxidation and reduction to oxidase inhibitors

The inhibition of mitochondrial respiration induces a reduction of plastoquinone in darkness [2]. It was proposed that this inhibition leads to a decrease in the ATP level in the cell that stimulates the

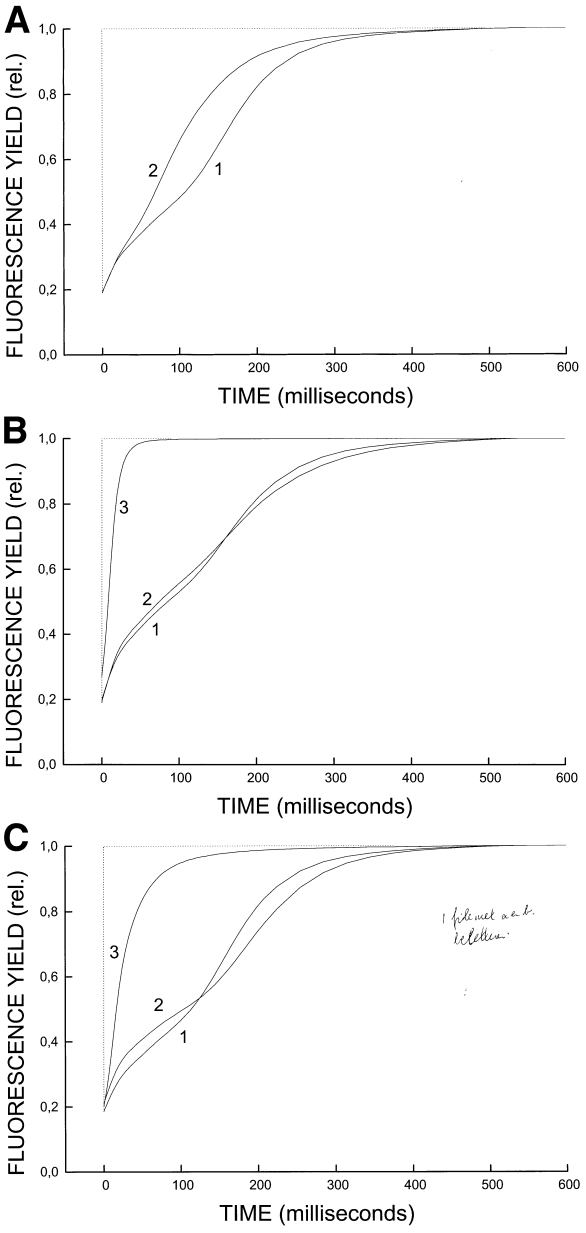


Fig. 2. Effect of azide, PG, and SHAM on the redox state of plastoquinone in darkness. Fluorescence kinetics of dark-adapted algae were obtained as depicted in Fig. 1A. (A) 1, control; 2, algae incubated for 2 min with 1 mM sodium azide. The comparison of the area over the two fluorescence rises indicates that azide induces a reduction of 25% of the plastoquinone pool. (B) 1, control; 2, algae incubated for 2 min with 1 mM PG; 3, algae incubated for 2 min with 1 mM PG plus 1 mM sodium azide. The comparison of the area over the fluorescence rises 2 and 3 indicates that the mixture of PG plus azide induces a reduction of 90% of the plastoquinone pool. (C) 1, control; 2, algae incubated for 2 min with 1 mM SHAM; 3, algae incubated for 2 min with 1 mM SHAM plus 1 mM sodium azide. The comparison of the area over the fluorescence rises 2 and 3 indicates that the mixture of SHAM and azide induces a reduction of 75% of the plastoquinone pool.

