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Reduction of the plastoquinone pool by exogenous NADH and NADPH in higher plant chloroplasts
Characterization of a NAD(P)H–plastoquinone oxidoreductase activity

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Abstract

Chlorophyll fluorescence measurements were performed on osmotically lysed potato chloroplasts in order to characterize the reactions involved in the dark reduction of photosynthetic inter-system chain electron carriers. Addition of NADH or NADPH to lysed chloroplasts increased the chlorophyll fluorescence level measured in the presence of a non-actinic light until reaching F_{max}, thus indicating an increase in the redox state of the plastoquinone (PQ) pool. The fluorescence increase was more pronounced when the experiment was carried out under anaerobic conditions and was about 50% higher when NADH rather than NADPH was used as an electron donor. The NAD(P)H–PQ oxidoreductase reaction was inhibited by diphenylene iodonium, *N*-ethylmaleimide and dicoumarol, but insensitive to rotenone, antimycin A and piericidin A. By comparing the substrate specificity and the inhibitor sensitivity of this reaction to the properties of spinach ferredoxin–$NADP^+$-reductase (FNR), we infer that FNR is not involved in the NAD(P)H–PQ oxidoreductase activity and conclude to the participation of rotenone-insensitive NAD(P)H–PQ oxidoreductase. By measuring light-dependent oxygen uptake in the presence of DCMU, methyl viologen and NADH or NADPH as an electron donors, the electron flow rate through the NAD(P)H–PQ oxidoreductase is estimated to about 160 nmol O_2 min^{-1} mg^{-1} chlorophyll. The nature of this enzyme is discussed in relation to the existence of a thylakoidal NADH dehydrogenase complex encoded by plastidial *ndh* genes.
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1. Introduction

During oxygenic photosynthesis, light energy is converted into chemical energy thanks to the cooperation of two reaction centers, photosystem II (PS II) and photosystem I (PS I) participating to coordinated electron transfer reactions. Different lines of evidence, based on fluorescence data [1] or flash-induced oxygen exchange measurements [2,3], have

Abbreviations: FNR, ferredoxin–$NADP^+$-reductase; FQR, ferredoxin–plastoquinone reductase; MV, methyl viologen; PQ, plastoquinone; PS, photosystem; DPI, diphenyleneiodonium; NEM, *N*-ethylmaleimide

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suggested that besides photosynthetic electron transfer, the thylakoid membrane may contain a respiratory electron transport chain, called chlororespiration. Chlororespiration would involve at least two other components: a protein complex reducing the PQ pool from a soluble stromal pool of electron donors and another complex involved in the oxidation of reduced PQ [4]. Presently, the nature of these two putative complexes remains to be established. Moreover, if chlororespiration has been well-documented in unicellular algae [1–5], its occurrence in higher plant chloroplasts is not clearly established. Garab et al. [6], by studying the effects of cyanide on the redox state of inter-system cytochromes, concluded to the existence of a cyanide-sensitive chlororespiratory oxidase in higher plants.

Complete sequencing of chloroplast genome from higher plants revealed the existence of coding sequences showing homologies with genes coding for subunits of the mitochondrial NAD(P)H dehydrogenase complex or complex I [7]. These genes (named *ndh* genes) have been shown to be transcribed [8] and the polypeptides products of some *ndh* genes have been found in thylakoid membranes [9–14]. By using non-denaturing gel electrophoresis, different authors have recently reported the existence of NAD(P)H dehydrogenase activity bands in higher plant chloroplasts [12,15]. Guedeney et al. [12] reported the existence of an activity band, specific for NADPH oxidation, which was recognized by antibodies directed against two *ndh* gene products (NDH-B and NDH-J). These authors concluded to the existence of a complex involving *ndh* gene products. Because the activity band cross-reacted with an antibody directed against FNR, it was suggested that an association between *ndh* gene products and FNR may allow the complex to use NADPH thanks to the diaphorase activity of this enzyme [12]. Cuello et al. [15] and Sazanov et al. [16] reported the existence of chloroplast activities specific for NADH oxidation. One of these activities was reported to co-migrate with a polypeptide recognized by an antibody directed against NDH-I [16]. The NAD(P)H dehydrogenase complex, by allowing electrons to enter the PQ pool from a soluble stromal pool, has been suggested to be involved in cyclic electron flow around PS I [17] or in chlororespiration, a respiratory electron transport chain identified in chloroplasts [1,2]. Another protein complex, the ferredoxin–PQ reductase (FQR), has been suggested to be involved in the reduction of PQ (for a review, see [18]). According to Bendall and coworkers [19,20], FQR would reduce PQ through an antimycin A-sensitive reaction. From a recent survey of the literature, Bendall and Manasse [18] concluded that the NADPH–PQ reductase activity of thylakoids is adequately explained by an electron transfer sequence involving FNR and FQR and that the participation of the NAD(P)H dehydrogenase complex to this reaction is doubtful. Clearly, both the nature of the protein complex involved in the dark reduction of the PQ pool as well as the existence of a chlororespiration in higher chloroplasts need further investigations to be clarified.

