THE ROYAL SOCIETY

doi 10.1098/rstb.2000.0714

Chlororespiration

Peter J. Nixon

Department of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AY, UK (p.nixon@ic.ac.uk)

The term 'chlororespiration' is used to describe the activity of a putative respiratory electron transfer chain within the thylakoid membrane of chloroplasts and was originally proposed by Bennoun in 1982 to explain effects on the redox state of the plastoquinone pool in green algae in the absence of photosynthetic electron transfer. In his original model, Bennoun suggested that the plastoquinone pool could be reduced through the action of a NAD(P)H dehydrogenase and could be oxidized by oxygen at an oxidase. At the same time an electrochemical gradient would be generated across the thylakoid membrane. This review describes the current status of the chlororespiration model in light of the recent discoveries of novel respiratory components within the chloroplast thylakoid membrane.

Keywords: respiration; oxygenic photosynthesis; complex I; alternative oxidase; thylakoid; cyclic photophosphorylation

1. BACKGROUND

In the pioneering paper by Bennoun (1982), variations in the dark redox state of the plastoquinone pool were determined by measuring the area above the chlorophyll fluorescence induction curve in cells of a photosystem (PS)-I-less mutant of the green alga *Chlamydomonas reinhardtii*. In the absence of oxygen, the plastoquinone pool became reduced (figure 1). Oxidation of plastoquinol was inhibited by various inhibitors of terminal oxidases (carbon monoxide, cyanide and azide but not salicylhydroxamic acid (SHAM), an inhibitor of the mitochondrial alternative oxidase). In contrast, for *Chlorella pyrenoidosa*, SHAM but not cyanide and carbon monoxide, inhibited reoxidation of the pool. In open-cell preparations, both NADPH and NADH could reduce the pool. Such evidence was interpreted in terms of a thylakoid-associated respiratory chain consisting of components that were analogous to those found in the mitochondrial respiratory chain. At that time there was some indication for the presence of a thylakoidal NADH-specific dehydrogenase in *C. reinhardtii* (Godde & Trebst 1980), but with hindsight mitochondrial contamination of the preparation could not be totally ruled out (Atteia *et al.* 1992). Shortly thereafter Bennoun showed through the use of mutants that chlororespiration in *C. reinhardtii* did not rely on the activity of the thylakoid cytochrome b_6f (cyt b_6f) complex (Bennoun 1983).

Similar effects could be observed in the higher plant tobacco (Garab *et al.* 1989). Although there is now ample evidence for non-photochemical reduction of the plastoquinone pool in a range of higher plants (Groom *et al.* 1993) the original conclusions concerning the presence of thylakoid terminal oxidases are less convincing for the reasons summarized below (Gans & Rebeillé 1990; Bennoun 1994, 1998).

2. MITOCHONDRIAL–PLASTID INTERACTIONS AND THE PROBLEMS DETECTING CHLOROPLAST TERMINAL OXIDASES

The interpretation of the early experiments was based on the unstated assumption that chloroplasts and mitochondria were not in redox communication so that effects on the redox state of the ubiquinone pool in the mitochondrion were presumed not to be relayed to the thylakoid membrane. However, chloroplasts, like mitochondria, possess a number of protein transporters within the inner envelope membrane that enable the selective passage of metabolites into and out of the organelle (Flügge 1998). Although there is no translocator of NAD(P)H, reducing equivalents can be transported indirectly (Heineke *et al.* 1991). For instance the oxaloacetate–malate antiporter allows the transport of malate, which can be oxidized by NAD^+- and $NADP^+$-dependent malate dehydrogenases to yield NADH and NADPH, respectively. This ability to transport reductant across the inner chloroplast membrane means that the chloroplast is in metabolic communication with the rest of the cell so that redox events in the mitochondrion can be communicated to the stromal compartment and thence to the thylakoid membrane. The concept of chloroplast–mitochondrial interaction at the metabolic level has been given a wide variety of experimental support including the isolation of a photoautotrophic suppressor of a chloroplast ATPase mutant of *Chlamydomonas* which is unable to synthesize chloroplast ATP at the thylakoidal ATP synthase and has to rely on the import of mitochondrially generated ATP (Lemaire *et al.* 1988) as well as the reduction of photosynthetic oxygen evolution following inhibition of mitochondrial respiration (Krömer *et al.* 1988).