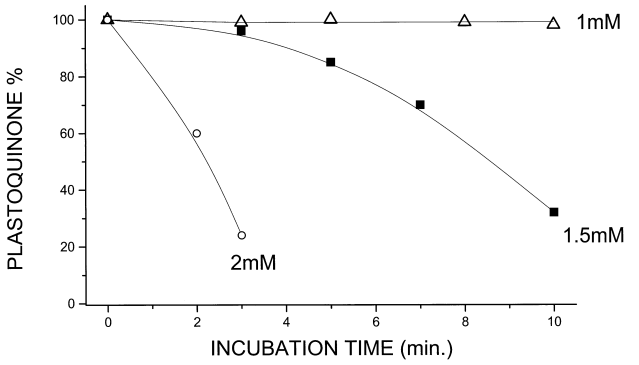


Fig. 3. Effect of increasing incubation time and concentration of PG on the redox state of plastoquinone. Algae were incubated in darkness with PG for various durations and fluorescence kinetics were recorded. The size of the plastoquinone pool was deduced from the area over the fluorescence rises. The concentrations of PG are indicated in the figure.

glycolytic pathway in the chloroplast, and hence increases the concentration of reductants able to reduce plastoquinone [19]. In order to completely inhibit mitochondrial respiration in plants, it is necessary to block both pathways of electron transport: the main one leading to the cytochrome *c* oxidase sensitive to cyanide and azide, and the alternative one leading to a quinol:oxygen oxidoreductase sensitive to PG and to salicylhydroxamic acid (SHAM) [20,21]. We have tested the sensitivity of our strains to PG and to SHAM in the absence or in the presence of azide. PG and SHAM were dissolved in ethanol, the final concentration of solvent was kept in all cases below 2 mM, and no solvent effect could be detected. Under our experimental conditions, only 25% of the plastoquinone pool was reduced upon addition of azide in darkness (Fig. 2A), and no significant reduction was observed upon addition of either 1 mM PG or SHAM (Fig. 2B,C, curves 1 and 2). The minor differences in the shape of the fluorescence rises 1 and 2 in Fig. 2B,C likely relate to the existence of domains in photosystem II with different rates of photoreduction and to their slight fluctuation in darkness [22]. Small fluctuations of the amounts of active photosystem II reaction centers may also occur in darkness and affect the fluorescence rise as well. The addition of a mixture of azide plus either PG or SHAM induced on the other hand a strong reduction of the plastoquinone pool (Fig. 2B,C, curves 3). It should be noted that the combination of azide plus PG induced a more pronounced reduction of plastoquinone than did the combination of azide plus SHAM. As shown in Fig. 3, PG alone at concentrations above 1 mM induced a slow reduction of the plastoquinone pool, whereas SHAM alone showed no effect on the redox state of the pool above 1 mM (for instance after 10 min of incubation at 2 mM, not shown). The effect of these inhibitors on the reoxidation of plastoquinol (chlororespiration) was observed following full reduction by light and 3 s of incubation in darkness (Fig. 4). The proportion of plastoquinol reoxidized in

