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Inhibitor studies on non-photochemical plastoquinone reduction and H_2 photoproduction in *Chlamydomonas reinhardtii*

Florence Mus, Laurent Cournac*, Véronique Cardettini, Amandine Caruana, Gilles Peltier

CEA Cadarache, DSV DEVM Laboratoire d'Ecophysiologie de la Photosynthèse, UMR 6191 CNRS-CEA, Aix-Marseille II, F-13108 Saint-Paul-lez-Durance Cedex, France

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Abstract

In the absence of PSII, non-photochemical reduction of plastoquinones (PQs) occurs following NADH or NADPH addition in thylakoid membranes of the green alga *Chlamydomonas reinhardtii.* The nature of the enzyme involved in this reaction has been investigated in vitro by measuring chlorophyll fluorescence increase in anoxia and light-dependent O_2 uptake in the presence of methyl viologen. Based on the insensitivity of these reactions to rotenone, a type-I NADH dehydrogenase (NDH-1) inhibitor, and their sensitivity to flavoenzyme inhibitors and thiol blocking agents, we conclude to the involvement of a type-II NADH dehydrogenase (NDH-2) in PQ reduction. Intact *Chlamydomonas* cells placed in anoxia have the property to produce H_2 in the light by a Fe-hydrogenase which uses reduced ferredoxin as an electron donor. H_2 production also occurs in the absence of PSII thanks to the existence of a non-photochemical pathway of PQ reduction. From inhibitors effects, we suggest the involvement of a plastidial NDH-2 in PSII-independent H_2 production in *Chlamydomonas*. These results are discussed in relation to the absence of *ndh* genes in *Chlamydomonas* plastid genome and to the existence of 7 ORFs homologous to type-II NDHs in its nuclear genome.

Keywords: Hydrogen production; Photosystem II; NADH dehydrogenase; Plastoquinone; *Chlamydomonas reinhardtii*

1. Introduction

In anaerobic conditions, the green alga *Chlamydomonas reinhardtii* is able to produce H_2 in the light thanks to the activity of a chloroplast Fe-hydrogenase, which catalyzes the reversible reduction of protons to H_2, using ferredoxin (Fd) as a redox partner [1]. This hydrogenase is inactivated by O_2 and its expression requires anaerobiosis [2]. H_2 photoproduction can be driven by electrons supplied by photochemical water splitting at PSII, which results in simultaneous production of H_2 and O_2 [3]. In this case, H_2 photoproduction is a transitory process which is inhibited by O_2 generated at PSII. Recently, it was shown that, in response to sulfur deprivation, H_2 photoproduction is induced and sustained during several days [4]. In such conditions, activity of oxygenic photosynthesis declines below the level of respiration, allowing *Chlamydomonas* cell cultures to reach anoxia. When PSII is inhibited, H_2 photoproduction can be driven by metabolic oxidation of organic compounds [5–7]. This pathway involves the PQ pool and the cytochrome (cyt) b_6f complex [8]. Both (PSII dependent and PSII independent) pathways of H_2 photoproduction require PSI activity [9]. Different electron carriers such as NAD(P)H [10], succinate [11] or reduced Fd [12] have been proposed to mediate the non-photochemical reduction of the PQ pool. Fd being the donor for the hydrogenase, PQ reduction by Fd cannot contribute to H_2 photoproduction. Consequently, NAD(P)H or succinate could be potential electron sources for PSII-independent H_2 production in chloroplast. Chlorophyll fluorescence measurements performed on open cells preparations [13], or oxygen exchange measurements performed on thylakoid suspensions [10], suggested the existence of a thylakoid

* Corresponding author. Tel.: +33 4 42 25 43 66; fax: +33 4 42 25 62 65.
E-mail address: laurent.cournac@cea.fr (L. Cournac).

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membrane-localized NAD(P)H oxidoreductase activity in *C. reinhardtii*. Evidence of an interaction of NAD(P)H with the photosynthetic electron transport chain has also been obtained in vivo by monitoring light-induced variations of blue-green fluorescence in photosynthetic mutants [14]. The NAD(P)H-PQ oxidoreductase has been reported to take part in chlororespiration [13,15] and in cyclic electron transport around PSI [16]. The nature of the enzyme reducing the PQ pool and the nature of the soluble stromal electron donor(s) remain to be established.

In higher plants chloroplasts and plastids, the presence of a functional NAD(P)H dehydrogenase, homologous to mitochondrial or bacterial complex I, has been demonstrated [17–20]. Most subunits of this complex are encoded by plastid genes [21,22]. Despite the existence of a NAD(P)H-PQ oxidoreductase activity in *C. reinhardtii* thylakoid membranes, homologues of chloroplast *ndh* genes could neither be detected in its chloroplast (NCBI accession no: NC_005353) [23] or nuclear (http://www.jgi.doe.gov/; *C. reinhardtii* release 2.0, February 14th, 2004) genomes. Bacteria [24,25], cyanobacteria [26–28], and mitochondria from various organisms, especially plants [29–32] and fungi [33–35], but not animals, contain alternative rotenone-insensitive NADH-quinone oxidoreductases (also called type-II NADH dehydrogenases or NDH-2s). In contrast with complex I, NDH-2s are monomeric enzymes and do not transfer protons across the membrane. Based on inhibitor effects, it has been proposed that, in addition to NDH-1, an NDH-2 activity could be present in higher plant chloroplasts [36].

In the present work, we investigated the nature of the enzyme involved in the non-photochemical reduction of PQ in *Chlamydomonas reinhardtii* by measuring electron transport activity in vitro and photobiological production of H_2 in vivo. Based on substrate specificity and on the effect of inhibitors, we conclude to the involvement of an NDH-2.