Further confirmation that the mitochondrion played a role in observations originally ascribed to chlororespiration came from using a strain of *C. reinhardtii* in which the

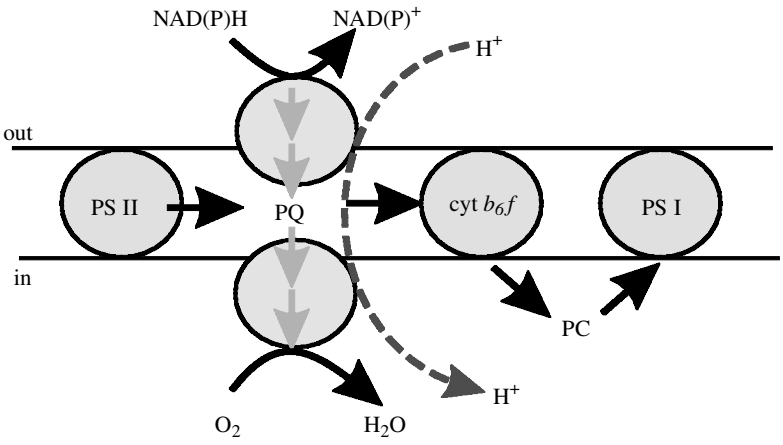

Figure 1. Original model for chlororespiration in *C. reinhardtii* as described by Bennoun (1982) and represented in Bennoun (1994). The transfer of electrons from NAD(P)H to O_2 is coupled to the translocation of protons. The putative respiratory chain and the photosynthetic electron transfer chain consisting of PS II, cyt b_6f, plastocyanin (PC) and PS I are connecting at the level of plastoquinone (PQ).

mitochondrial cytochrome bc_1 complex was resistant to myxathiazol (Bennoun 1994). Oxidation of the chloroplast plastoquinol pool in the dark was now found to be insensitive to myxathiazol suggesting that the inhibitors of the putative chloroplast oxidase were in fact inhibiting the mitochondrial oxidase which then caused the indirect reduction of the chloroplast plastoquinone pool. Because of mitochondrial–chloroplast interactions, mitochondrial oxidases are in principle able to drive the oxidation of the plastoquinone pool through reverse electron flow from plastoquinol to NAD(P)$^+$ at a proton pumping NAD(P)H dehydrogenase, with the reaction driven by the proton electrochemical gradient across the membrane. Alternatively inhibition of mitorespiration, which would lead to lowered levels of cellular ATP, may stimulate glycolysis in the chloroplast resulting in enhanced levels of NAD(P)H and an increase in the rate of reduction of the plastoquinone pool through the action of NAD(P)H dehydrogenase(s) (Gans & Rebeillé 1990). However, the plastoquinone pool still became reduced at low oxygen concentrations when mitorespiration was relatively uninhibited (Bennoun 1994). This observation prompted speculation that chloroplasts possess a terminal oxidase but with a lower affinity for oxygen than mitochondrial oxidases. Indeed, experimental support for such an oxidase has recently been obtained (Cournac *et al.* 2000). A further modification to the original scheme of chlororespiration is that chlororespiration in *C. reinhardtii* is now no longer thought to contribute to the thylakoid membrane potential in the dark. Instead a novel ATP-driven ion pump quite distinct from the ATPase may fulfil this role (Bennoun 1994; Rappaport *et al.* 1999).

3. A CURRENT MODEL FOR CHLORORESPIRATION IN HIGHER PLANT CHLOROPLASTS

Despite the fact that the original data (and possibly later results from higher plants) can no longer be interpreted unambiguously, there are now biochemical and genetic data to support the concept of chlororespiration. First, the so-called Ndh complex found in higher plant chloroplasts, but not yet in *Chlamydomonas*, appears to fulfil the role of the NAD(P)H dehydrogenase. Second, a protein, designated IMMUTANS, with strong sequence similarities to the alternative oxidases of plant mitochondria has been detected in chloroplasts of *Arabidopsis thaliana* (Wu *et al.* 1999; Carol *et al.* 1999). It is plausible that these two components together with plastoquinone may form a chloroplast respiratory chain (figure 2). Additional or alternative plastoquinone reductase and oxidase activities may be present depending on the species and physiological state.