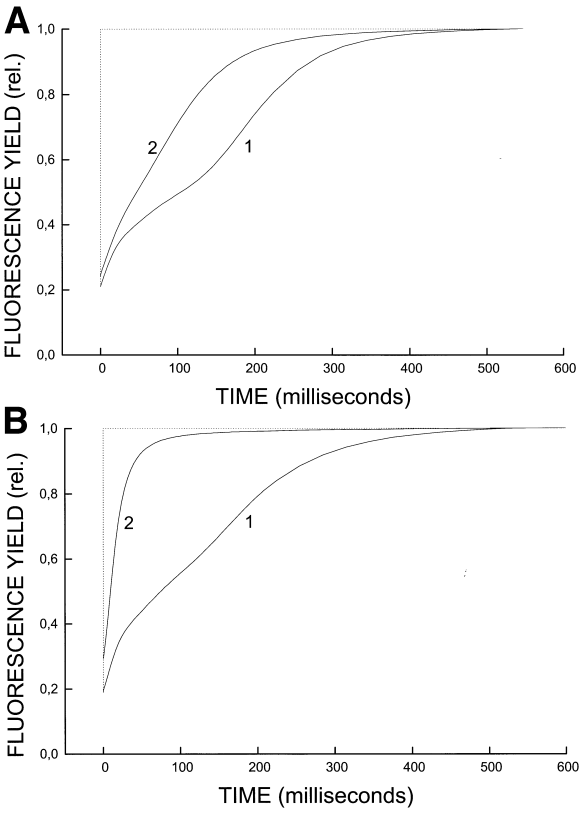


Fig. 4. Effect of PG and SHAM on the reoxidation of the plastoquinol pool in darkness following reduction by light. Fluorescence kinetics of dark-adapted algae were obtained as in Fig. 1A. 1, dark-adapted; 2, preilluminated for 600 ms to achieve full reduction of the plastoquinone pool and then incubated in darkness for 3 s to allow partial reoxidation of plastoquinol. (A) 1, algae incubated for 2 min with 1 mM SHAM. The comparison of kinetics 1 and 2 shows that 59% of the plastoquinol pool was reoxidized within the 3 s dark period compared to 58% in the control of Fig. 1A. (B) 1, algae incubated for 2 min with 1 mM PG. The comparison of kinetics 1 and 2 shows that 17% of the plastoquinol pool was reoxidized within the 3 s dark period compared to 59% in the presence of SHAM.

darkness was not altered by the presence of SHAM whereas it was strongly decreased by the presence of PG, though solvent conditions were equal. In similar experiments, azide did not affect the proportion of plastoquinol reoxidized in darkness (not shown). The same sensitivity to oxidase inhibitors was observed in *C. sorokiniana* S14 for the oxidation and reduction of the plastoquinone pool in darkness (not shown).

3.3. Sensitivity of plastoquinol oxidation and reduction to partial anaerobiosis

The effect of partial anaerobiosis was tested on the redox state of the plastoquinone pool in darkness and on the reoxidation of plastoquinol following reduction by light. For this purpose, algae were equilibrated with a mixture of air and argon. Dark-adapted algae were equilibrated at 1.45% air for 7 min and their light-induced fluorescence kinetics was recorded (Fig. 5, curve 1). The area over the fluorescence rise indicates that plastoquinone remained largely oxidized under these conditions. Following light reduction, the algae were incubated for 25 s in darkness and then submitted to a second illumination (Fig. 5, curve 2). The comparison of the area over the two fluorescence rises indicates that, during the 25 s dark time, 17% of the plastoquinol pool was reoxidized. On the other hand, 86% was reoxidized when the same experiment was performed at 2.55% air (not shown). Dark-adapted algae were equilibrated with various mixtures of air and argon for 7 min and their light-induced fluorescence was recorded. The comparison of the area over their fluorescence rises indicates that the plastoquinone pool remained largely oxidized down to 1.45% air and became completely reduced at 0% air (insert of Fig. 5). The kinetics of reoxidation of plastoquinol in darkness were measured for various mixtures of air and argon. For this purpose, after an equilibration with a given mixture of air and argon during 7 min, the algae were submitted to two illuminations separated by various dark periods. The extent of plastoquinol reoxidation reached during this dark time was deduced from the area over the two fluorescence rises and plotted as a function of the dark time (similar to Fig. 1B). The half-times (τ) of these kinetics are shown in the insert of Fig. 5. It is quite clear that upon decreasing the oxygen content in the mixture, the inhibition of the reoxidation of plastoquinol comes while the pool is still largely oxidized in darkness.

