THE ROYAL SOCIETY

doi 10.1098/rstb.2000.0705

Flexibility in photosynthetic electron transport: a newly identified chloroplast oxidase involved in chlororespiration

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Besides electron transfer reactions involved in the 'Z' scheme of photosynthesis, alternative electron transfer pathways have been characterized in chloroplasts. These include cyclic electron flow around photosystem I (PS I) or a respiratory chain called chlororespiration. Recent work has supplied new information concerning the molecular nature of the electron carriers involved in the non-photochemical reduction of the plastoquinone (PQ) pool. However, until now little is known concerning the nature of the electron carriers involved in PQ oxidation. By using mass spectrometric measurement of oxygen exchange performed in the presence of ^{18}O-enriched O_2 and *Chlamydomonas* mutants deficient in PS I, we show that electrons can be directed to a quinol oxidase sensitive to propyl gallate but insensitive to salicyl hydroxamic acid. This oxidase has immunological and pharmacological similarities with a plastid protein involved in carotenoid biosynthesis.

Keywords: chlororespiration; quinol oxidase; chloroplast; oxygen; *Chlamydomonas*

1. INTRODUCTION

During photosynthesis, two photosystems (PS II and PS I), coupled through an electron transfer chain, transform light energy to chemical energy. Besides this main electron transport pathway, called the 'Z' scheme of photosynthesis, alternative pathways such as cyclic electron transport around PS I (Arnon 1955; Heber & Walker 1992; Ravenel *et al*. 1994) and a respiratory chain called chlororespiration (Bennoun 1982; Peltier *et al*. 1987) have been identified in thylakoid membranes. Recent work has supplied some clues on the molecular properties of electron carriers involved in alternative pathways. First, a NAD(P)H dehydrogenase complex (Ndh), encoded by plastidial *ndh* genes, has been characterized in thylakoid membranes (Guedeney *et al*. 1996; Sazanov *et al*. 1998). Inactivation of *ndh* genes by plastid transformation was simultaneously performed by different laboratories (Burrows *et al*. 1998; Shikanai *et al*. 1998; Kofer *et al*. 1998; Cournac *et al*. 1998). It was shown that the Ndh complex is involved in the non-photochemical reduction of plastoquinones (PQ) occurring in the dark after a period of illumination and it was further suggested that this complex is involved in cyclic electron flow around PS I and in chlororespiration. Although not characterized at a molecular level, the existence of other activities, such as ferredoxin quinone reductase activity (Bendall & Manasse 1995; Endo *et al*. 1998) or non-electrogenic NAD(P)H dehydrogenase activity—different from the Ndh complex and involved in PQ reduction (Corneille *et al*. 1998)—have been reported in thylakoids.

If the nature of electron carriers involved in non-photochemical reduction of the PQ pool appears better understood, the nature of electron carriers involved in plastoquinol oxidation remains a subject of controversy. Recently, a homologue to mitochondrial alternative oxidase has been simultaneously characterized in *Arabidopsis* thylakoid membranes by two different laboratories (Carol *et al*. 1999; Wu *et al*. 1999). This enzyme, which is encoded by the nuclear gene *immutans*, has been shown to be essential during carotenoid biosynthesis and it was assumed that it might catalyse plastoquinol oxidation and be involved in chlororespiration. In contrast, based on experiments performed *in vitro*, Casano *et al*. (2000) recently proposed a chlororespiration model in which plastoquinol oxidation would be achieved by a plastidial peroxidase, H_2O_2 being used as an electron acceptor.

In order to elucidate the nature of the chlororespiratory oxidase, we have used photosynthetic mutants of the green alga *Chlamydomonas* and performed mass spectrometric measurements. Mass spectrometry, using ^{18}O-labelled O_2,

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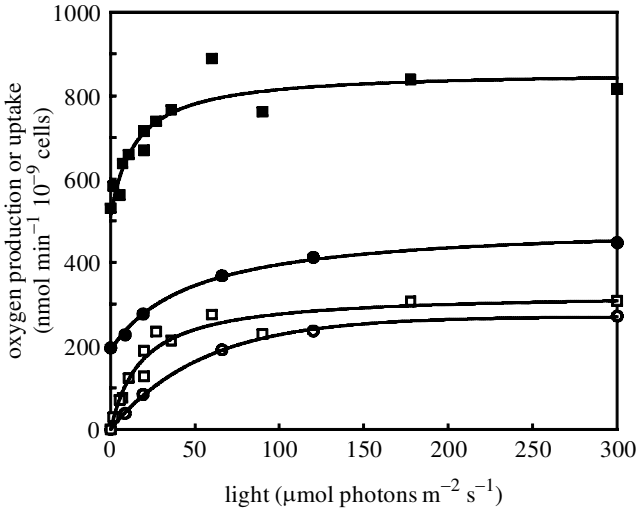