In the present work, we have addressed these questions by studying the reactions involved in the entry of electrons in the PQ pool in osmotically lysed potato chloroplasts. By studying the properties of chlorophyll fluorescence increase, measured in dim light following NADH or NADPH addition, we conclude that a rotenone-insensitive NAD(P)H:(PQ-acceptor) oxidoreductase different from FNR and FQR, is involved in the reduction of the PQ pool. We estimate the electron flow through this complex to about 160 nmol O_2 min^{-1} mg^{-1} chlorophyll.

2. Experimental procedures

2.1. Plant material – Solanum tuberosum L.

Plants cv. ''Haig'' were originating from in vitro plantlets and grown on compost in a phytotron (23°C day/15°C night, 12 h photoperiod, light intensity 350 $\mu E\, m^{-2}\, s^{-1}$, 60–80% relative humidity) as previously described [21]. Plantlets were irrigated using a half-diluted nutrient solution [22]. Experiments were performed on leaves sampled from 1-month-old plants.

2.2. Chloroplast isolation

Leaves were harvested at the end of the night period and intact chloroplasts were isolated at 4°C on a percoll gradient according to a modification of the method described by Mills and Joy [23]. About 15 g of leaves were blended for 2 s in 25 ml of medium a

containing 330 mM sorbitol, 50 mM Tricine–NaOH (pH 7.8), 2 mM EDTA, 1 mM $MgCl_2$, 2 mM ascorbic acid and 5 mM DTT, using a Polytron homogenizer (Poly Labo, Strasbourg, France). After filtration through two layers of 250 μm and two layers of 60 μm nylon net, followed by centrifugation ($2000 \times g$, 5 min at 4°C), the crude extract was resuspended in medium A (DTT free) and layered onto a Percoll step gradient formed with two layers of medium A (DTT free) containing 90 and 40% (v/v) of Percoll, respectively. After centrifugation ($3500 \times g$, 10 min at 4°C), intact chloroplasts were recovered from the 40/90% Percoll interphase, washed with medium A, pelleted at $2000 \times g$ for 5 min at 4°C and osmotically lysed by resuspension in 10 mM $MgCl_2$ and 1 mM PMSF for 30 min. Lysed chloroplasts were homogenized in a storage buffer containing 0.25 M sucrose, 10 mM $MgCl_2$, 5 mM NaCl, 5 mM Hepes, 2.5 mM sodium phosphate (pH 7.5), 50% (v/v) glycerol, 1 mM PMSF and used for fluorescence measurements. In order to study the effect of different inhibitors on the same chloroplast preparation, aliquots of the chloroplast preparation were stored at −20°C. In these conditions the NAD(P)–PQ oxidoreductase activity, estimated from chlorophyll fluorescence measurements, was unaffected for at least two weeks. For fluorescence measurements, broken chloroplasts were diluted 10-fold in 1 ml of an assay medium containing 0.25 M sucrose, 30 mM $MgCl_2$, 5 mM NaCl, 5 mM Hepes, 2.5 mM sodium phosphate (pH 7.5) and 1 mM PMSF. Final chlorophyll concentration (between 40–50 $\mu g\,ml^{-1}$) was measured according to Lichtenhaler and Wellburn [24].

2.3. Chlorophyll fluorescence measurements

Chlorophyll fluorescence was measured at 25°C using a pulse modulated amplitude fluorimeter (PAM 101–103, Walz, Effeltrich, Germany). The optic fiber of the fluorimeter was placed in contact to the glass tube of the reaction chamber (1 ml reaction volume) of an O_2 electrode (Hansatech, Kings's Lynn, England). 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 a 800 ms saturating pulse (about 8000 μmol photons $m^{-2}\,s^{-1}$). The maximal photochemical efficiency of PS II $(F_m - F_0)/F_m$ measured on chloroplast samples was 0.7 (±0.05, 30 experiments). Anaerobiosis was achieved by addition of glucose (20 mM) and glucose oxidase ($2\,mg\,ml^{-1}$) to the chloroplast suspension about 15 min before measurements. In some experiments, far-red illumination was supplied to the chloroplast suspension using a Schott illuminator (KL1500) equipped with a fiber optic and a far-red filter (RG 730, Schott, Mainz, Germany). Irradiance of the resulting far-red light was about $50\,W\,m^{-2}$ measured with a Li-Cor (Lincoln, Nebraska, USA) radiometer (LI-185B/LI-200SB).

2.4. Measurement of FNR activity

The diaphorase activity of FNR was measured by following O_2 uptake in the presence of methyl viologen (MV) according to a modified procedure of the method described by Böger [25]. The reaction medium contained 500 μM MV, ferredoxin ($50\,\mu g\,ml^{-1}$) and purified FNR from spinach leaves ($40\,\mu g\,ml^{-1}$). The reaction was carried out at 25°C in the same assay medium as described above and in the presence of NADH or NADPH as electron donors. O_2 consumption rates were determined using an Hansatech O_2 electrode.