2. Results

2.1. Measurement of a NAD(P)H dehydrogenase plastoquinone reductase activity in Chlamydomonas thylakoids

PSII-independent electron flow from exogenous electron donors to PSI was assayed in *C. reinhardtii* thylakoid membranes by measuring O_2 uptake rates in the presence of methyl viologen (MV, a self-oxidizing PSI acceptor) and DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea). In these experimental conditions, PQ pool cannot be reduced by PSII which is inhibited by DCMU, and the light-dependent O_2 uptake catalyzed by MV reflects the ability of PSI to reduce MV using intersystem electrons donors. In the following experiments, myxothiazol (2 μM, a cyt *bc* inhibitor) and salicylhydroxamic acid (SHAM 0.8 mM, a mitochondrial alternative oxidase inhibitor) were added in order to prevent respiratory O_2 uptake from possible contaminating mitochondria. In these conditions, several electron donors (succinate, malate, glycolate, NADH, NADPH) were tested for their ability to provide reducing equivalents to the intersystem electron transport chain. Only NADH (Fig. 1A) and NADPH (not shown) were found to induce a significant light-driven O_2 uptake in the presence of DCMU and MV. A similar experiment was performed in the absence of DCMU using thylakoids from a PSII-deficient mutant of *C. reinhardtii* (FUD7), and similar O_2 exchange patterns were

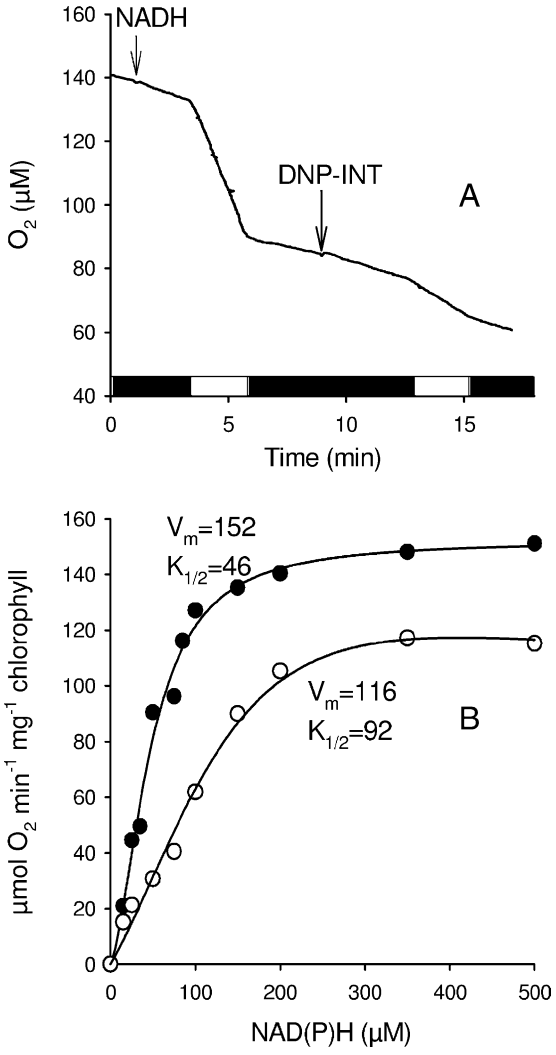

Fig. 1. Effect of exogenous addition of NAD(P)H on DNP-INT sensitive, light dependent, PSII-independent electron flow. O_2 uptake measurements on *C. reinhardtii* thylakoid membranes were performed with a Clark electrode in presence of MV, SOD, catalase, DCMU, myxothiazol and SHAM. (A) Effect of an addition of NADH (200 μM) and DNP-INT (10 μM). Dark periods are represented on the *x*-axis by black bars and the light period by a white bar. NADH and DNP-INT additions are represented by arrows. NADH-induced, DNP-INT-sensitive, light-dependent, PSII-independent electron flow was calculated as the difference between light-induced O_2 uptake rates in the presence and in the absence of DNP-INT. It was about 151 nmol O_2 min^{-1} mg^{-1} chlorophyll in this experiment. (B) Effect of varying NADH (●) and NADPH (○) concentrations on DNP-INT sensitive, light-dependent, PSII-independent electron flow.

observed (data not shown). NADH and NADPH-induced, light-dependent, PSII-independent electron flows were sensitive to inhibitors of the cyt b_6f [37,38]: 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl ether (DNP-INT, Fig. 1A) and 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB, not shown). DNP-INT was the most efficient, inhibiting both NADH and NADPH photooxidation by about 60–70%. The residual DNP-INT insensitive, light-induced O_2 uptake (likely due to unspecific cyt b_6f-independent oxidation) was subtracted to that observed in the absence of DNP-INT in the following experiments. NADH and NADPH-induced, DNP-INT-sensitive, light-dependent, PSII-independent electron flows, calculated by this way, will be named "NADH-dependent flow" and "NADPH-dependent flow".

The effect of varying NAD(P)H concentrations was determined (Fig. 1B). Apparent K_m ($K_{1/2}$) values for NADH and NADPH were 46 μM and 92 μM, respectively. The maximal activities (V_m) in the presence of NADH and of NADPH were around 150 and 120 nmol min^{-1} mg^{-1} chlorophyll, respectively. Pharmacological characteristics of NADH-dependent flow and NADPH-dependent flow were investigated in the following experiments at NAD(P)H concentrations close to saturation, (i.e., 200 μM NADH and 350 μM NADPH, respectively). In these conditions, we have tested the effect of the uncoupling agent FCCP (2 μM). NADH-dependent flow and NADPH-dependent flow were insensitive to this compound (not shown) indicating that NAD(P)H oxidation rate was not limited by proton gradient.