Figure 1. Photosynthetic oxygen evolution measured in intact *Chlamydomonas* cells. Measurements were performed in two independent PS-I-deficient strains showing different chlorophyll contents. Circles, mutant strain *psaAΔ*1, 0.44 mg chlorophyll 10^{-9} cells. Squares, mutant strain *psaBΔ*7, 2.3 mg chlorophyll 10^{-9} cells. Rates of PS II oxygen production (deduced from $^{16}O_2$ enrichment of the medium) and of oxygen uptake (deduced from $^{18}O_2$ depletion of the medium) are plotted versus the illumination intensity. Open circles, *psaAΔ*1 production; closed circles, *psaAΔ*1 uptake; open squares, *psaBΔ*7 production; closed squares, *psaBΔ*7 uptake.

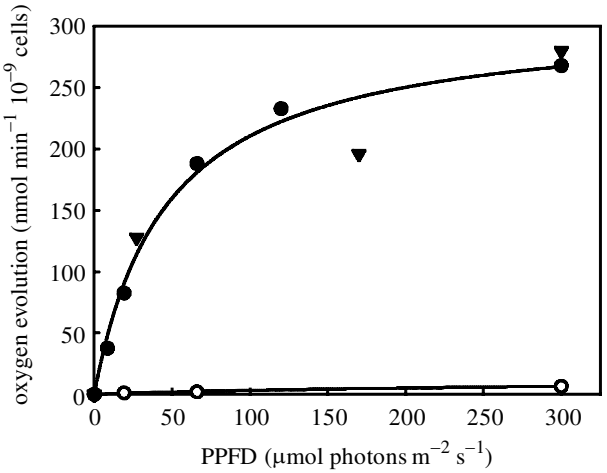

Figure 2. Effect of DCMU and loss of the cyt b_6f complex on the PS-II-driven O_2 production measured in intact *Chlamydomonas* cells using $^{18}O_2$ to label dissolved oxygen and monitoring $^{16}O_2$ (produced by PS II) and $^{18}O_2$ (taken up) with mass spectrometry. Closed circles, mutant strain *psaAΔ* deficient in PS I; open circle, mutant strain *psaAΔ* in the presence of 10 μM DCMU; closed triangles, double mutant *psaAΔ petAΔ* deficient in PS I and in the cyt b_6f complex.

is a powerful way to determine whether electrons produced at PS II (measured as unlabelled O_2 from water photolysis) are diverted towards O_2 or to another electron acceptor. By performing such measurements in *Chlamydomonas* preparations lacking either the PS I complex or the cytochrome (cyt) b_6f complex, we show that electrons provided by PS II can be diverted at a significant rate towards a chloroplast quinol oxidase. Based on the similarity of immunological (Cournac *et al*. 2000) and pharmacological properties between the *immutans* encoded plastid terminal oxidase (PTOX) in *Arabidopsis* and the plastoquinol oxidizing activity in *Chlamydomonas*, we propose the involvement of a quinol oxidase in chlororespiration.

2. EXPERIMENTAL PROCEDURES

Chlamydomonas reinhardtii cells were grown on a tris–acetate–phosphate medium (TAP). Algal cultures were maintained at 25 °C under continuous agitation and low illumination (about 1 μmol photons m^{-2} s^{-1}). The wild-type strain used in this work was isolated as a *mt*$^+$ segregant of a cross between two strains isogenic to the 137c strain (Harris 1989). The original deletions of *psaA* and *psaB* (chloroplast genes which encode essential subunits of PS I) were made in this strain as previously reported (Fischer *et al*. 1996). Marker recycling allowed subsequent transformations to delete the chloroplast *petA* gene, which encodes for an essential subunit of cyt b_6f (Cournac *et al*. 2000).

Prior to thylakoid isolation, the cells were harvested, centrifuged (600 *g*, 5 min) and washed once with 15 mM HEPES-KOH, pH 7.2. After centrifugation in the washing medium (600 *g*, 5 min), the pellet (around 5×10^8 cells) was resuspended in 10 ml buffer A (0.3 M sorbitol, 50 mM HEPES-KOH, pH 7.8, 2 mM EDTA, 5 mM $MgCl_2$) supplemented with 1% bovine serum albumin (BSA). Thylakoids were obtained through disruption in a French press chamber of the cells at 5000 psi in buffer A + 1% BSA (two runs). After disruption, broken or intact cells and heavy parts were discarded by centrifugation (600 *g*, 3 min). The supernatant was then centrifuged at 3000 *g*. The pellet (thylakoid fraction) was resuspended in 300–500 μl buffer A (without BSA) and stored on ice until used in the experiments. Oxygen exchange assays were conducted in buffer A without BSA.