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

The fluorescence assay medium was supplemented with 10 μM DCMU, 500 μM MV, 1000 units ml^{-1} of catalase and 500 units ml^{-1} of superoxide dismutase. Catalase and superoxide dismutase were added to the assay medium to insure a constant stoichiometry between photosynthetic electron flow and O_2 uptake rates, and to scavenge the activated O_2 species. Chemicals and enzymes were incubated about 5 min in the dark before the onset of illumination. Light-dependent O_2 uptake rates were determined as the difference between uptake rates measured in the dark (partly due to the activity of FNR) and in the light.

All chemicals and enzymes were purchased from Sigma, except catalase which was purchased from Merck. Piericidin A was kindly supplied by Dr. A. Dupuis (CEA/Grenoble, DBMS, France).

3. Results

3.1. Reduction of the PQ pool by exogenous NADH or NADPH

We first investigated the ability of NADH and NADPH to reduce the PQ pool, by performing chlorophyll fluorescence measurements on osmotically lysed potato chloroplasts. The fluorescence level determined under an extremely weak (non-actinic) light intensity is related to the redox state of Q_A, the first quinone acceptor of PS II, and depends on the equilibrium between Q_A and the PQ pool. The relation between the redox state of Q_A and the redox state of the PQ pool has been studied by Diner [26]. Based on the data of Diner, Groom et al. [27] estimated that Q_A reaches 10% reduction when the PQ pool is 50% reduced and 50% reduction when 90% of the PQ pool is in the reduced state. When Q_A is fully oxidized, the measured fluorescence level represents the basal antenna fluorescence (F_0). Addition of 2 mM NADH to the chloroplast suspension provoked an increase in the apparent F_0 level of chlorophyll fluorescence (Fig. 1(A)). Similar patterns were observed when 2 mM NADPH were added to the chloroplast preparation (data not shown). The fluorescence rise was reversed by supplying an additional far-red illumination to excite PS I (Fig. 1) or by adding an electron acceptor such as oxidized diaminodurene to the chloroplasts suspension (data not shown), thus showing that the fluorescence increase observed in these conditions is due to an increase in the redox state of the PQ pool. When the same experiment was carried out under anaerobic conditions (in the presence of glucose/glucose oxidase), the fluorescence increase was more pronounced and the maximum fluorescence level (F_{max}) was reached in a few minutes (Fig. 1(B)). This suggests that under aerobic conditions, a significant part of electrons entering the PQ pool are used for O_2 reduction, presumably through the PQ oxidation pathway of chlororespiration [6]. Fig. 2(A) shows that both NADH and NADPH can efficiently reduce the PQ pool of potato chloroplasts, although NADH appears about 50% more efficient when comparing the maximum fluorescence increase rates obtained in the presence of saturating substrate concentrations. Some authors have suggested that FNR, thanks to its diaphorase activity, might be involved in the non-photochemical reduction of PQ [18]. Fig. 2(B) shows the diaphorase activity of spinach FNR measured by following O_2 uptake rate in the presence of MV and

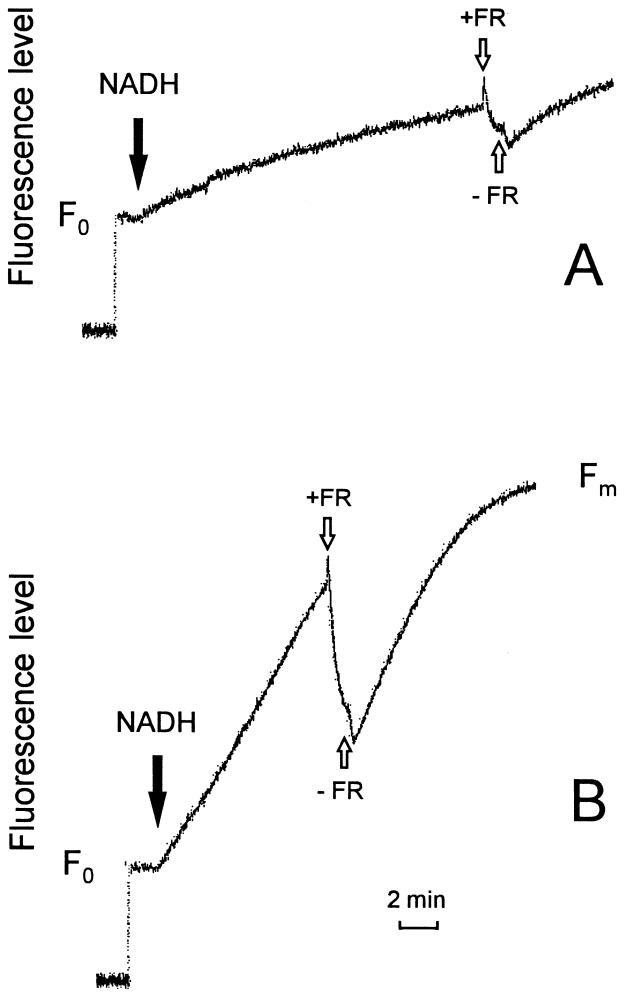

Fig. 1. Effect of exogenous addition of 2 mM NADH on the chlorophyll fluorescence level measured in dim modulated light on osmotically lysed potato chloroplasts. (A) measurements were performed under aerobic conditions; (B) measurements performed under anaerobic conditions following the addition of glucose/glucose oxidase to the chloroplast suspension. When indicated by arrows, illumination with far-red light was turned on (⇓) and off (⇑). Note that immediately after switching on far-red light a rapid increase in fluorescence was observed. This fast component, which was also observed when switching far-red light off was attributed to the excitation of PS II by the far-red light beam.