The ability of NAD(P)H to reduce the plastoquinone pool was also investigated by performing chlorophyll fluorescence measurements on dark-adapted thylakoid membranes in anaerobiosis. Anaerobic conditions were applied here in order to prevent dark reoxidation of the PQ pool. The chlorophyll fluorescence emission provides a non-invasive method for studying photosynthetic electron transfer in thylakoid membranes. The fluorescence level is related to the redox state of Q_A, the first quinone acceptor of PSII, which in the dark depends on the equilibrium between Q_A and the PQ pool. Variations in fluorescence level account then indirectly for the redox state of the plastoquinone pool in the dark [36]. When the quinone pool is fully oxidized, the measured fluorescence level under a non-actinic light represents the basal antenna fluorescence (F_0). In anaerobic conditions, addition of NADH (200 μM) to *C. reinhardtii* thylakoid membranes provoked a slow increase of the chlorophyll fluorescence level, indicating a reduction of the PQ pool (Fig. 2). A similar pattern was observed in the presence of NADPH (350 μM). When the same experiment was carried out in the presence of FCCP (2 μM), the NAD(P)H-induced chlorophyll fluorescence increase was more pronounced and the maximum fluorescence level (F_m) was reached in a few minutes (Fig. 2). As PQ reduction was not limited by proton gradient in our thylakoid preparations (see above), the stimulation of fluorescence increase by FCCP can probably not be attributed to an increase in PQ reduction rate. Rather, it can be interpreted as the fact that FCCP, by a mechanism that remains to be clarified, accelerates equilibration between the PSII fluorescence signal and PQ redox state, making the conditions more appropriate for assaying PQ reduction. In the following experiments, NAD(P)H fluorescence increase and its sensitivity towards inhibitors will be checked in the presence of FCCP.

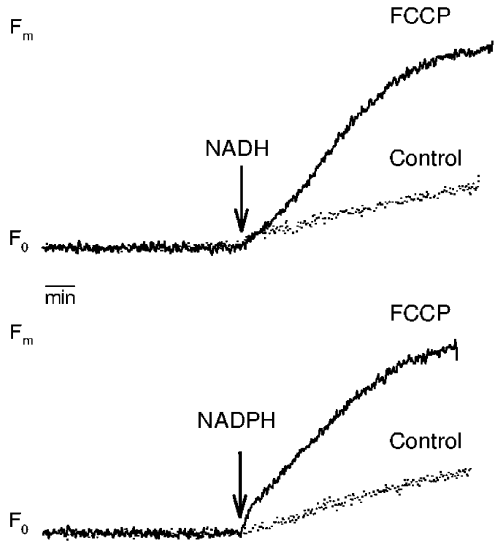

Fig. 2. Effect of exogenous addition of NADH and NADPH on chlorophyll fluorescence level, with or without FCCP. Fluorescence measurements were performed under anaerobic conditions on the *C. reinhardtii* membranes, using a pulse modulated amplitude fluorometer connected to the Hansatech Clark electrode vessel. Basal (F_0) and maximal (F_m) fluorescence, the levels of which are indicated on the graphs, were determined prior to anaerobiosis onset, F_0 being measured in the dark and F_m under a saturating white light pulse. $(F_m - F_0)$/Fm was about 0.75 in these extracts. Anaerobiosis was achieved by addition of glucose and glucose oxidase to the thylakoid suspension about 15 min before measurements. When added, FCCP (2 μM) was incubated 15 min before the addition of NADH (200 μM) or NADPH (350 μM).

2.2. Inhibitors effects on NADH dehydrogenase plastoquinone reductase activity

In order to investigate the nature of the NAD(P)H-plastoquinone oxidoreductase activity in *Chlamydomonas* thylakoids, we studied the effect of different chemicals. NADH- and NADPH-dependent flows were insensitive to rotenone (up to 100 μM), a classic inhibitor of mitochondrial complex I [39] and to TTFA (100 μM), which acts on the ubiquinone reducing side of the succinate dehydrogenase complex [39] and also on ferredoxin-NADP-reductase (FNR) [40]. No inhibition was observed with antimycin A (5 μM), a compound previously reported to inhibit FQR-mediated reduction of PQ [41].

By contrast, as shown in Fig. 3, both NADH- and NADPH-dependent flows were inhibited by several compounds known to impair rotenone insensitive NADH:ubiquinone oxidoreductases. DPI, a flavoenzyme inhibitor [42],

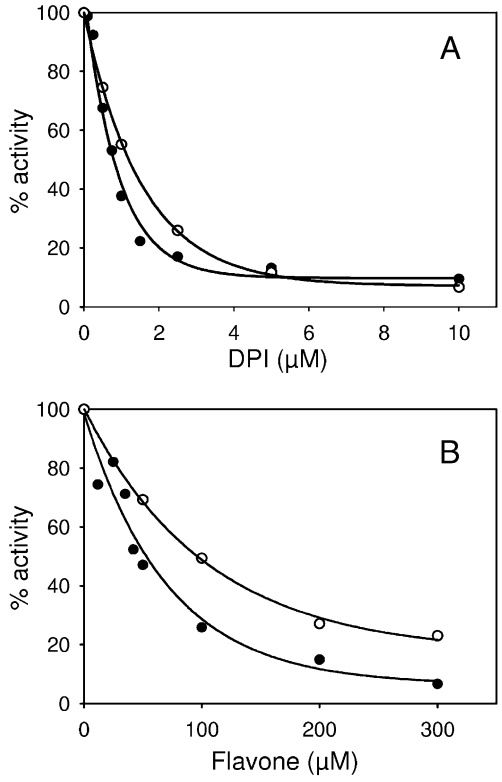

Fig. 3. Effects of DPI and flavone on NAD(P)H-dependent flows. O_2 uptake measurements were performed on *C. reinhardtii* thylakoid membranes by a Clark electrode in the presence of MV, SOD, catalase, DCMU, myxothiazol, SHAM and 200 μM NADH (●) or 350 μM NADPH (○). Effects of DPI (A) and of flavone (B).

was a powerful inhibitor of both NADH- and NADPH-dependent flows (Fig. 3A). Half-maximal inhibition was obtained at DPI concentration of 0.74 μM and 1.2 μM when NADH and NADPH were, respectively, used as substrates. Maximal inhibition by DPI was about 90%. Flavone, an other flavoenzyme inhibitor, has been reported to inhibit specifically the activity of the internal NDH-2 of *S. cerevisiae* mitochondria [43,44]. In *Chlamydomonas* thylakoids, flavone inhibited NADH- and NADPH-dependent flows, respectively, with an $I_{50(NADH)}$ of 74 μM and an $I_{50(NADPH)}$ of 117 μM (Fig. 3B). Maximal effect of flavone was about 80–90%. The effects of N-ethylmaleimide (NEM) and p-chloromercuribenzoate (pCMB), sulfhydryl group modifiers acting on cystein residues and affecting both NDH-1 [45] and NDH-2 [29], were also investigated. $I_{50(NADH)}$ and $I_{50(NADPH)}$ were, respectively, obtained at 67 μM and 221 μM for NEM (Fig. 4A), and at 11 μM and 26 μM for pCMB (Fig. 4B). Maximal effects of these two compounds were of about 80–90%.