Thylakoid membranes were resuspended in buffer A up to 1.5 ml in the measuring chamber. For measuring O_2 exchange on whole cells, algal cultures were harvested in exponential growth phase, centrifuged, washed and resuspended in buffer A. One and a half millilitres of the suspension was placed in the measuring chamber: a Clarke electrode-type thermostated and stirred cylindrical vessel (Hansatech, Norfolk, UK) fitted onto a mass spectrometer connecting device. Dissolved gases were directly introduced in the ion source of the mass spectrometer (model MM 14-80, VG instruments, Cheshire, UK) through a Teflon membrane as described in Cournac *et al*. (1993). For O_2 exchange measurements, the sample was sparged with N_2 to remove $^{16}O_2$, and $^{18}O_2$ (95% ^{18}O isotope content, Euriso-Top, Les Ulys, France) was then introduced to achieve an O_2 concentration in solution close to that in equilibrium with normal air. Light was supplied by a fibre-optic illuminator (Schott, Main, Germany) and neutral filters were used to vary light intensity. Unless specified, experiments shown here were performed at 300 μmol photons m^{-2} s^{-1} incident light. All gas exchange measurements were performed at 25 °C. The chloroplastic extracts were used as quickly as possible after extraction.

The portion of the *Arabidopsis immutans* cDNA coding for the entire mature peptide (PTOX) was PCR-amplified and inserted in the *Escherichia coli* expression vector pQE31 (Qiagen, Courtaboeuf, France) as described elsewhere (Cournac *et al*. 2000; Josse *et al*. 2000). The recombinant membrane protein PTOX which possesses a 6 His-tag was

Table 1. *Effect of electron acceptors on oxygen exchange*

(Measured in intact cells or thylakoids of the *psa AΔ* and *psaAΔ petAΔ* strains. *E*, photosynthetic O_2 evolution; δU, light-induced oxygen uptake (uptake in the light − uptake in the dark).)

	psaAΔ				*psaAΔ petAΔ*			
	nmol O_2 min^{-1} mg^{-1} chlorophyll				nmol O_2 min^{-1} mg^{-1} chlorophyll			
	intact cells		thylakoids		intact cells		thylakoids	
treatment	*E*	δU	*E*	δU	*E*	δU	*E*	δU
control	520	560	120	130	570	510	140	170
FeCN	620	640	670	80	550	590	170	160
DCBQ	980	10	900	0	1100	30	930	10

produced in *E. coli*. After induction, cells were lysed and membranes were recovered upon centrifugation at 100 000 *g* for 1 h. Pelleted membranes were resuspended in tris–HCl 0.2 M, pH 7.5, sucrose 0.75 M. Oxygen consumption was measured in a Clark O_2 electrode chamber (Hansatech). A typical assay contained 100 g membrane protein in the following buffer: tris–maleate 50 mM, pH 7.5, KCl 10 mM, $MgCl_2$ 5 mM, EDTA 1 mM, decyl-plastoquinone 0.2 mM.

3. RESULTS

PS-I-deficient algae obtained by inactivation of *psaA* or *psaB* genes were illuminated in the presence of ^{18}O-labelled O_2, and O_2 exchange was determined by mass spectrometry by following concentration changes in $^{18}O_2$ and $^{16}O_2$. As previously reported in nuclear mutants deficient in PS I (Peltier & Thibault 1988) or in plastid mutants (Cournac *et al*. 1997), significant O_2 evolution by PS II was measured, this phenomenon being accompanied by a simultaneous stimulation of O_2 uptake (figure 1). In these conditions, no change in the apparent respiration rate was observed, since light-dependent O_2 production and light-stimulated O_2 uptake are of the same amplitude. Light-dependent oxygen evolution was measured in different PS-I-deficient mutants. The maximal (light-saturated) activity was variable when expressed on a chlorophyll basis (from 120–600 nmol O_2 min^{-1} mg^{-1} chlorophyll), but was more constant when normalized to the cell number (250–350 nmol O_2 min^{-1} 10^{-9} cells) or to the protein amounts (8–13 nmol O_2 min^{-1} mg^{-1} protein), probably reflecting differences in chlorophyll contents between strains. Figure 1 shows O_2 exchange data in two strains with different chlorophyll contents. Comparable rates of maximal electron transfer activity were reached by both strains, but strains with higher chlorophyll contents were found to be more efficient at low light intensities. Note that the maximum rate of O_2 evolution in PS-I-deficient mutants represented about 10% of the maximal O_2 production rate measured in wild-type cells (not shown). The PS-II-dependent O_2 production was previously reported to be strongly affected by inhibition of mitochondrial respiration (Peltier & Thibault 1988; Cournac *et al*. 2000). However, we found that the light-driven activity of PS II was unaffected by the increase in respiration consecutive to acetate addition (data not shown) or by the level of basal respiration observed in different mutant strains (see figure 1). In contrast, the PS-II-dependent activity was found to vary during the algal cell cycle. Maximal activity was present during exponential growth, but severely decreased during the stationary phase (data not shown).

DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), an inhibitor blocking photosynthetic electron transfer between Q_A (the primary quinone acceptor of PS II) and Q_B (the secondary quinone acceptor, which exchanges with the PQ pool), strongly inhibited the PS-II-driven O_2 evolution (figure 2). Also, the PS-II-dependent electron flow was observed in the absence of the cyt b_6f complex in a *Chlamydomonas* double mutant *psaAΔ petAΔ* lacking both PS I and cyt b_6f (figure 2). Similar results were obtained in the single mutant (*petAΔ*) deficient in cyt b_6f or in the presence of 1 μM dibromothymoquinone (DBMIB), a cyt b_6f inhibitor (not shown). We conclude from these data that the PQ pool, but not the cyt b_6f complex, is involved in the PS-II-dependent pathway.

In order to determine the maximal PS II activity present in thylakoids of PS-I-deficient mutants, we measured photosynthetic O_2 evolution in the presence of artificial electron acceptors like 1,5-dichlorobenzoquinone (DCBQ) or potassium ferricyanide (FeCN) (table 1). In the presence of DCBQ, PS II activity was increased, indicating that PS II was not limiting the electron transport activity. In parallel, the light stimulation of O_2 uptake was completely suppressed. A similar effect was observed in whole cells and in a double mutant lacking PS I and the cyt b_6f complex (table 1). An increase in O_2 evolution was also observed in thylakoids of PS-I-deficient mutants when using FeCN as an electron acceptor. This effect was accompanied by a *ca*. 40% diminution of the light-induced stimulation of O_2 uptake (table 1). However, FeCN had no significant effect on the PS-II-dependent O_2 evolution in intact cells, which is explained by the fact that this compound cannot enter intact cells. Interestingly, FeCN has no significant effect on O_2 exchange rates measured in thylakoids from the *Chlamydomonas* strain lacking both PS I and the cyt b_6f complex (*psaAΔ petAΔ*, table 1).

A gene (*immutans*) encoding a plastid protein (PTOX) showing a high homology with the mitochondrial alternative oxidase, was recently discovered in *Arabidopsis thaliana* (Carol *et al*. 1999; Wu *et al*. 1999). As it was not easy to assay oxidase activity in *Arabidopsis* chloroplasts,