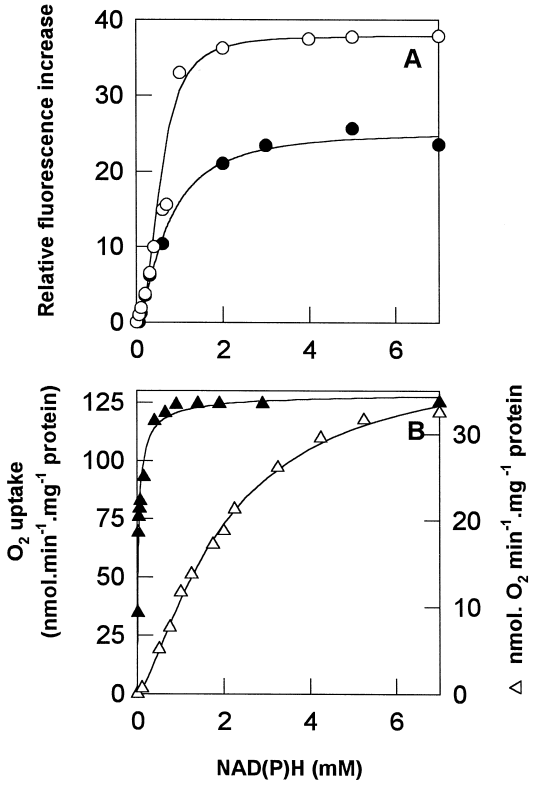

Fig. 2. Effect of NADH and NADPH concentrations on the rate of apparent chlorophyll fluorescence increase rate measured on osmotically lysed potato chloroplasts (A) and on the activity of purified spinach FNR (B). (A) Experiments were carried out under anaerobic conditions as described in Fig. 1. The initial slope of fluorescence increase was measured following addition of NADH (○) or NADPH (●) and plotted versus added concentrations of NADH or NADPH; (B) FNR activity measured as O_2 uptake in the presence of MV and NADH (△) or NADPH (▲).

using either NADPH or NADH as a substrate. As already reported [28], and in contrast to the non-photochemical reduction of PQ (Fig. 2(A)), FNR has a much higher affinity for NADPH than for NADH.

3.2. Inhibitor effects on the chlorophyll fluorescence rise

We then investigated the effects of different inhibitors on the chlorophyll fluorescence increase measured under anaerobic conditions. We chose anaerobic conditions to get rid of any interference with the PQ oxidation pathway and to insure that the observed inhibitors effects could be readily interpreted through an action on the enzymatic system involved in the reduction of PQ. As shown in Fig. 3(A) and (B), both NADH and NADPH-induced reduction of PQ were severely inhibited by 0.5 mM *N*-ethylmaleimide (NEM), a sulfhydryl group modifier acting on cysteine residues. On the other hand, FNR activity measured in the presence of NADPH needed much higher NEM concentrations (more than 3 mM) for full inhibition and was virtually insensitive to NEM when NADH was used as a substrate. The effect of diphenyleneiodonium (DPI), a flavoenzyme inhibitor [29], was also investigated. DPI strongly inhibited FNR activity at rather low concentrations (around 5 μM), whereas much higher concentrations (about 50 μM) were required to inhibit the chlorophyll fluorescence increase measured following NADH or NADPH additions (Fig. 4). When NADH