4. RESPIRATORY COMPLEXES OF THE THYLAKOID MEMBRANE

(a) *The Ndh complex*

(i) *Identification of the Ndh complex*

The possibility that the chloroplast may contain an NADH dehydrogenase was given impetus in 1986 with the identification in two chloroplast genomes (Shinozaki *et al.* 1986; Ohyama *et al.* 1986) of open reading frames (ORFs) that showed significant sequence similarities to subunits of the mitochondrial and eubacterial respiratory NADH:ubiquinone oxidoreductase (also known as complex I or type I NADH dehydrogenase) (Fearnley & Walker 1992). These ORFs were consequently designated *ndh* genes (NADH dehydrogenase), which have now been identified in cyanobacteria and in the chloroplast genomes of some but not all green algae (Turmel *et al.* 1999). The absence of *ndh* genes from other sequenced plastomes could reflect transfer of *ndh* genes to the nucleus or deletion from the organism. In the case of black pine, of the 11 *ndh* genes usually found in land plants, four have been lost from the plastome and seven remain as pseudogenes (Wakasugi *et al.* 1994).

Despite little evidence at the time to suggest a role for the 11 *ndh* genes in encoding a chloroplast analogue of complex I (Fearnley & Walker 1992), recent biochemical (Sazanov *et al.* 1998*a*) and genetic (Burrows *et al.* 1998; Shikanai *et al.* 1998; Kofer *et al.* 1998) studies have provided strong evidence for this assignment. The low abundance of the Ndh complex in the chloroplast, estimated to be at 1.5% of the levels of photosystem (PS) II in tobacco (Burrows *et al.* 1998), helps to explain why it had escaped detection for so long.

The biochemical analysis of the Ndh complex is still at a preliminary stage. A 550 kDa Ndh complex with an associated NADH:ferricyanide oxidoreductase activity has been isolated from pea thylakoids (Sazanov *et al.* 1998*a*). Of the estimated 16 subunits, five have been so far assigned to plastid Ndh proteins on the basis of