Inhibitor effects were also investigated on NAD(P)H-induced chlorophyll fluorescence increase (Fig. 5 for NADPH, similar patterns with NADH—not shown) in the presence of FCCP (2 μM). NADH and NADPH-induced chlorophyll fluorescence increases were insensitive to rotenone (100 μM), TTFA (100 μM) and antimycin A (5 μM)—not shown. Both NADH and NADPH-induced chlorophyll fluorescence increases were sensitive to DPI (10 μM), NEM (500 μM) and pCMB (150 μM). Maximal inhibitions of NADH and NADPH-induced chlorophyll fluorescence increase rates were observed in presence of DPI. pCMB still had a strong inhibitory effect while NEM only partially inhibited chlorophyll fluorescence increase. A saturating light pulse was applied at the beginning and at the end of these experiments and showed that Fm was not quenched, indicating that the differences observed in fluorescence levels were actually due to inhibition of PQ reduction and not to global quenching phenomena.

No inhibition was found in the presence of flavone (300 μM); instead, a stimulation of the chlorophyll fluorescence rise was observed in presence of NAD(P)H (Fig. 5B for NADPH, similar pattern with NADH—not shown). In order to check whether this stimulation of chlorophyll fluorescence rise was due to PQ reduction, 2s far red (FR) light was applied in order to transiently reoxidize the PQ pool [36]. Reoxidation of the PQ pool by far red light had a much lower effect on the fluorescence signal in the presence of flavone than in control. This, together with the slow fluorescence rise observed before NADPH addition (Fig. 5B), indicates an effect of flavone on PSII or on PQ reoxidation by PSI. This implies that flavone has significant effects on the electron transport chain and therefore that the

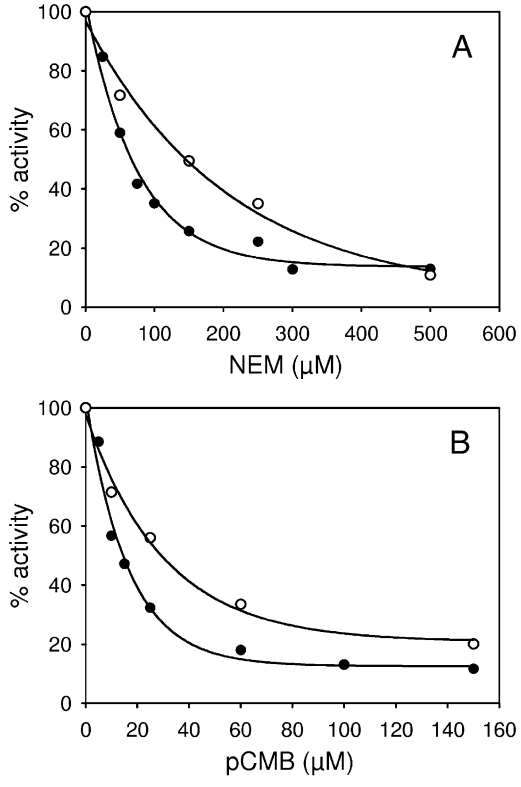

Fig. 4. Effects of NEM and pCMB on NAD(P)H-dependent flows. O_2 uptake measurements were performed on *C. reinhardtii* thylakoid membranes by a Clark electrode in the presence MV, SOD, catalase, DCMU, myxothiazol, SHAM and 200 μM NADH (●) or 350 μM NADPH (○). Effects of NEM (A) and of pCMB (B).