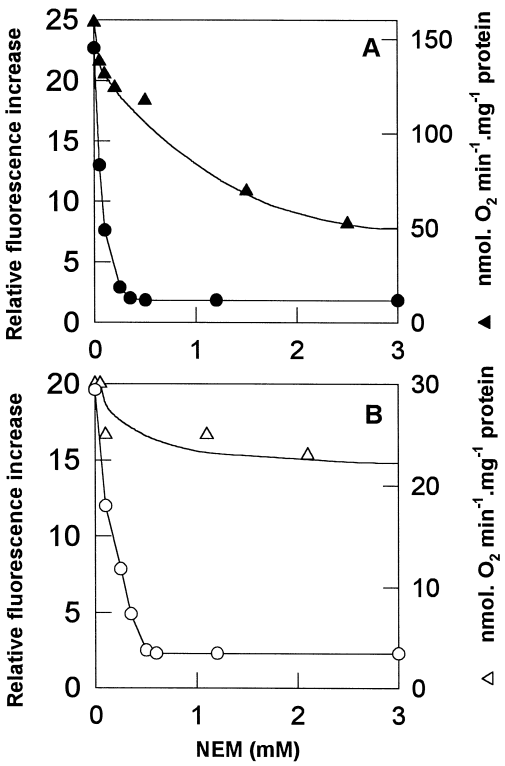

Fig. 3. Effect of NEM on the rate of apparent chlorophyll fluorescence increase measured in osmotically lysed potato chloroplasts and on the activity of spinach FNR measured in the presence of NADPH (A) or NADH (B). Fluorescence increase measured under anaerobic conditions following addition of 2 mM NADH (○) or 2 mM NADPH (●); activity of purified spinach FNR measured as O_2 uptake in the presence of MV and 2 mM NADH (△) or 2 mM NADPH (▲).

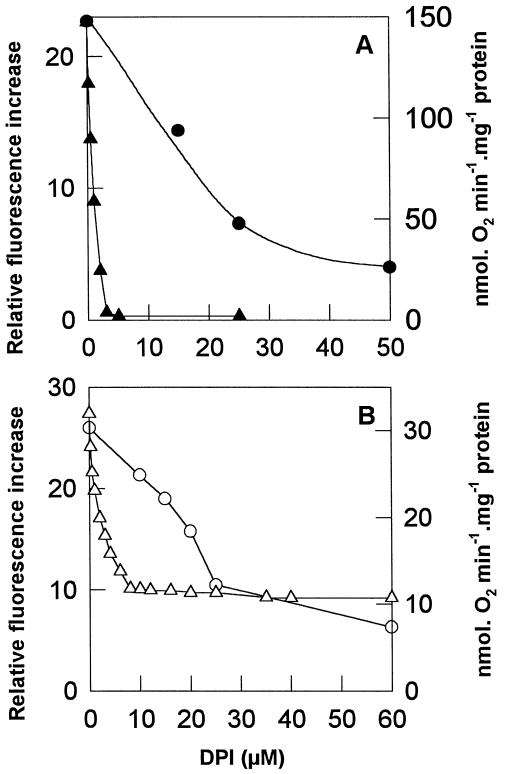

Fig. 4. Effect of DPI on the rate of apparent chlorophyll fluorescence increase measured in osmotically lysed potato chloroplasts and on the activity of spinach FNR measured in the presence of NADPH (A) or NADH (B). Fluorescence increase measured under anaerobic conditions following addition of 2 mM NADH (○) or 2 mM NADPH (●); activity of purified spinach FNR measured as O_2 uptake in the presence of MV and 2 mM NADH (△) or 2 mM NADPH (▲).

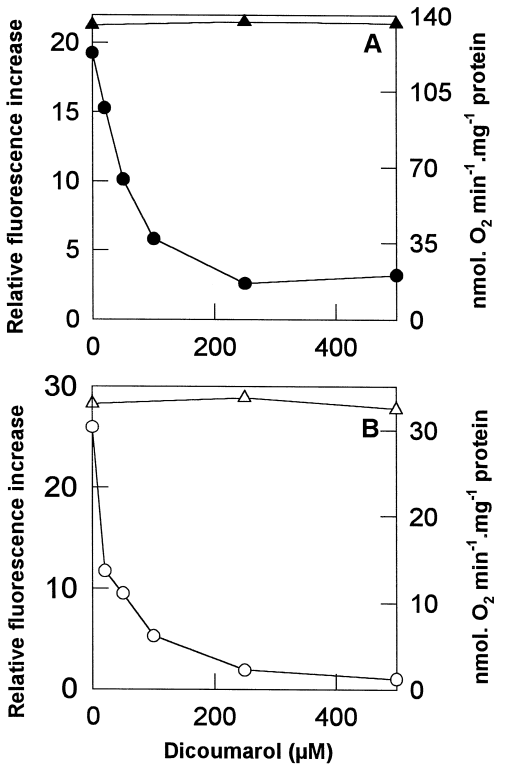

Fig. 5. Effect of dicoumarol on the rate of apparent chlorophyll fluorescence increase measured in osmotically lysed potato chloroplasts and on the activity of spinach FNR measured in the presence of NADPH (A) or NADH (B). Fluorescence increase measured under anaerobic conditions following addition of 2 mM NADH (○) or 2 mM NADPH (●); activity of purified spinach FNR measured as O_2 uptake in the presence of MV and 2 mM NADH (△) or 2 mM NADPH (▲).

was used as a substrate, a significant part (about 30%) of the reaction catalyzed by FNR was insensitive to DPI. The effect of dicoumarol, a potent inhibitor of DT-diaphorase, a one subunit NAD(P)H:quinone oxidoreductase [30], was also investigated (Fig. 5). Both NADH- or NADPH-induced reduction of PQ were inhibited by 250 μM dicoumarol, whereas this compound had no significant effect on FNR activity (Fig. 5). In contrast, we found that the dark reduction of PQ was not inhibited and even stimulated by antimycin A (at 20 μM final concentration), a compound previously reported to inhibit FQR-mediated reduction of PQ [20]. Also, the non-photochemical reduction of PQ was insensitive to rotenone (1 mM) and to piericidin A (2 μM), both potent inhibitors of complex I (Table 1).