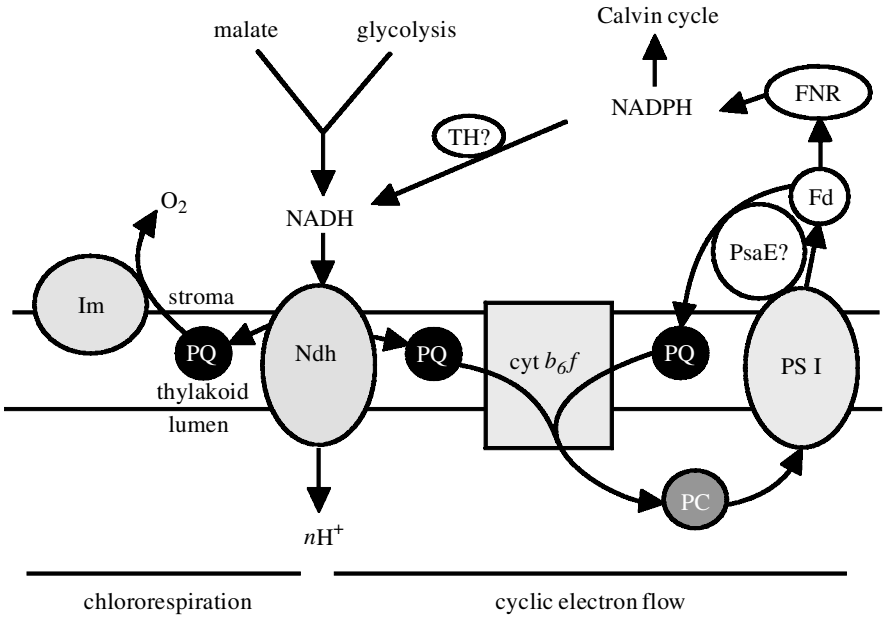

Figure 2. A possible dual role for the Ndh complex in both cyclic electron flow and chlororespiration (adapted from Sazanov *et al.* 1998*a*). Ndh-mediated cyclic electron flow around PS I involves ferredoxin (Fd), FNR, NADPH, NADH, the Ndh complex, plastoquinone, cyt b_6f, and plastocyanin (PC). nH^+, an unknown number of protons pumped. Electron transfer from NADPH to NAD^+ may be catalysed directly by a transhydrogenase (TH) or indirectly via substrate cycles. A ferredoxin-mediated cyclic pathway, possibly catalysed by the PsaE protein is also shown. In this scheme, chlororespiration is considered to involve the Ndh complex and IMMUTANS (Im) although other components cannot be excluded.

immunoblotting and microsequencing. More recently, an NADH-specific dehydrogenase has been immunopurified from barley using antibodies specific for NdhA (Casano *et al.* 2000) but has yet to be characterized in detail. For cyanobacteria, a 376 kDa hydrophilic subcomplex of the Ndh complex, which displays an NADPH:ferricyanide oxidoreductase activity, has been isolated from *Synechocytis* PCC 6803 (Matsuo *et al.* 1998). Of the nine subunits present, only NdhH could be assigned. The degree of similarity in subunit composition between the cyanobacterial and plastid Ndh complexes awaits clarification.

(ii) *Substrate specificity of the Ndh complex* in vitro *and* in vivo

The enzyme activity of the Ndh complex has been assayed mainly through the use of pyridine nucleotides as electron donors and artificial electron acceptors such as water-soluble quinones and ferricyanide, which probably accept electrons from the iron–sulphur clusters within the complex rather than from the natural quinone binding site (Sazanov *et al.* 1998*a*). Reduction of plastoquinone is, however, suggested from experiments involving reconstitution of the Ndh complex into liposomes (Casano *et al.* 2000).

Biochemical characterization of the Ndh complex has proved difficult because of its low abundance in chloroplast thylakoid membranes, its lability, the lack of a convincing inhibitor, possible mitochondrial contamination in chloroplast samples and the presence in thylakoid membranes of multiple NAD(P)H dehydrogenase activities, including a high level of NADPH:ferricyanide oxidoreductase activity due to FNR (ferredoxin:$NADP^+$ oxidoreductase). This latter difficulty may explain why FNR has been suggested to be a component of the Ndh complex (Guedeney *et al.* 1996). Consequently it is extremely difficult to assign enzyme activities within the thylakoid membrane to the Ndh complex without additional purification or verification. The instability of the Ndh complex also raises the possibility that the activity of the Ndh complex and its possible role in cyclic photophosphorylation may have been overlooked in early experiments.

Attempts to study the Ndh complex in tobacco thylakoid membranes, for which there are engineered *ndh* knock-out mutants available to help assign the activities of the complex, have proved difficult because of the apparent instability of the complex (Endo *et al.* 1998). Such behaviour may explain why reduction of the plastoquinone pool in isolated higher plant thylakoid membranes using NAD(P)H is so low (Rich *et al.* 1998).

The nature of the stromal reductant oxidized by the isolated detergent-solubilized plastid Ndh complex, of different degrees of purification, is still under debate with there being evidence for a specificity for NADH (Sazanov *et al.* 1998*a*; Elortza *et al.* 1999; Casano *et al.* 2000), NADPH (Guedeney *et al.* 1996) or both NADH and NADPH (Quiles & Cuello 1998; Funk *et al.* 1999). For an isolated subcomplex of the *Synechocystis* 6803 Ndh complex, a specificity for NADPH has been reported (Matsuo *et al.* 1998) while for the complex in thylakoid membranes, NADPH, NADH and reduced ferredoxin have all been implicated as substrates (Mi *et al.* 1995).