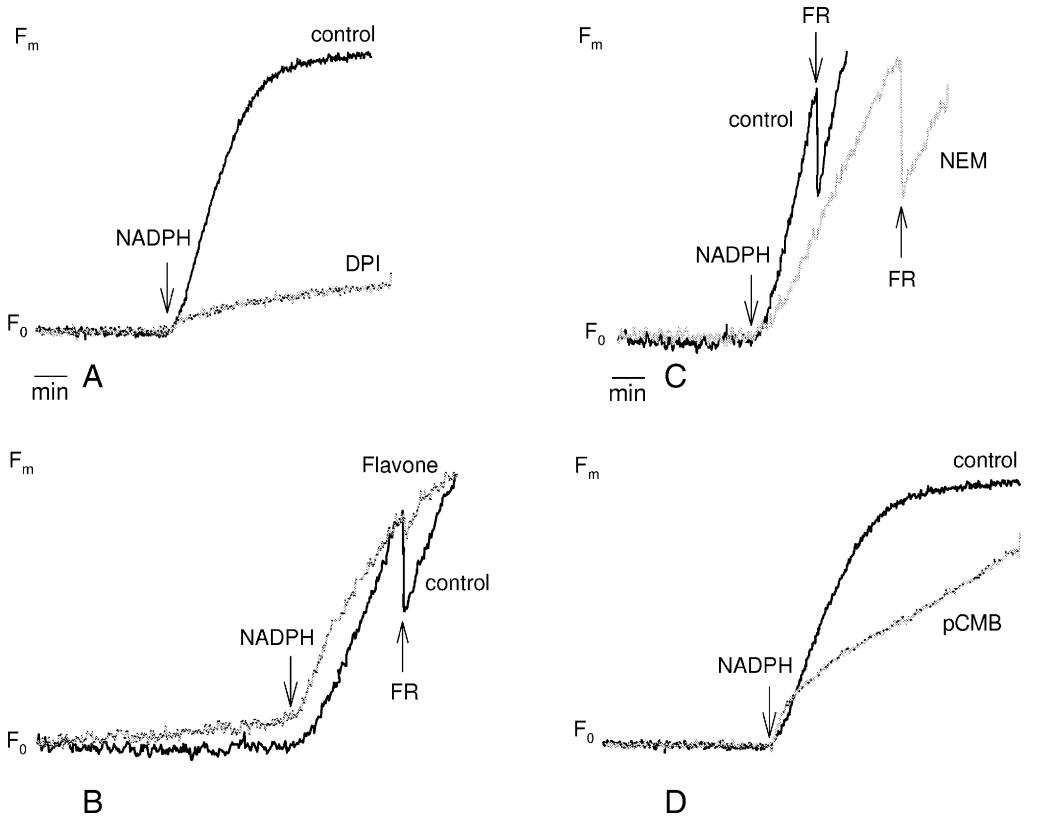

Fig. 5. Effect of DPI, Flavone, NEM and pCMB on NADPH-induced chlorophyll fluorescence increase in presence of 2 μM FCCP. Chlorophyll fluorescence measurements were performed under anaerobic conditions on the *C. reinhardtii* thylakoid membranes, using a pulse-modulated fluorometer. F_0 and F_m levels are figured on the graphs. Anaerobiosis was achieved by addition of glucose and glucose oxidase to the thylakoid suspension about 15 min before measurements. FCCP and chemical inhibitors were incubated 15 min before the addition of NADPH (350 μM, indicated by arrow). In some experiments, 2s far red (FR) light was applied when indicated.

inhibition of NAD(P)H-dependent flows due to this compound cannot be specifically assigned to an impact on the PQ reducing enzyme. In order to test if the limitation in the effect of NEM on fluorescence increase was due to a similar interaction, we also applied FR light (Fig. 5C for NADPH, similar pattern with NADH—not shown). In this case, FR effect was the same than in control and no drift was observed before NAD(P)H addition.

2.3. NADH dehydrogenase plastoquinone reductase and PSII-independent photoproduction of H_2

Measurements of H_2 exchange were performed by mass spectrometry on intact *C. reinhardtii* cells. In anaerobic conditions, dark adapted *C. reinhardtii* cells produced H_2 at a low rate due to fermentative metabolism. H_2 production is strongly stimulated by light during the first minutes of illumination. When PSII is inhibited by DCMU, a lower, but significant H_2 photoproduction is observed (Fig. 6). This production was strongly (>90%) inhibited by 1 μM DBMIB or 20 μM DNP-INT (not shown), indicating that the PSII-independent pathway of H_2 production involved electron transfer through the PQ pool and cyt b_6f.

The effects of NDH inhibitors were then studied on H_2 photoproduction in the presence or in the absence of

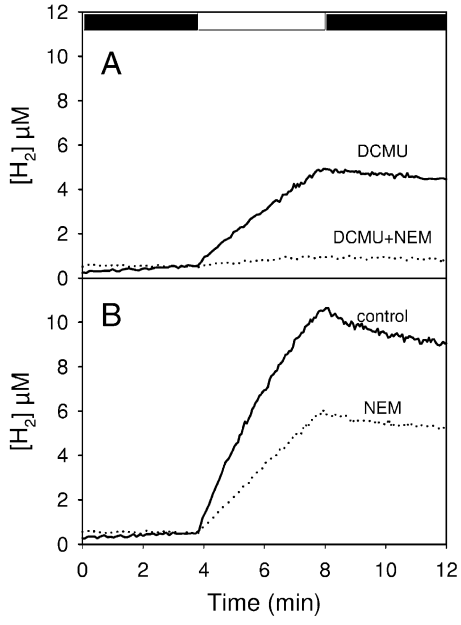

Fig. 6. Mass spectrometric measurements of H_2 exchange during dark–light transient in wild type *C. reinhardtii* cells adapted to dark anaerobic conditions. (A) Effect of NEM on light-dependent H_2 production in the presence of DCMU. (B) Effect of NEM on light-dependent H_2 production. Dark periods are represented on the *x*-axis by black bars and light period by white bar.

DCMU. A typical experiment using NEM is shown in Fig. 6. DCMU-insensitive H_2 photoproduction measured was almost completely inhibited by NEM (Fig. 6A). In the absence of DCMU, inhibition of H_2 production by NEM was about 40% (Fig. 6B), suggesting that the PSII-dependent pathway of H_2 photoproduction is less sensitive to this compound than the PSII-independent one. Interestingly, the sum of the rates of NEM-insensitive (PSII dependent) H_2 photoproduction and DCMU-insensitive (PSII independent) H_2 photoproduction roughly equaled the rate observed in the control, suggesting that both pathways contribute to H_2 production in normal conditions. DPI also inhibited DCMU-insensitive H_2 production (Fig. 7).

In order to test the specificity of NEM and DPI, we measured the effects of these inhibitors on the hydrogenase activity (assessed using the H/D exchange reaction), photosynthesis and respiration, and compared these effects to those observed on H_2 photoproduction (Fig. 7). DPI only slightly inhibited photosynthesis and had no significant effect on respiration. However, this compound strongly inhibited the hydrogenase activity (H–D exchange velocity decreased to 40 nmol min^{-1} mg^{-1} Chl at 5 μM DPI vs. 3000 nmol min^{-1} mg^{-1} Chl in control) which reached values close to that measured for H_2 photoproduction, therefore, indicating that H_2ase activity is probably limiting in these conditions. NEM strongly affected both respiration and photosynthesis, but in contrast to DPI had a much smaller effect on the hydrogenase activity (H–D exchange velocity around 1500 nmol min^{-1} mg^{-1} Chl, much greater than H_2 production rates, suggesting that the catalytic capacity of the enzyme is not limiting). Inhibition of mitochondrial respiration might be due an effect of this compound on the mitochondrial electron transport chain at the Complex I level for instance. Interestingly, while NEM strongly inhibited photosynthesis, electron transfer from PSII to hydrogenase, H_2 production measured in the absence of DCMU was only partly affected (the amplitude of inhibition matching that of the PSII-independent pathway alone). This indicates that the site of photosynthesis inhibition by NEM is located after Fd (maybe at FNR). We conclude from this experiment that the effect of NEM on PSII-independent H_2 production is not explained by an impairment of electron transfer from PQ to PSI or an inhibition of the hydrogenase, but is more likely due to an inhibition of the PQ-reducing step. Interestingly, the I50 of inhibition of PSII-independent H_2 photoproduction by NEM (around 100 μM) matches the I50 of NAD(P)H-driven PQ reduction on thylakoid preparations. In contrast, rotenone (20 μM), antimycin A (up to 60 μM) and pCMB (1 mM) were found to have no effect on H_2 production (not shown).