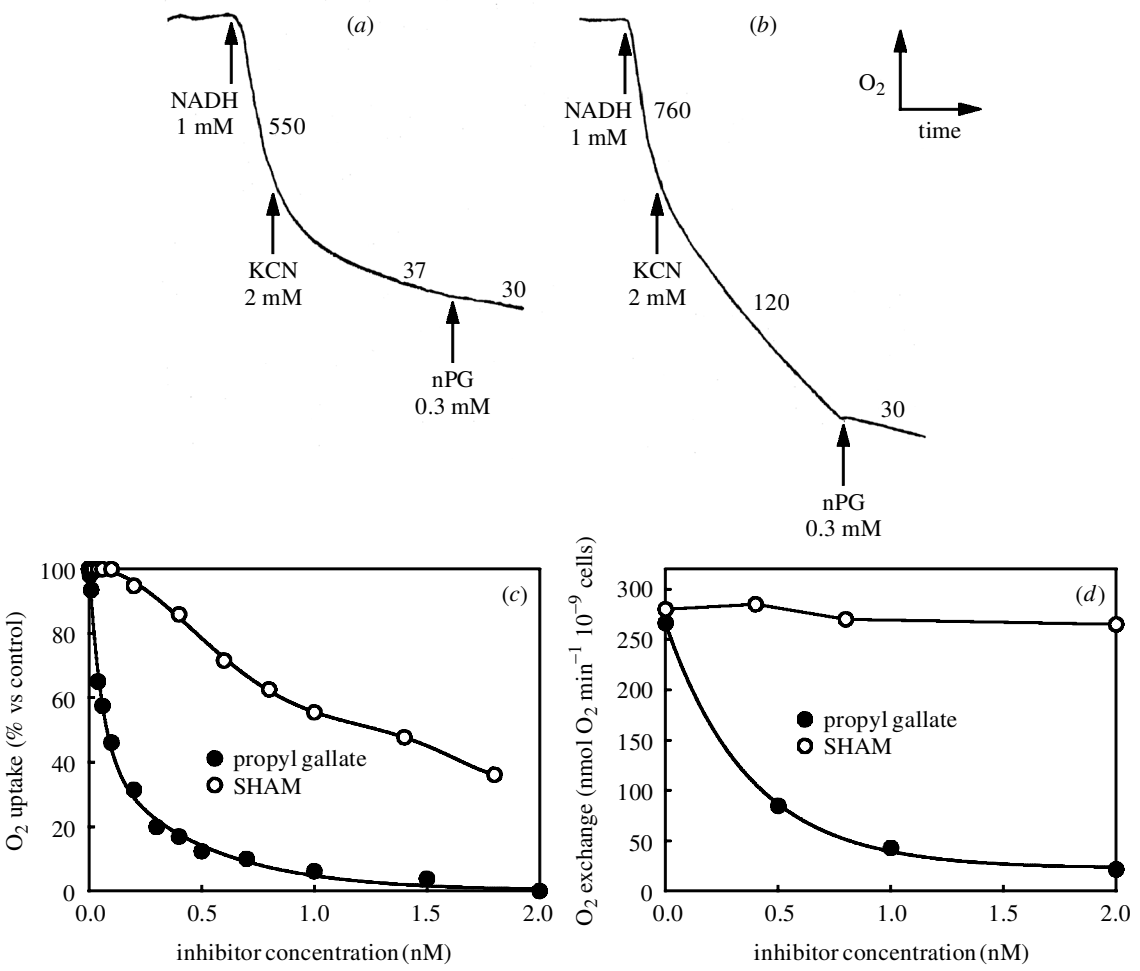

Figure 3. (*a*,*b*) Effects of sequential addition of KCN and propyl gallate on O_2 uptake induced by addition of NADH in membranes of *E. coli* from (*a*) control cells and (*b*) cells expressing the PTOX protein. O_2 uptake are given in nmol min^{-1} mg $protein^{-1}$. (*c*) Sensitivity of the PTOX-induced O_2 uptake to propyl gallate and SHAM in membranes of *E. coli*. (*d*) Sensitivity of PS-II-driven O_2 exchange to propyl gallate and SHAM in *Chlamydomonas* mutants deficient in PS I.

due to the low abundance of PTOX in chloroplasts and to the possible occurrence of mitochondrial cross-contamination, PTOX was produced as a recombinant protein in *E. coli*. After induction of the chimeric gene, the oxidase activity of membrane preparations was assayed by adding NADH and measuring oxygen consumption. KCN (1 mM) was used to inhibit oxygen consumption due to the cytochrome oxidase pathway (Josse *et al.* 2000). Expression of PTOX in *E. coli* membranes conferred a significantly higher cyanide-resistant oxygen consumption (figure 3*a*,*b*). Propyl gallate and salicylhydroxamic acid (SHAM) are well-known inhibitors of the mitochondrial alternative oxidase. The PTOX-dependent and cyanide-resistant oxidase activity was sensitive to propyl gallate (figure 3*a*–*c*), but at least ten times less sensitive to SHAM (figure 3*c*). The PS-II-dependent activity of PS-I-inactivated mutants showed comparable sensitivity to propyl gallate and was insentive to SHAM up to 2 mM (figure 3*d*).

4. DISCUSSION

(a) *Characteristics of photosynthetic electron transport in PS-I-deficient mutants*

In agreement with previous findings (Peltier & Thibault 1988; Cournac *et al.* 1997; Redding *et al.* 1999), results shown in this paper show that significant electron transport activity occurs from PS II to O_2 in PS-I-deficient *Chlamydomonas* mutants. Based on the effect of DCMU and on measurements performed in strains lacking the cyt b_6f complex, we conclude that the electron flow between PS II and molecular O_2 involves the thylakoid PQ pool, but not the cyt b_6f complex. Due to its electronic requirements and to its insensitivity to relative oxygen species (ROS) scavengers, PQ oxidation has been concluded to involve an enzymatic process reducing molecular O_2 into water (Cournac *et al.* 2000). As demonstrated here using an artificial electron acceptor for PS II (DCBQ), the activity of oxidase limits PS-II-dependent O_2 evolution in the absence of PS I. This explains why the maximal rates of O_2 evolution in PS-I-deficient cells are five to 20 times lower than that in wild-type cells, where PS I and cyt b_6f cooperate to reoxidize the PQ pool. However, light saturation curves of PS II activity indicate that PS-II-driven electron transport is limited by chlorophyll content at low light, and by oxidase content at high light. This suggests that oxygen uptake is not directly dependent on chlorophyll and is not related to chlorophyll photo-oxidation, further supporting the involvement of an enzymatic process in plastoquinol oxidation.