3.3. Electron flow involved in the non-photochemical reduction of intersystem electron acceptors

We then attempted to determine the electron flow rate involved in the reduction of PS I electron donors

Table 1
Effect of antimycin A, rotenone and piericidin A on the rate of chlorophyll fluorescence increase measured following the addition of 2 mM NADH to osmotically lysed potato chloroplasts under anaerobic conditions (glucose/glucose oxidase)

	Relative rate of chlorophyll fluorescence increase
Control	100
Antimycin A (20 μM)	331
Rotenone (1 mM)	98
Piericidin A (2 μM)	96

by measuring the light-dependent O_2 uptake rate in the presence of DCMU and MV. In these conditions, PS II cannot reduce the PQ pool and the light-dependent O_2 uptake rate reflects the ability of PS I to reduce O_2 using intersystem electron donors. As shown on Fig. 6(A), a light-dependent O_2 uptake of about 300 nmol O_2 min^{-1} mg^{-1} chlorophyll is measured when NADPH is used as an electron donor. In the presence of NADH, the O_2 uptake rate is lower (about 200 nmol O_2 min^{-1} mg^{-1} chlorophyll). Apparent affinities for NADH or NADPH appear somewhat different depending on the method used: around 1 mM when using the chlorophyll fluorescence increase and around 100 μM when the estimation was based on O_2 uptake measurements. This difference is likely due to the fact that at the equilibrium, the redox state of Q_A is lower than the redox state of the PQ pool (see above), thus leading to an under-estimation of the redox state of the PQ and of the apparent substrate affinities when using the method based on chlorophyll fluorescence measurements. The NADH-dependent reaction is inhibited by more than 80% by 1 mM dicoumarol, whereas the NADPH-dependent reaction was only inhibited by 20% by this compound (Fig. 6(B)). Similarly, the NADH-dependent reaction was inhibited by 85% by 2 mM NEM, whereas the NADPH reaction was only inhibited by 40% (Fig. 6(C)). If we compare these inhibitor effects to that observed on the fluorescence rise (Figs. 3–5), it clearly appears that the NADH-dependent O_2 uptake reaction (Fig. 6) and the NADH-induced fluorescence increase (Figs. 1–5) are similarly inhibited by dicoumarol and NEM, while the NADPH-dependent O_2 uptake reaction appears relatively insensitive to these compounds.

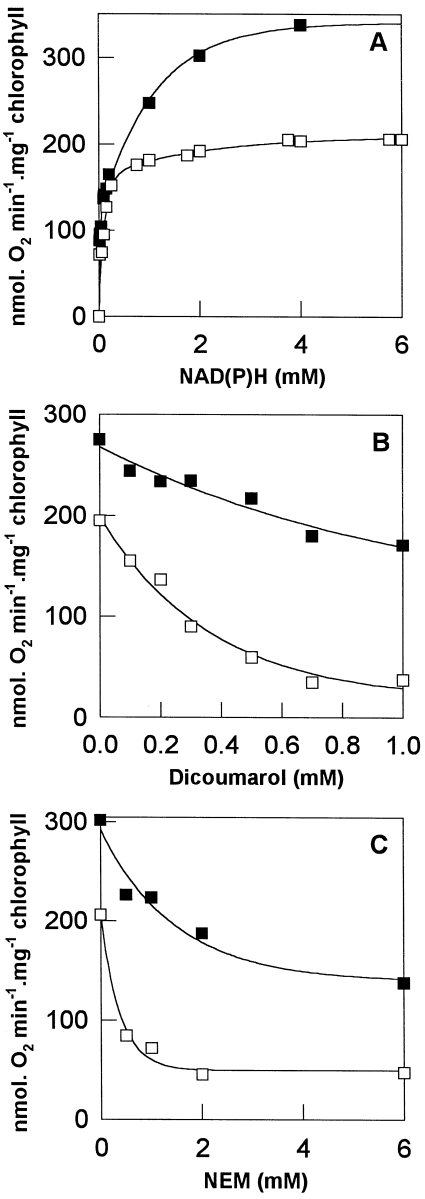

Fig. 6. Light-induced oxygen uptake measured in osmotically lysed potato chloroplasts in the presence of DCMU, MV and NADH (□) or NADPH (■) as electron donors. (A) effect of varying NADH or NADPH concentrations; (B) effect of dicoumarol on the reaction measured in the presence of 2 mM NADH or 2 mM NADPH; (C) effect of NEM on the reaction measured in the presence of 2 mM NADH or 2 mM NADPH.