Uncouplers have been shown to stimulate PSII-independent H_2 photoproduction (Cournac et al., 2002). We have also checked the effect of inhibitors on H_2 photoproduction in the presence of FCCP (2 μM). Addition of FCCP significantly stimulated H_2 production in controls and in the presence of DCMU. In the presence of FCCP, similar patterns of inhibition by NEM were observed (not shown data).

3. Discussion

The green alga *Chlamydomonas reinhardtii* is able to use both NADH and NADPH as electron donors for PQ reduction within its photosynthetic electron transport chain. We propose, based on the pharmacological analysis performed in this study, that NAD(P)H-dependent PQ reduction is likely achieved through a rotenone-insensitive NDH-2 rather than through a NDH-1. Indeed, the absence of *ndh* genes in *Chlamydomonas* chloroplast genome [23] and the insensitivity of the NAD(P)H-PQ oxidoreductase activity to rotenone (this study) argue against the participation of a bacterial-type complex I in PQ reduction in *Chlamydomonas*, unlike described by Godde and Trebst [10]. A possible explanation for previous findings by these authors might be a contamination by mitochondrial membranes (which are difficult to avoid during thylakoid preparation). Such membranes could easily interact with

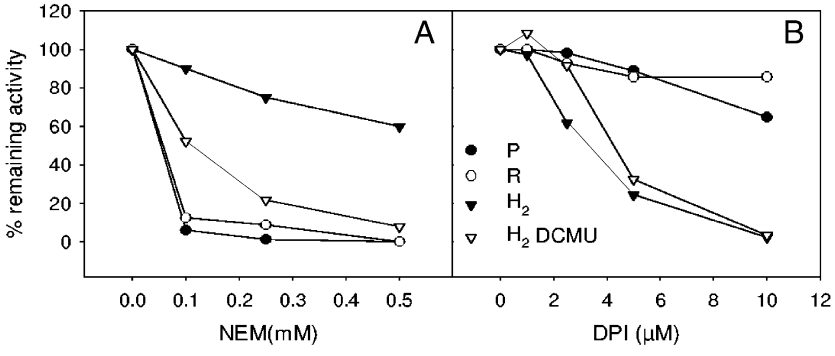

Fig. 7. Effects of NEM (A) and DPI (B) on photosynthetic and respiratory O_2 exchange rates and on H_2 production measured by mass spectrometry in intact *Chlamydomonas* cells. P: photosynthetic O_2 production in the light; R: respiratory O_2 consumption in the dark; H_2: initial H_2 production measured under anaerobic conditions in the light in the absence of DCMU; H_2 DCMU: initial H_2 production measured under anaerobic conditions in the light in the presence of DCMU. Results are expressed as percent of controls. Average exchange rates in the absence of inhibitors were: 2100, 375: 775 and : 175 nmol min^{-1} mg Chl^{-1} for photosynthesis, respiration, H_2 production in the absence or presence of DCMU, respectively.

PSI, for instance through cyt c (which is reduced by the mitochondrial electron transport chain and can act as a donor for PSI). This was prevented in our experiments by addition of myxothiazol.

The thylakoid NAD(P)H-PQ oxidoreductase activity from *Chlamydomonas* thylakoids had a better affinity for NADH than for NADPH, but comparable maximal velocities using both substrates. Thylakoid NAD(P)H-PQ oxidoreductase was sensitive to DPI and flavone, flavoenzyme inhibitors known to inhibit both mitochondrial NDH-1 [46,47] and also some NDH-2s [43,48]. While DPI inhibited both NAD(P)H-induced light-dependent O_2 uptake and NAD(P)H-induced chlorophyll fluorescence increase, flavone inhibited NAD(P)H-induced light-dependent O_2 uptake but not NAD(P)H-induced chlorophyll fluorescence increase. Perturbations of the fluorescence signal by flavone suggested a direct effect of flavone on the photosynthetic electron transport chain (either by altering PSII fluorescence or by inhibiting PQ reoxidation by PSI) which would explain this behavior. This is also in agreement with the observation that flavone affects photosynthetic electron transport in vivo (not shown). Thiol-blocking reagents NEM and pCMB, previously reported to inhibit eukaryotic and prokaryotic NDH-2s [29,49–51] were also able to inhibit thylakoid NAD(P)H-PQ oxidoreductase activity of *Chlamydomonas*, indicating the involvement of cysteine residues in the protein activity. pCMB was more efficient than NEM in inhibiting both NAD(P)H-dependent flows and fluorescence rises but had no effect on intact cells, likely due to a limitation in pCMB diffusion through membranes. It can be noted that NADH-PQ oxidoreductase activity is more sensitive to NEM and pCMB than NADPH-PQ oxidoreductase activity. It has been reported that NADPH could reduce the PQ pool in thylakoid preparations via FNR, directly [52] or indirectly via Fd reduction and subsequent PQ reduction by FQR activity [53], both activities having been described as involved in cyclic electron transfer around PSI. Also, NEM [54] and DPI [36] have been reported to inhibit FNR activity in presence of NADPH. In our experiments, the affinity of the NAD(P)H-PQ oxidoreductase was higher for NADH than for NADPH, thus arguing against a major participation of the FNR, at least for the NADH-mediated flow. Another indication against the involvement of FNR in the activities reported in this paper is the insensitivity of NAD(P)H oxidation to TTFA, a FNR inhibitor [40]. This conclusion relies on the assumption that PQ reductase activity of *C. reinhardtii* FNR is also sensitive towards TTFA, which remains to be demonstrated: the possibility that a significant part of the NADPH-dependent flow could depend on this enzyme cannot then be totally discarded. Also, the insensitivity of NAD(P)H-PQ oxidoreductase to antimycin A indicates that FQR is not involved in this activity. This does not exclude the presence of FQR in our preparations. But most probably it is not detected in our assays as we do not add ferredoxin.