It is possible that *in vivo* the Ndh complex may be rather promiscuous, interacting with a number of different reductants (including for example reduced ferredoxin) depending on plant species and physiological state. The resulting holocomplex(es) containing the plastid *ndh* gene products plus the as yet uncharacterized electron input module(s) (Friedrich *et al.* 1995) may be rather

unstable. The identity of the subunits that constitute the subcomplex involved in NAD(P)H oxidation is particularly intriguing. As yet no obvious homologues of the NADH-binding subunits found in other complex I species have been identified (Friedrich *et al.* 1995).

(iii) *Phenotype of plastid* ndh *mutants*

A number of plastid *ndh* knock-out mutants have been constructed in tobacco (Burrows *et al.* 1998; Shikanai *et al.* 1998; Kofer *et al.* 1998). Despite initial controversy (Maliga & Nixon 1998; Koop *et al.* 1998), the consensus now appears that under normal growth conditions loss of the Ndh complex does not result in an obvious growth defect (Burrows *et al.* 1998; Shikanai *et al.* 1998). Possibly other activities can substitute for the Ndh complex under these growth conditions. Plastid *ndh* mutants do, however, appear to be more sensitive to the effects of high light damage, the reason for which is not yet clear (Endo *et al.* 1999).

(iv) *A possible dual role for the Ndh complex*

The probable activity of the Ndh complex as an NAD(P)H:plastoquinone oxidoreductase has led to the idea that it may have a dual function in photosynthetic systems (Burrows *et al.* 1998). In the light, one role for the Ndh complex may be to catalyse cyclic electron flow around PS I (either directly by providing an electron transfer pathway or indirectly through appropriate redox poising of the intersystem electron transfer chain to allow ferredoxin-mediated cyclic electron transfer), whereas in the dark, the Ndh complex may function in chlororespiration. Figure 2 summarizes the possible electron pathways involving an NADH-specific Ndh complex of the chloroplast. By analogy to the situation in purple bacteria, it is also possible that the Ndh complex may also catalyse the reverse reaction in which the plastoquinol-mediated reduction of $NAD(P)^+$ to NAD(P)H is coupled to the proton-motive force.

Experimental data to support a role in cyclic electron flow first came from studies on cyanobacterial *ndh* mutants (Mi *et al.* 1992; Yu *et al.* 1993) and more lately from analysis of chloroplast *ndh* mutants (Burrows *et al.* 1998; Shikanai *et al.* 1998). However, the convergence of respiratory and cyclic electron flows at the plastoquinone pool means that it is difficult to determine the contribution of each of these pathways to the reduction of PS I.

Further circumstantial evidence to support a role for the Ndh complex in cyclic electron flow has come from the finding that Ndh proteins are located in the stromal lamellae close to PSI (Nixon *et al.* 1989) and show elevated levels in the bundle sheath cells of C_4 plants, which lack PSII and carry out high levels of cyclic photophosphorylation (Kubicki *et al.* 1996). In this latter case, it is possible that a significant electron transfer pathway involves the oxidation by the Ndh complex of NAD(P)H derived from malate dehydrogenation rather than the oxidation of NAD(P)H produced by PS I activity. A role for the Ndh complex outside photosynthesis is also suggested from its detection in non-photosynthetic tissue in higher plants (Berger *et al.* 1993). Evidence for a role of the Ndh complex in the reduction of the plastoquinone pool in the dark has come from analysis of tobacco *ndh* mutants subjected to heat stress (Sazanov *et al.* 1998*b*).

(b) *Other plastoquinone reductases*

In chloroplast thylakoid membranes the reduction of the plastoquinone pool by NADPH is stimulated by ferredoxin (Mills *et al.* 1979; Rich *et al.* 1998). Rather than an Ndh-catalysed reaction, the possible pathway may involve the membrane-bound FNR-mediated reduction of ferredoxin by NADPH followed by reduction of the plastoquinone pool by a so-far uncharacterized ferredoxin-quinone reductase (FQR) activity. In principle NADPH can be produced in the dark in the chloroplast by the pentose phosphate pathway. The contribution of FQR to chlororespiration is still hypothetical and requires a greater understanding of FQR and its regulation in the dark.

Also thought to be associated with the thylakoid membrane is a glycolate:quinone oxidoreductase (Goyal & Tolbert 1996), a succinate dehydrogenase (Willeford *et al.* 1989) and an Ndh-independent NADH dehydrogenase (Cornac *et al.* 1998). Further work is needed to clarify these activities.