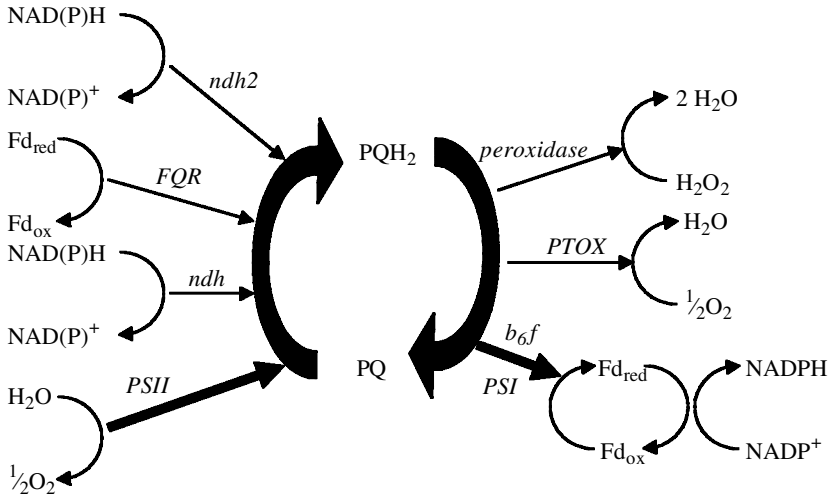

Figure 4. Schematic representations of the different plastoquinone (PQ) reduction and plastoquinol (PQH_2) oxidation pathways now evidenced in thylakoid membranes. Fd_{red}, reduced ferredoxin; Fd_{ox}, oxidized ferredoxin; ndh, complex I-like NAD(P)H dehydrogenase; ndh2, alternative NADH dehydrogenase; FQR, ferredoxin–quinone reductase; PTOX, plastid terminal oxidase (the quinol oxidase described in this paper).

Interestingly, we found that FeCN could accept electrons from PS II in PS-I-deficient strains containing cyt b_6f, but not in cyt b_6f-depleted strains. This shows that FeCN can interact with the intersystem photosynthetic electron transport chain, probably at the level of cyt *f* as previouly reported (Wood & Bendall 1976). This also indicates that the cyt b_6f complex of PS-I-deficient mutants keeps the ability to oxidize plastoquinol and to compete efficiently with the quinol oxidase.

The influence of various inhibitors has given us clues as to the nature of the chloroplast oxidase involved in this plastoquinol oxidation. Cyanide, which has been reported to impair chlororespiration (Buchel & Garab 1995; Bennoun 1982; Peltier *et al.* 1987) or cyanobacterial quinol oxidases (Howitt & Vermaas 1998; Buchel *et al.* 1998), had no effect unless very high concentrations were used (Cournac *et al.* 2000). The absence of effect of FeCN on plastoquinol oxidation in the cyt b_6f-deleted strain (table 1) also precludes the involvement of a soluble transporter such as soluble cytochromes, since FeCN can interact with such cytochromes, as shown in mitochondria (Hoefnagel *et al.* 1995).

(b) *Similarities between the* Chlamydomonas *plastoquinol oxidase and PTOX*

In plant mitochondria, quinol oxidation can be accomplished either by the cyt bc_1 complex (cyanide-sensitive pathway), or directly to molecular O_2 through an alternative oxidase (cyanide-insensitive pathway). Alternative oxidases have been reported to be inhibited by compounds such as SHAM or propyl gallate (Siedow 1980). We found that propyl gallate, but not SHAM, inhibited the PS II-to-O_2 electron flow in *C. reinhardtii* mutants deficient in PS I. Interestingly, Berthold (1998) reported the existence of different mutant forms of the *Arabidopsis thaliana* mitochondrial alternative oxidase that are resistant to SHAM but remain sensitive to propyl gallate, thus showing that sensitivity to these two inhibitors is separable.