The inhibitor response pattern of non-photochemical reduction of the PQ pool in *C. reinhardtii* thylakoids favors the hypothesis of the involvement of either a NDH-2 able to use both NADH and NADPH or of two enzymes, one being specific for NADH and the other for NADPH. Complete sequencing of nuclear genome from *C. reinhardtii* also revealed the existence of 7 encoding sequences showing homologies with known genes encoding for NDH-2s in other organisms. These seven ORF have been numbered as C_310108, C_1170009, C_5950001, C_1890016, C_1450028, C_1450029, C_270109 from the last total sequence release (http://genome.jgi-psf.org/chlre2/chlre2.home.html). The predicted proteins show part or totality of the characteristic conserved motifs of NDH-2s [55]: NADH binding motif (6/7), FAD binding motif (5/7), and other conserved unassigned motifs (5/7). Among them, some (C_310108, C_1170009, C_1890016, C1450028, C1450029) are predicted as potentially chloroplast-targeted using the ChloroP prediction program (available on http://www.infobiogen.fr). The TargetP program calculates scores for potential presence of mitochondrial (mTP), chloroplastic (cTP) or secretory pathway (SP) signal peptides in proteic sequences. Using this program, two sequences were found to yield a higher score for chloroplast signaling prediction: C_310108 (cTP: 0.606; mTP: 0.485) and C_1170009 (cTP: 0.649; mTP: 0.513). However, these predictions have to be taken with caution since they have been designed for plant proteins and target peptides characteristics in *Chlamydomonas* do not completely match those of higher plants [56]. Research is actually conducted to identify and characterize the actual products of these genes and their addressing. We have also searched for orthologs of other bacterial proteins known to exhibit NAD(P)H/quinone oxidoreductase activity, but we did not find any sequence with significant similarity with one of these protein types in the current release of the genome. It cannot be excluded, however, that an entirely novel type of NAD(P)H/PQ oxidoreductase could be present in *Chlamydomonas* thylakoids. In *Arabidopsis thaliana*, recent subcellular localization of the 7 *ndh2* gene products identified from the nuclear genome sequence was studied using fusion of their N-terminal targeting peptide to green fluorescent protein. It was concluded that all the corresponding gene products were exclusively addressed to mitochondria [32]. This questioned the existence of NDH-2s in higher plant chloroplasts, as suggested by Corneille et al. [36]. In any case, it will be of great interest to develop a similar approach in *Chlamydomonas*.

The physiological role of NAD(P)H-PQ oxidoreductase in the thylakoid membranes of *C. reinhardtii* remains unclear. Bennoun postulated [13] the presence, in *Chlamydomonas* thylakoid membranes, of an electrogenic respiratory electron transport chain “chlororespiration”, involving an NAD(P)H-PQ oxidoreductase activity. But if the NAD(P)H-PQ oxidoreductase present in *Chlamydomonas* is a NDH-2 (this study), and if the O_2 reducing enzyme is an alternative oxidase [15,57], the so-called chlororespiratory

electron transfer would not be electrogenic. Its role could rather be that of adjusting redox balance in the dark (dissipation of excess reducing power) or in the light (poising of PQ redox state for adjusting cyclic vs. linear electron flow and then tuning the ATP/NADPH balance, see ref. [15]). Some authors suggested that NDH-1 in plants could directly catalyze electron transfer around PSI, besides FQR [58,59]. In *Chlamydomonas* also, the coexistence of two pathways for this cyclic flow was also proposed, one (antimycin A sensitive), corresponding to a Fd-mediated cyclic electron flow catalyzed by Fd-PQ reductase (FQR), the other (NEM sensitive) involving a NAD(P)H-dehydrogenase [16].

DCMU-treated *Chlamydomonas* cells can support a substantial PSII-independent H_2 photoproduction, after a period of anaerobic incubation in the dark [60–62]. Although some of the electron transfer activities they evidenced might have been due to mitochondrial contamination, Godde and Trebst [10] reported that photosynthetically active chloroplast particles from *Chlamydomonas* are able to evolve H_2 in the light at the expense of NADH, and suggested that NADH-dehydrogenase present in the photosynthetic membranes of *C. reinhardtii* might participate in H_2 photobiological production. Our findings suggest that a chloroplast NDH-2 is more likely involved in the PSII-independent pathway of H_2 photoproduction. Its contribution, in the conditions of our experiments, is of the same magnitude as that of the PSII-dependent pathway. In the case of H_2 production induced by conditions of sulfur deprivation [4], Antal et al. demonstrated that PSII activity is essential for significant H_2 production on the long term [63] and suggested that the PSII-independent pathway would be marginal. Nevertheless, the potential for biotechnological H_2 photoproduction through NDH-2 activity might be significant, provided that a strategy could be designed to sustain it on a long term at rates close to those observed in our short-term experiments.

4. Materials and methods

4.1. Strains and growth conditions

Chlamydomonas reinhardtii 137c wild type cells were grown in Tris-acetate phosphate medium [64] or in a mineral medium of same nutrient composition except it was devoid of acetate. Algae cultures were maintained at 25 °C under continuous agitation (120 rpm) and illumination of 100 μmol photon m^{-2} s^{-1}.