4. Discussion

The results presented in this paper show that exogenous NADH and NADPH can be used as electron donors to the PQ pool in higher-plant chloroplasts. Both the substrate specificity of this reaction which is almost as efficient for NADH or NADPH and inhibitor effects indicate that FNR is likely not involved in this reaction. Indeed, as already reported in the literature, the diaphorase activity of spinach FNR is much more efficient when using NADPH rather than NADH as a substrate [28]. Also, the NAD(P)H–PQ oxidoreductase activity is inhibited by concentrations of NEM (500 μM) and dicoumarol (250 μM) which have no effect on the diaphorase activity of

FNR. On the other hand, FNR is inhibited by rather low DPI concentrations (around 5 μM), whereas significantly higher concentrations (around 30 μM) are required to inhibit the NAD(P)H–PQ oxidoreductase activity. Taken together, these results argue against a direct participation of FNR in the dark reduction of PQ and favor the existence of a NAD(P)H–PQ oxidoreductase. Another possibility, that we consider very unlikely, would be that membrane-bound and soluble FNR are characterized by different properties (different substrate specificity and different inhibitor effects). The absence of inhibition of the dark reduction of PQ by antimycin A (Table 1) shows that FQR, which has been reported to be inhibited by this compound [18], is likely not involved in this reaction under the experimental conditions used in this study. This conclusion is in apparent contradiction with the data of Mills et al. [31] reporting the involvement of FNR in the electron transfer reaction from NADPH to PQ in osmotically lysed spinach chloroplasts as well as the inhibition of this phenomenon by antimycin A. Experimental conditions used by these authors were however somewhat different from the conditions used here. Indeed, experiments were performed under aerobic conditions, therefore, allowing oxidation of reduced PQ through the chlororespiration PQ oxidation pathway. This probably explains why only 50% reaction centers could be closed upon NADPH addition [31]. Also, addition of exogenous ferredoxin was required to observe the NADPH-dependent PQ reduction which was not the case in the present work. Recently, by studying the transient increase in chlorophyll fluorescence observed after saturating illumination in spinach chloroplasts, Mano et al. [32] reported the sensitivity of this reaction to antimycin A and concluded to a participation of both cytochrome b_{559} and FNR. However, this reaction was not observed after an hypo-osmotic treatment unless ferredoxin was added. It clearly appears that depending on the experimental conditions used, different pathways of non-photochemical PQ reduction can be induced. The stimulation of PQ reduction by antimycin A (Table 1) was somewhat surprising: this effect might be explained, as already reported in *Chlamydomonas* [33], by an effect of this compound on the PQ oxidation pathway of chlororespiration.

The molecular nature of the NAD(P)H–PQ oxidoreductase activity reported here is an important question to clarify. A set of eleven genes, homologous to genes encoding subunits of the mitochondrial NADH dehydrogenase (complex I) and called *ndh* genes, have been found in the plastid genome of higher plants [7,34]. Some *ndh* gene products have been localized in thylakoid membranes [9–14] and recently, Guedeney et al. [12] and Sazanov et al. [16] supplied evidence for the participation of different *ndh* gene products to a NAD(P)H dehydrogenase complex. Friedrich et al. [35], by comparing chloroplast *ndh* gene sequences with bacterial and cyanobacterial genes encoding subunits of the NADH dehydrogenase complex, concluded that the chloroplast NADH dehydrogenase complex likely resembles bacterial-type complexes. In mitochondria or in bacteria, the activity of complex I has been reported to be strongly inhibited by rotenone or piericidin A [36]. The site of action of rotenone has been reported to be located at the level of hydrophobic subunits of the complex [37], which are rather well-conserved between cyanobacteria and chloroplasts. Berger et al. [38] reported that the cyanobacterial complex was sensitive to rotenone, although rather high rotenone concentrations (40 mM) were used in this study. Interestingly, these authors observed that a significant part of the NADH-oxidizing activity measured in *Synechocystis* PCC6803 was insensitive to rotenone and suggested the existence of a rotenone-insensitive NADH dehydrogenase. Recently, full sequencing of the cyanobacterial genome revealed the presence of a coding sequence homologous to the rotenone-insensitive NADH–quinone oxidoreductase of *Saccharomyces cerevisiae* [39]. The insensitivity of the NAD(P)H–PQ oxidoreductase activity to rotenone (at 1 mM final concentration) and piericidin A (at 2 μM final concentration) would argue against the participation of a bacterial-type complex I. However, the rotenone sensitivity of the NADH dehydrogenase coded by *ndh* genes has not been established and the cyanobacterial complex has been reported to be inhibited by extremely high rotenone concentration (40 mM). Prokaryotic-type complex I have been reported to be much more labile than mitochondrial complexes, thus resulting in loss of activity during purification procedures [40]. One may consider that the hypo-osmotic choc used to permeabilize chloroplasts might have affected both the integrity and activity of the chloroplast complex. In these condi-

tions, remaining NAD(P)H–PQ oxidoreductase activity might be due to a rotenone-insensitive enzyme.