(c) *IMMUTANS: a possible chloroplast thylakoid alternative oxidase*

An important clue in understanding the molecular basis of chlororespiration has come recently from the study of *immutans* a variegated mutant of *Arabidopsis thaliana* (Wu *et al.* 1999; Carol *et al.* 1999). The *IMMUTANS* gene product shows a high degree of sequence similarity to mitochondrial alternative oxidases, is targeted to chloroplast membranes and displays a cyanide-resistant oxidase activity upon expression in *E. coli* consistent with a role as a thylakoid quinol oxidase (Cournac *et al.* 2000). The interesting phenotype observed in the *IMMUTANS* mutant is thought to be a reflection of the ability of chloroplasts within sectors of the plant to be able to desaturate phytoene in the carotenoid biosynthetic pathway, a step that requires oxidized plastoquinone as an electron acceptor. In the white sectors the lack of IMMUTANS leads to an overreduced plastoquinone pool in developing chloroplasts and a reduced ability to synthesize carotenoid, which under the prevailing light conditions causes photo-oxidative damage and bleaching of pigment. The green sectors also lack IMMUTANS but are thought to survive the light stress sufficiently well to accumulate PS I (or other oxidants of the plastoquinone pool) and so allow carotenoid synthesis to proceed, which further protects the chloroplast from light damage. On the basis of immunoblotting experiments, an IMMUTANS homologue is also thought to be located in the chloroplast of *C. reinhardtii* (Cournac *et al.* 2000). The presence of IMMUTANS transcripts in roots also would argue for a role in other plastid types (Carol *et al.* 1999). Recently a plastoquinol peroxidase has also been suggested to be a component of chlororespiration (Casano *et al.* 2000). Whether IMMUTANS also has a peroxidase activity deserves further attention.

(d) *Other possible thylakoid reductases and terminal oxidases predicted from analysis of cyanobacteria*

Examination of the complete genome sequence for the cyanobacterium *Synechocystis* PCC 6803 has revealed a number of potential plastoquinone reductases and oxidases that may have homologues in chloroplasts. In addition to the *ndh* genes, there are three ORFs encoding

potential type 2 NADH dehydrogenases, designated *ndbA-C* (Howitt *et al.* 1999). This class of dehydrogenase consists of single subunits, lacks iron–sulphur centres and does not pump protons across the membrane. Because the activity of the *Synechocystis ndb* gene products appears to be low under laboratory growth conditions it has been suggested that they may act as redox sensors and serve a regulatory function (Howitt *et al.* 1999). Succinate dehydrogenases are also found in *Synechocystis* 6803 (Cooley *et al.* 2000) and as mentioned above possibly in the chloroplast of green algae (Willeford *et al.* 1989).

The genome of *Synechocystis* 6803 also contains three sets of genes for terminal respiratory oxidases: a cytochrome aa_3-type cytochrome *c* oxidase (CtaI), a possible cytochrome *bo*-type quinol oxidase (CtaII) and a putative cytochrome *bd* quinol oxidase (Cyd). Recent conclusions based on the analysis of engineered mutants are that CtaI is the major oxidase in thylakoid membranes, Cyd is mainly located in the cytoplasmic membrane and that CtaII plays only a minor role in cellular respiration under the conditions tested (Howitt & Vermaas 1998). Interestingly there is no obvious cyanobacterial homologue of IMMUTANS.

(e) *Role of plastid respiratory complexes*

(i) *Photoprotection*

Recent studies on a tobacco *ndhB* null mutant indicate a role for the Ndh complex in protecting tobacco from extreme high light (Endo *et al.* 1999). The mechanism is unknown but may reflect the ability of the Ndh complex to oxidize stromal reductant and thus act as an emergency electron sink for photosynthetic electron transport, which, unless present, would lead to the generation of reactive oxygen species (ROS) in the stroma. Interestingly expression of the NdhA subunit increases in response to photo-oxidative stress (Martín *et al.* 1996).