4.2. Isolation of a thylakoid membrane fraction

Chlamydomonas cultures grown on Tris acetate phosphate medium were harvested in exponential growth phase, centrifuged, washed in 35 mM HEPES–NaOH buffer, pH 7.2 and resuspended in 12 mL of buffer A (50 mM Tricine–NaOH,10 mM NaCl, 5 mM $MgCl_2$; pH 8) supplemented with 1% bovine serum albumine *w*/*v*,1 mM benzamidine, 1 mM phenyl methyl sulfone fluoride. The following operations were carried out in the dark at 4 °C. Cells were disrupted by two cycles of a chilled French pressure cell (2000 p.s.i.). The homogenate was centrifuged at 500×*g* for 5 min. The pellet, containing unbroken cells, was discarded. The supernatant was centrifuged at 10,000 g for 10 min to collect thylakoid membranes. Thylakoid membranes were resuspended in 250–500 μL of buffer A and stored on ice in the dark.

4.3. Chlorophyll concentration measurements

Chlorophyll extraction was performed in 80% *v*/*v* acetone/H_2O, and chlorophyll concentration was calculated from absorption measurements at 663 and 645 nm [65]. In thylakoid preparations, chlorophyll concentrations were set between 30 and 60 μg Chl mL^{-1}. For gas exchange measurements in vivo, chlorophyll concentrations were set between 20 and 30 μg mL^{-1} in the measurement vessel.

4.4. Measurement of light-dependent O_2 uptake in the presence of MV and DCMU

Light-dependent O_2 uptake rates were measured in presence of DCMU and MV using a Clark electrode (DW2/2, Hansatech, King's Lynn, England). The reaction medium contained 50 μL of thylakoid membrane suspension and 950 μL of buffer A (pH 7.2). The medium was supplemented with 50 μM MV, 25 μM DCMU, 4 μM myxothiazol, 800 μM SHAM, 1000 units mL^{-1} catalase and 500 units mL^{-1} superoxide dismutase (SOD), about 5 min before the onset of illumination. Catalase and SOD were added to the assay medium to scavenge the reactive oxygen species and to ensure a constant stoichiometry between photosynthetic electron flow and O_2 uptake rates. The reaction was carried out at 25 °C in the presence of NADH or NADPH as electron donors. Chemical inhibitors were incubated about 15 min before measurements. Light-dependent O_2 uptake rates were determined as the difference between uptake rates measured in the dark and in the light (50 μmol photons m^{-2} s^{-1}).

4.5. Chlorophyll fluorescence measurements

Chlorophyll fluorescence was measured at 25 °C, in anaerobic conditions using a Clark electrode (DW2/2, Hansatech, King's Lynn, England) coupled with a pulse modulated amplitude fluorometer (PAM 101–103, Walz, Effeltrich, Germany). The optic fiber of the fluorometer was placed close to the glass tube of the electrode reaction chamber (1 mL reaction volume: 50 μL of thylakoid membrane suspension and 950 μL of buffer A, pH 7.2). Non-actinic modulated light (650 nm, 1.6 kHz) was used to determine the chlorophyll fluorescence level F_0. The

maximal chlorophyll fluorescence level (F_m) was measured under 1 s saturating pulse (about 1000 μmol photons m^{-2} s^{-1}). The maximal photochemical efficiency of PSII was calculated by the ratio: $(F_m - F_0)/F_m$. Anaerobiosis was achieved about 15 min before measurements by addition to the thylakoid membrane suspension of glucose (10 mM) and glucose oxidase (2 mg mL^{-1}) in the presence of catalase (1000 units mL^{-1}).

4.6. Mass spectrometric measurements of gas exchange

Chlamydomonas cultures grown on mineral medium were harvested in exponential growth phase, centrifuged, washed and resuspended in 35 mM HEPES–NaOH buffer, pH 7.2. For mass spectrometric measurements, the cell suspension (1.5 mL) was placed in the measuring chamber (adapted from an Hansatech electrode chamber). The bottom of the chamber was sealed with a Teflon membrane allowing dissolved gases to be directly introduced through a vacuum line into the ion source of the mass spectrometer (model MM 8–80, VG Instruments, Cheshire, UK). The chamber was thermostated at 25 °C and the cell suspension was continuously stirred by a magnetic stirrer. Light (300 μmol photons m^{-2} s^{-1}) was supplied to the suspension by an optic fiber illuminator (Schott, Main, Germany). Inhibitors were added using a Hamilton syringe, about 10 min before experiments. Measurements of H_2 and O_2 production or uptake rates, as well as hydrogenase activity assays by H^+/D_2 exchange were performed as described previously [66]. Net production or uptake rates are simply calculated from time derivation of concentrations. Calculations of hydrogenase activities are based on the hydrogenase property to catalyze atom exchange between protons or deuterons from the medium and H or D atoms from dissolved hydrogen species. Hydrogenase activity estimation from D_2 decay and H_2/HD production rates has recently been reformulated [67], our calculations were based on this work. The spectrometer scans gas abundances (H_2, HD, D_2, O_2, CO_2, selected in function of the experiment) by automatically adjusting the magnet current to the corresponding mass peaks ($m/e = 2$, 3, 4, 32, 44). During experiments, mass peaks are recorded every 2 s for fast kinetics measurements, or every 10 s for measuring slower kinetics, each mass peak measurement taking about 0.5 s. The amperometric signal collected by the spectrometer is directly proportional to the gas concentration in the chamber, the proportionality coefficient varying from one gas to the other according to the ionization properties of the corresponding gas. In order to calculate gas exchange rates from variations in concentrations, a correction from the gas consumption by the mass spectrometer was applied. In our experimental conditions, consumption of dissolved gases by the mass spectrometer, assayed in a cell-free buffer, showed first order kinetics with constants around 0.09 min^{-1} for H_2, 0.088 for HD, 0.086 for D_2, 0.024 min^{-1} for O_2 and negligible for CO_2.

4.7. Chemicals and enzymes

All chemicals and enzymes were purchased from Sigma, except SHAM which was purchased from Aldrich and DNP-INT which was generously provided by Pr. A. Trebst. Most inhibitors stock solutions were made in methanol, except pCMB and rotenone (in dimethylenesulfoxide). Substrates (NADH, NADPH, malate, succinate, glucose), and enzymes (SOD, catalase, glucose oxidase) were dissolved in water.

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