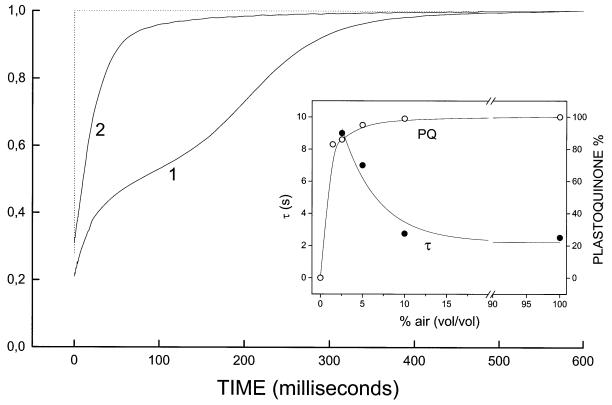


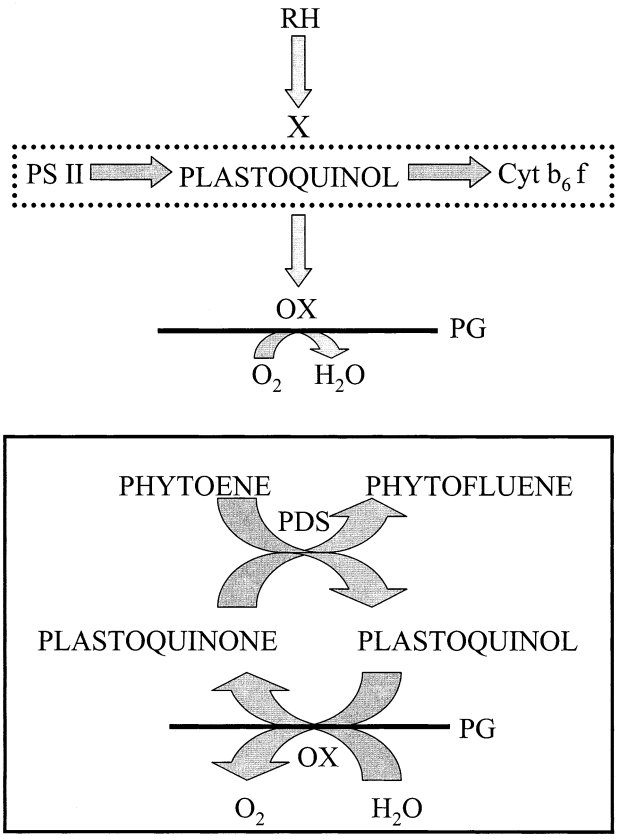
Fig. 5. Reoxidation of the plastoquinol pool in darkness following reduction by light. Fluorescence induction kinetics were measured in whole cells equilibrated for 7 min with a mixture of argon and air at 1.45% air. 1, dark adapted; 2, preilluminated for 600 ms to achieve full reduction of the plastoquinone pool and incubated in darkness for 25 s in order to allow reoxidation of plastoquinol. The comparison of kinetics 1 and 2 shows that 17% of the plastoquinol pool was reoxidized within the 25 s dark period. The kinetics of plastoquinol reoxidation were achieved by varying the dark time between the two illuminations, and the half-time τ of this kinetics was measured. This was repeated for various mixtures of argon and air and the insert shows the evolution of τ with the air content of the mixture. The insert also shows the evolution of the proportion of oxidized plastoquinone as a function of the air content of the mixture. This was deduced from the area over the fluorescence rise of the dark-adapted algae.

4. Discussion

4.1. Features of the plastoquinol:oxygen oxidoreductase

The plastoquinone pool during dark adaptation is submitted to a reduction by endogenous reductants and to an oxidation by molecular oxygen. The relative amount of oxidized plastoquinone present at a given time can be revealed by the chlorophyll fluorescence kinetics in mutants lacking the cytochrome b_6f complex. The oxygen consumption attributed to plastoquinol oxidation, that is to say to chlororespiration, can be deduced from these fluorescence kinetics. Such a method is invaluable since direct mea-

surements of oxygen concentration fail to distinguish the relative uptake due to mitochondrial respiration from that due to chlororespiration. Because of the occurrence of mitochondrial–chloroplast interactions [2], it has been difficult to prove that, as predicted by the model of chlororespiration, the oxygen consumption related to plastoquinone oxidation takes place in the chloroplast compartment rather than in the mitochondrial one. It was recently proposed that the plastoquinol:oxygen oxidoreductase predicted from chlororespiration was responsible for a light-induced photosystem II-dependent oxygen uptake sensitive to PG [9]. In order to reinvestigate this problem, we studied the effect of different oxidase inhibitors on the plastoquinol oxidation and reduction in darkness. According to the model of chlororespiration, a reduction of plastoquinone in darkness can indicate either an increase in the input of electrons to the plastoquinone pool or a decrease of their output, so that both possibilities should be permanently appreciated (Scheme 1). As previously mentioned, a reduction of the plastoquinone pool occurred when mitochondrial respiration was inhibited, because of the increase in the input of electrons to the pool. Indeed, a large reduction of plastoquinone was rapidly observed upon addition of a mixture of either azide plus SHAM or azide plus PG, indicating that both pathways of mitochondrial respiration need to be blocked (Fig. 2B,C). When these inhibitors were used independently, none of them induced a noticeable reduction of the plastoquinone because none of them was able to induce a complete block of the mitochondrial respiration. The combination of either azide plus SHAM or azide plus PG did not lead to additive effects on the redox state of the plastoquinone pool. This shows that the mitochondrial synthesis of ATP should decrease sufficiently before an appreciable increase of reducing power can occur in the chloroplast. Though both SHAM and PG did inhibit the mitochondrial alternative oxidase, there are some differences in their effects (Fig. 2). First, a mixture of azide plus PG induced a more pronounced reduction of the plastoquinone than did a mixture of azide plus SHAM. Second, unlike SHAM, PG induced a reduction of the plastoquinone in darkness upon increasing its concentration (Fig. 3). These observations support the conclusion that PG inhibited additionally the output of electrons