Recently, two laboratories simultaneously reported the existence, in *Arabidopsis thaliana*, of a gene (*immutans*) coding for a plastid protein (PTOX) showing homology with mitochondrial alternative oxidases (Carol *et al.* 1999; Wu *et al.* 1999). Based on the phenotype of mutants affected in the *immutans* gene, it was concluded that PTOX is involved in carotenoid biosynthesis, more particularly in phytoene desaturation. The authors proposed a model in which PTOX would catalyse reoxidation of plastoquinol to PQ, using O_2 as a terminal acceptor. We have shown that PTOX, when expressed in *E. coli*, confers a KCN-insensitive quinol oxidase activity. In this assay, the plastid oxidase PTOX is sensitive to propyl gallate and much less sensitive to SHAM. Interestingly, PTOX appears to be more resistant to both inhibitors than mitochondrial alternative oxidase (Berthold 1998). Figure 3 indicates that both PTOX and the *Chlamydomonas* plastoquinol oxidase have similar sensitivities towards propyl gallate. Both activities show resistance towards SHAM, but PTOX appears significantly more sensitive. Differences in SHAM sensitivity can be explained by different hypotheses.

(i) The oxidases are not exactly the same, and the *Chlamydomonas* type is more resistant to SHAM.
(ii) The O_2 uptake in *E. coli* membranes is more sensitive to SHAM than in thylakoids, some modifications of its properties being induced by the expression system (a chimeric gene in a bacterial context).

Based on similar effects of inhibitors on PTOX and PS-II-driven electron flow, we conclude that the enzyme responsible for plastoquinol oxidation in *Chlamydomonas* is closely related to PTOX. This conclusion is further supported by immunological data (Cournac *et al.* 2000).

(c) *Oxygen, reactive oxygen species and chlororespiration*

We have concluded from our experiments that the major part of chloroplast O_2 uptake is due to the activity of a quinol oxidase that uses molecular O_2 as an electron acceptor and is sensitive to propyl gallate but insensitive to cyanide. Such a sensitivity to inhibitors appears contradictory to the involvement in chlororespiration of a cyanide-sensitive oxidase, as concluded by different authors (Bennoun 1982; Peltier *et al.* 1987; Buchel & Garab 1995). On the other hand, the use of molecular O_2 as a terminal acceptor is not consistent with the model of chlororespiration recently proposed by Casano *et al.* (2000). Indeed, based on experiments performed on an

in vitro reconstructed system, these authors proposed that plastoquinol oxidation was achieved by a plastid peroxidase using H_2O_2 as a terminal acceptor.

We cannot exclude at this stage the existence of different pathways of non-photochemical oxidation of plastoquinols, one involving a quinol oxidase and the other a peroxidase. According to Casano *et al*. (2000), the participation of a peroxidase might explain the cyanide sensitivity through an inhibition of superoxide dismutase. These different pathways might be differentially regulated depending on the environmental conditions. One might expect that the peroxidase pathway, provided that its existence is confirmed *in vivo*, would be associated with conditions generating ROS such as stress or senescence. On the other hand, PTOX would be involved in reactions occurring during the early biogenesis of chloroplasts (see Carol *et al*. 1999). This would be consistent with the higher plastoquinol oxidation activity observed during active phases of division. In this respect, it would be interesting to determine whether the peroxidase pathway is triggered during phases of senescence or in stress conditions.

It seems now likely that just as the non-photochemical PQ reduction pathways are diverse, so too are the chloroplastic O_2 (or ROS) uptake pathways (figure 4). Unravelling the molecular basis of these activities and their physiological significance will be an exciting task for the future.

The authors thank P. Carrier, Dr B. Dimon and J. Massimino (Commissariat à l'Energie Atomique) for skilful technical assistance throughout the experiments.

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Discussion

J. Barber (*Department of Biochemistry, Imperial College of Science, Technology and Medicine, London, UK*). Have you estimated the stoichiometric level of the PQ oxidase and the NAD complex in normal chloroplasts relative to the major complexes such as PS I, PS II and cyt b_6f?

G. Peltier. Sazanov *et al.* (1996) have estimated that the Ndh complex of pea chloroplasts represented less than 0.2% of total thylakoid membrane proteins (about one complex every 100 photosynthetic chains). It is therefore clearly a minor component of thylakoid membranes compared with the major complexes such as PS I, PS II or cyt b_6f. We have not yet estimated the amounts of the chlororespiratory oxidase present in thylakoid membranes. However, one may speculate that, like the Ndh complex, it represents a minor component of thylakoid membranes. This probably explains why these enzymes have not been discovered earlier.

K. Niyogi (*Department of Plant and Microbial Biology, University of California, Berkeley, USA*). Have you found any evidence for reverse electron flow through the Ndh complex?