Under moderate light intensities but conditions of water stress, when the levels of electron acceptors for linear flow are limited, chloroplast *ndh* mutants also show a reduced ability to quench fluorescence non-photochemically during the early stages of the induction of photosynthesis in dark-adapted plants (Burrows *et al.* 1998). Such behaviour is consistent with a role for the Ndh complex under conditions when the Calvin cycle enzymes are not fully activated and there is a build up of reductant in the stroma. Cyclic electron flow (or perhaps chlororespiration) under these conditions would contribute to the pH gradient across the thylakoid enhancing energy-dependent quenching (q_E), thus downregulating PS II, as well as removing stromal reductant, which could lead to the generation of ROS.

Since mutants depleted in the mitochondrial alternative oxidase are known to produce enhanced levels of ROS (Maxwell *et al.* 1999), it is possible that IMMUTANS may also act as an emergency electron sink, in this case to prevent overreduction of the plastoquinone pool and concomitant damage to PS II.

(ii) *Regulation*

The reduction state of the plastoquinone pool plays an important role in the regulation of photosynthetic electron transport at many levels from plastid gene expression (Pfannschmidt *et al.* 1999) to the distribution of light energy between the two photosystems (in so-called state transitions). The ability of stromal reductant to reduce the plastoquinone pool via respiratory complexes may thus allow the metabolic needs of the cell to be coordinated with photosynthetic electron transport. For instance when the ratio of ATP to NADPH produced by the light reactions needs to be increased, such as to accommodate a change in the nitrogen source available for algal growth, a state transition is induced in the dark to favour cyclic electron transport around PS I upon re-illumination (Turpin & Bruce 1990).

The pyridine nucleotide pool and plastoquinone also act as coenzymes in a number of metabolic steps and so need to be regenerated. Thus the Ndh complex may have a role in recycling $NAD(P)^+$ during for instance starch mobilization, and IMMUTANS is probably needed to reoxidize plastoquinol for carotenoid biosynthesis and possibly other biosynthetic steps.

(iii) *Energy transduction*

For *C. reinhardtii* chloroplasts, where there is little evidence for the presence of the Ndh complex, chlororespiration may involve a homologue of IMMUTANS and a non-proton-pumping NADH dehydrogenase. Such a chain may be poorly electrogenic and so contribute little to ATP synthesis in the dark (Cournac *et al.* 2000). For higher plant chloroplasts where the Ndh complex is probably able to contribute to the proton-motive force, a role in ATP production in the dark or the maintenance of a pH gradient is more likely. The rate of chlororespiratory electron transfer in the dark is, however, quite small with rates in sunflower estimated to be at only about 0.3% of the light-saturated photosynthetic electron flow (Feild *et al.* 1998). However, this level may still be important physiologically, for example, in the dark recovery of plants from photoinhibition through *de novo* protein synthesis. Preliminary support for this idea has come from analysis of tobacco *ndh* mutants (Burrows 1998).

Chlororespiration is unlikely to make a significant contribution to ATP synthesis in the light in mature chloroplasts but in immature or non-photosynthetic plastids, the contribution may become important. However, the Ndh component of the chain may have a role in cyclic electron flow in the light as described above, possibly directly or by poising the intersystem electron transfer chain in an appropriate state for cyclic electron transfer via ferredoxin-mediated pathways.

5. CONCLUSIONS AND FUTURE PROSPECTS

The recent identification of the Ndh complex and IMMUTANS provides the first strong biochemical evidence in favour of the presence of both a respiratory dehydrogenase and oxidase within the thylakoid membrane of higher plant chloroplasts. Whether they can function together as a true respiratory chain requires verification. Analysis of chlororespiration has been and will continue to be hindered by the low abundance of the respiratory complexes in chloroplasts and the difficulty of excluding direct and indirect effects of mitorespiration. Given that IMMUTANS and the Ndh complex are found in non-green tissues, it may be more appropriate to use the term plastid respiration rather than chlororespiration

to describe this phenomenon. Future work must be directed at characterizing the subunit composition and structure of these and other respiratory complexes, their substrate specificity and regulation, and their physiological importance.

The author thanks the Biotechnology and Biological Sciences Research Council and The Royal Society for financial support.

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