Scheme 1. Redox interactions of the plastoquinone pool. (Upper) The general scheme of chlororespiration. In the light, plastoquinone is a redox carrier of the photosynthetic electron transport chain located between photosystem II reaction centers (PS II) and cytochrome b_6f complex. In the dark, a complex X mediates the reduction of plastoquinone by an internal reductant RH. Plastoquinol is reoxidized at the expense of molecular oxygen via an oxidase OX sensitive to PG. In higher plants, the complex X is probably the chloroplast NADH:plastoquinone oxidoreductase. In algae, the nature of this complex is not known. One possibility is that the flavoprotein phytoene desaturase (PDS) would mediate this reduction (see below). (Lower) The link with carotenoid biosynthesis. Several steps of the desaturation process in the biosynthetic pathway of carotenoid synthesis require free energy: For instance the transition from phytoene to phytofluene achieved by PDS and associated with a reduction of plastoquinone. The oxidation of the plastoquinol is achieved by a plastoquinol:oxygen oxidoreductase (OX) sensitive to PG in an exergonic reaction. From a thermodynamic point of view, the desaturation step is made possible because it is coupled with the plastoquinol oxidation. This coupling can take place because both reactions share the plastoquinone pool as a common intermediate.

from the pool whereas SHAM did not, and that the concentration used (1 mM) was not saturating for this effect.

The rate of reoxidation of the plastoquinol formed after light reduction also depends on the rate of both the input and output of electrons prevailing in darkness. The inhibition of this dark reoxidation, induced by PG but not by SHAM (Fig. 4), is not due to a difference in the input because both drugs affect mitochondrial respiration in the same way and thus induce the same indirect effect on the input. It rather follows from a difference in the output of electrons from the pool. Contrary to the mitochondrial respiration, the dark reoxidation of the plastoquinone pool was not sensitive to azide (not shown). Since the pattern of inhibition of plastoquinone oxidation and reduction in darkness differs from that of mitochondrial respiration, mitochondrial oxidases cannot be responsible for the oxygen consumption linked to plastoquinol oxidation. It rather confirms the presence of a plastoquinol:oxygen oxidoreductase inhibited by PG but not by azide or SHAM. The same conclusion also applies to *C. sorokiniana* which showed the very same sensitivity to oxidase inhibitors as *C. reinhardtii*. However, it was previously reported that SHAM could induce a rapid reduction of the plastoquinone in *Chlorella pyrenoidosa* [1]. It is thus possible that the plastoquinol:oxygen oxidoreductase has different sensitivity to oxidase inhibitors according to species. One can notice in this respect that, in *A. thaliana*, single residues control the sensitivity of the alternative oxidase to SHAM independently of that to PG [23].

Our experiments show that plastoquinone remained largely oxidized in darkness down to 1.45% air (Fig. 5), indicating that mitochondrial respiration was operating properly at this relative oxygen concentration. On the other hand, following light reduction, the dark reoxidation of plastoquinol was inhibited below a level of 10% air. Thus, the oxygen concentration in a 1.45% air:argon mixture was limiting the plastoquinol oxidation but not the mitochondrial respiration. These results indicate the presence of a specific plastoquinol:oxygen oxidoreductase different from the mitochondrial oxidases. In agreement with a previous report [2], this oxidase has a lower affinity for oxygen than mitochondrial oxidases.