G. Peltier. No, we have no evidence for this occurrence of reverse electron flow through the Ndh complex. Initially, the inhibition of the PS-II-dependent O_2 evolution by respiratory inhibitors observed in intact *Chlamydomonas* cells from PS-I-deficient mutants was interpreted by the generation of NAD(P)H through an energy-dependent reverse electron transfer occurring through a putative chloroplast Ndh complex and a transfer of reducing equivalents from the chloroplast to the mitochondria (Peltier & Thibault 1988). However, as shown here, and as recently published by Cournac *et al.* (2000), PS-II-dependent O_2 evolution could be measured in chloroplasts from PS-I-deficient mutants and was insensitive to respiratory inhibitors. We have concluded from these data that the PS-II-dependent O_2 evolution observed in PS-I-deficient mutants is due to a diversion of electrons towards a chloroplast oxidase. The inhibition of the PS-II-dependent electron flow by respiratory inhibitors would be explained by a competition between PS II and stromal donors for the reduction of the PQ pool. Moreover, it now seems clear that the plastid genome of most unicellular algae lacks *ndh* genes. In *Chlamydomonas*, non-photochemical reduction of the PQ pool is probably achieved by a non-electrogenic enzyme (for a review, see Cournac *et al.* 2000). This argues against the existence of a reverse electron flow, which would be only possible with an electrogenic complex. In higher plants, such a possibility cannot be excluded, since the Ndh complex is probably electrogenic, but no evidence for such a mechanism has been obtained until now.

C. H. Foyer (*Department of Biochemistry and Physiology, IACR-Rothamsted, UK*). The role of the alternative oxidase in the mitochondrial electron transport chains is considered to be prevention of over-reduction of the PQ pool and hence uncontrolled electron drainage to oxygen. Would you consider that a possible role of the chloroplast oxidase is to prevent over-reduction of the PQ pool and hence photoinhibition?

G. Peltier. Such a role should be considered. It is clear from our experiments that in PS-I-deficient *Chlamydomonas* mutants electrons can be diverted towards the chloroplast oxidase. Whether this reaction occurs *in vivo* in the presence of active PS I remains to be answered. One may speculate that in conditions where PS I is partially inhibited, for instance during introduction of photosynthesis, where electron acceptors are lacking, or during low temperature photoinhibition, diversion towards the oxidase may prevent over-reduction of the PQ pool.

A. Laisk (*Department of Plant Physiology, Tartu University, Estonia*). Is chloroplast Ndh a proton translocating enzyme? The background of my question is that with G. Edwards we measured quantum yields of C_4 plants and found them to be 15% higher than possible considering the known efficiency of cyclic electron transport. The discrepancy could be resolved with the assumption that proton-translocating Ndh participates in the cyclic electron flow in C_4-plant bundle-sheath chloroplasts.

G. Peltier. Based on the homology between plastid Ndh genes and bacterial genes encoding subunits of the NADH dehydrogenase complex, it seems likely that the chloroplast Ndh complex involved in chlororespiration and cyclic electron flow around PS I is a proton-translocating enzyme. In C_4 plants, Kubicki *et al.* (1996) have reported strong expression of Ndh genes in bundle-sheath chloroplasts. Possibly, the participation of such a proton-translocating complex to cyclic electron flow around PS I may explain increases in quantum yields.

H. C. P. Matthijs (*Department of Microbiology, University of Amersterdam, The Netherlands*). Professor Badger asked about the role of Ndh 1 in PS I cyclic, and pointed to the fact that Ndh 1, in addition to a role in PS I cyclic,

may be directly linked to CO_2 uptake. To this I added that in a Ndh-1-less mutant of the cyanobacterium *Synechocystis* which cannot grow in low CO_2 condition, growth on low CO_2 can be restored after (NaCl) stress. In this stress, PS I cyclic activity increases two- to three-fold, flavodoxin and FNR induction up to 20–30 times. This shows an intimate relationship between PS I cyclic and CO_2 uptake (Jeanjean *et al.* 1998).

G. Peltier. Our recent studies on Ndh-inactivated mutants (Horvath *et al.* 2000), have shown a role of the Ndh complex during photosynthesis under low CO_2 concentration, for instance during a stomatal closure induced by water limitation. Our interpretation is that under such conditions the requirement of photosynthetic CO_2 fixation for ATP is higher. To fix one CO_2, an ATP–NADPH ratio of 1.5 is needed under non-photorespiratory conditions, but under photorespiratory conditions this ratio increases up to 1.65. We proposed that cyclic electron flow around PS I mediated by the Ndh complex is a putative CO_2 concentrating mechanism similar to that occurring in cyanobacteria or algae. In this respect, the existence in the chloroplast genome of an open reading frame encoding a protein sharing homologies with a cyanobacterial and *Chlamydomonas* protein involved in CO_2 concentrating mechanisms is rather intriguing. However, until now, such a mechanism has not been evidenced in higher plant chloroplasts.

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