The existence of rotenone-insensitive NADH–quinone oxidoreductase has been reported in animals [30] and in plants [41]. This enzyme, also referred as DT-diaphorase, is a flavoprotein which catalyzes an obligatory two electron-reduction of quinones and uses either NADH or NADPH as electrons donors. In plants, this enzyme is mostly soluble, although some activity has been found associated to the membrane fractions [41]. In contrast to the rat liver enzyme which is very sensitive to dicoumarol, the tobacco NAD(P)H–QR has been reported to be weakly inhibited by this compound (about 30% of inhibition at 100 μM – see Ref. [41]). The plant complex has also been reported to be characterized by a similar and quite low affinity (around 380 μM) for both NADH and NADPH [41]. Most of the properties of the non-photochemical reduction of PQ by exogenous NADH or NADPH reported here would be consistent for the participation of a DT-diaphorase: (i) rather low and comparable affinities for NADH and NADPH (around 100 μM – see Fig. 6), (ii) sensitivity to the flavoenzyme inhibitor DPI, (iii) sensitivity to dicoumarol, (iv) insensitivity to rotenone and piericidin A, (v) sensitivity to NEM, the plant NAD(P)H–QR containing two 21.4 kDa subunits linked by a disulfide bridge [41]. Future work will be needed to demonstrate the activity of the NADH dehydrogenase complex encoded by *ndh* genes and to elucidate the protein complexes involved in the non-photochemical reduction of PQ in higher plant chloroplasts.

Similar effects of dicoumarol and NEM both on the NADH-dependent reduction of PQ and on the NADH-dependent O_2 uptake, indicate that both NADH-dependent reactions are mediated by the same activity. Accordingly, the electron flow rate involved in the reduction of PQ can be ascribed to the dicoumarol-sensitive component of the oxygen uptake rate measured in the presence of NADH (about 160 nmol O_2 min^{-1} mg^{-1} chlorophyll – see Fig. 6). On the other hand, the light-dependent O_2 uptake reaction measured in the presence of NADPH is most likely due to the superimposition of two different phenomena. The NADH–PQ oxidoreductase activity responsible for the fluorescence increase, likely drives part of the NADPH-dependent oxygen uptake reaction. This component of the reaction, sensitive to dicoumarol and NEM (Fig. 6), can be estimated to about 60% of the NADH-dependent reaction (on the basis of Fig. 2), that is about 120 nmol O_2 min^{-1} mg^{-1} chlorophyll (corresponding to 60% of 200 nmol O_2 min^{-1} mg^{-1} chlorophyll). The other component of the NADPH-dependent reaction, mainly insensitive to NEM and dicoumarol (Fig. 6(B) and (C)), was found to be inhibited by low (5 μM) DPI concentrations (data not shown), thereby indicating the participation of FNR in this phenomenon. Interestingly, the electron pathway involving FNR and NADPH, would not involve reduction of PQ since low DPI concentration had no significant effect on the fluorescence rise (Figs. 3–5). This phenomenon might possibly be explained by an entry of electrons in the inter-system chain via FNR and the cytochrome b_6/f complex or by the light-activation of FNR [42], which would result in an increase of the MV-mediated O_2 uptake through the diaphorase activity of this enzyme.

Evidence for chlororespiration, defined as a respiratory electron transport chain located in thylakoid membranes, has been mainly obtained in green unicellular algae from fluorescence or oxygen exchange measurements [1,2]. Although no chloroplastic *ndh* genes have been detected in green algae, the discovery of *ndh* genes in the chloroplast genome of higher plants was taken as an evidence for the existence of a chlororespiration in higher plant chloroplasts. Some attempts to detect chlororespiration in higher plants have been reported earlier [6,43]. Garab et al. [6] reported that cyanide or CO interact with cytochromes of the photosynthetic electron transport chain and concluded to the existence of a terminal oxidase. More recently, Gruszecki et al. [43] claimed that flash-induced oxygen uptake measured by using ^{18}O-enriched O_2 and mass spectrometry could be interpreted by the existence of a similar pathway to that studied in green algae [1,2]. It should be however stressed that the mass spectrometric data of Gruszecki et al. [43] are quite different from that reported by Peltier et al. [2] in the green alga *Chlamydomonas*. Indeed, Gruszecki et al. [43] reported the existence of a PS I-mediated flash-induced O_2 consumption, whereas Peltier et al. [2] observed a flash-induced inhibition of a respiratory process. If the flash-induced inhibition can be interpreted by a transitory inhibition by PS I of a respiratory process, the flash-

induced O_2 stimulation reported by Gruzsecki et al. [43] can be more adequately explained by the existence of a PS I-mediated O_2 consumption occurring during Mehler-type reactions and this study cannot be regarded as supplying a conclusive evidence for the existence of a chlororespiration in higher plant chloroplasts [44]. In the present study, we show that electrons originating from a soluble NADH or NADPH pool can enter the PQ pool. Moreover, the effects of anoxia on fluorescence transients recorded upon NADH or NADPH addition clearly show the existence of a substantial electron flow from reduced PQ to O_2. We therefore conclude, as already suggested by Garab et al. [6], that higher plant chloroplast contain a chlororespiratory activity and propose that depending on experimental conditions, chlororespiration may involve either a complex I-like NADH dehydrogenase or a DT-diaphorase-like enzyme.

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