4.2. Plastoquinol oxidation in darkness and carotenoid biosynthesis

A plastidial protein, IM, homologous to mitochondrial alternative oxidases was identified in *A. thaliana* and in several plants. It was proposed that the desaturation steps involved in carotene biosynthesis (for instance the transition from phytoene to phytofluene) would be coupled to a reduction of plastoquinone and to a reoxidation of plastoquinol via the IM protein. Upon expression in *E. coli*, this IM protein conferred a cyanide-resistant but PG-sensitive electron transport to isolated membranes [9–13]. Since both chlororespiration and carotenoid biosynthesis would require a plastoquinol:oxygen oxidoreductase sensitive to PG, it is tempting to consider that both processes involve the very same oxidase, i.e. that plastoquinol oxidation in darkness reveals the process of carotenoid biosynthesis. In higher plants, the amount of the terminal oxidase IM detectable in thylakoid membranes of mature leaves is very low compared to that of the photosynthetic complexes [4–6]. Interestingly, the half-time of plastoquinol oxidation in darkness is in the order of 15 s in spinach and tobacco leaves (P. Joliot, personal communication). This very low rate of plastoquinol oxidation is similar to those observed in algae reaching the stationary phase and could relate to the low level of IM observed. It is not known whether the falloff in plastoquinol oxidation observed in algae during the stationary phase of growth (Fig. 1B) relates to a decreased level of IM. Western blots of IM during the growth cycle might answer this question and indeed an antibody raised against the IM protein of *A. thaliana* was shown to react with a thylakoid membrane preparation of *C. reinhardtii* [9]. However, this antibody might also react with the homologous mitochondrial alternative oxidase, and mitochondrial membranes from *C. reinhardtii* are known to co-purify with thylakoids [24]. Therefore, the question raised by this falloff remains open at present. We noticed previously that, following light reduction, plastoquinol oxidation in darkness proceeded very slowly in open-cell preparations in comparison with intact cells [1]. This behavior would fit well with that of an oxidase involved in a biosynthetic process, since such a process very likely requires co-factors that would be lost upon cell breakage. The tentative

identification of the terminal oxidase involved in carotenoid biosynthesis with that of chlororespiration will surely suggest many new experiments and raises the following considerations.

(i) As we saw, a high rate of plastoquinol oxidation in darkness was reported only in algae in the exponential growth phase. To avoid confusion, the term chlororespiration should be restricted to processes with a fast rate of oxidation (half-time of 1–3 s). One should keep in mind that, even in that case, the rate of oxygen uptake that is concerned is over one order of magnitude smaller than that due to mitochondrial respiration. The very low rates of plastoquinol oxidation observed in various cases (half-time over 10 s) might be due to processes of a different nature.

(ii) We mentioned that the proteins involved in the reduction of plastoquinone in darkness in algae remained unknown. Based on the present model, one could tentatively include among the possible candidates the desaturases involved in the pathway of carotene biosynthesis (Scheme 1). These flavoproteins transfer electrons from the carotene precursors to plastoquinone [13].

(iii) The present model is closer to the original model of chlororespiration than it may appear (Scheme 1). The steps of desaturation of the carotene precursors are coupled to a respiratory redox pathway, namely to the reduction of plastoquinone and the reoxidation of plastoquinol via a terminal oxidase IM. Moreover, this oxidase is homologous to the alternative oxidases involved in mitochondrial respiration. Last but not least, in non-photosynthetic tissues of higher plants, carotenoid accumulation requires respiratory redox pathways as well [13].

Respiratory processes are most generally devoted to the chemiosmotic synthesis of ATP, an energy source available for a wide variety of biological processes. This synthesis is a striking example of the high sophistication that biological processes can reach. However, energy can be directly recovered from respiratory redox pathways in less advanced ways. For instance, detoxification processes that eliminate a variety of harmful organic compounds make use of the sole reducing power involved in respiratory redox pathways. In carotenoid biosynthesis, the direct recovery of energy from respiratory redox pathways would not be so simple. The highly endergonic reaction of carotene desaturation would be permitted on account of its coupling to the highly exergonic reaction of plastoquinol oxidation (Scheme 1). Such a coupling requires a common intermediary to both reactions: in this case the plastoquinone pool.

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