Characterization of Nda2, a Plastoquinone-reducing Type II NAD(P)H Dehydrogenase in Chlamydomonas Chloroplasts

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Electron transfer pathways associated to oxygenic photosynthesis, including cyclic electron flow around photosystem I and chlororespiration, rely on non-photochemical reduction of plastoquinones (PQs). In higher plant chloroplasts, a bacterial-like NDH complex homologous to complex I is involved in PQ reduction, but such a complex is absent from Chlamydomonas plastids where a type II NAD(P)H dehydrogenase activity has been proposed to operate. With the aim to elucidate the nature of the enzyme-supporting non-photochemical reduction of PQs, one of the type II NAD(P)H dehydrogenases identified in the Chlamydomonas reinhardtii genome (Nda2) was produced as a recombinant protein in Escherichia coli and further characterized. As many type II NAD(P)H dehydrogenases, Nda2 uses NADH as a preferential substrate, but in contrast to the eukaryotic enzymes described so far, contains non-covalently bound FMN as a cofactor. When expressed at a low level, Nda2 complements growth of an E. coli lacking both NDH-1 and NDH-2, but is toxic at high expression levels. Using an antibody raised against the recombinant protein and based on its mass spectroscopic identification, we show that Nda2 is localized in thylakoid membranes. Chlorophyll fluorescence measurements performed on thylakoid membranes show that Nda2 is able to interact with thylakoid membranes of C. reinardtii by reducing PQs from exogenous NADH or NADPH. We discuss the possible involvement of Nda2 in cyclic electron flow around PSI, chlororespiration, and hydrogen production.

Higher plant thylakoid membranes contain a multisubunit Ndh complex resembling bacterial complex I, which mediates plastoquinone (PQ)3 reduction from stromal pyridine nucleotide (NADH or NADPH) electron donors and therefore participates in the establishment of cyclic electron transfer around Photosystem I (PSI) and chlororespiration (1–3). Although significant activity of non-photochemical PQ reduction was reported in Chlamydomonas reinhardtii thylakoids (4, 5), it is generally considered that a type I Ndh complex is absent from algal chloroplasts (2). Indeed, most microalgal species, such as C. reinhardtii, and some gymnosperms species, such as Pinus thunbergii, do not harbor plastid ndh genes encoding hydrophobic subunits of the type I Ndh complex and, at least in the case of C. reinhardtii, the corresponding genes are absent from the nuclear genome, therefore, questioning the nature of the enzyme involved in non-photochemical reduction of PQs (2, 5).

Non-photochemical reduction of PQs is involved in several processes, which have been rather well-documented in C. reinhardtii, including chlororespiration (2, 6), cyclic electron flow around PSI (7), and state transition (8, 9). More recently, non-photochemical reduction of PQs has also been involved in the ability of algae to sustain hydrogen production during the anaerobic phase of stanch to hydrogen conversion (10, 11), this reaction considered as a limiting step of the process (10).

In this context, unraveling the nature of the enzymes involved in non-photochemical reduction of PQs in C. reinhardtii, appears as an important goal toward elucidating chlororespiration, cyclic electron transfer pathways operating around PSI, as well as mechanisms of hydrogen production. Based on pharmacological and physiological studies, we previously proposed that a NDH-2 type enzyme might be responsible for this activity in C. reinhardtii thylakoids (5). Type II NAD(P)H-dehydrogenases are monomeric enzymes that contain a flavin cofactor and, unlike type I NAD(P)H dehydrogenases (complex I), are non-proton pumping enzymes. Numerous sequences encoding putative NDH-2 have been found in various organisms, including bacteria, yeasts, and plants (12–14). The Arabidopsis thaliana genome contains three gene families, in total seven reading frames, encoding NDH-2 homologs (15). Based on GFP localization studies and in vitro protein import assays, six of these putative A. thaliana NDH-2 were located in mitochondria (15, 16).

In silico screening revealed the presence of several putative NDH-2 in the C. reinhardtii genome (17). One of them (Nda2) was produced as a recombinant protein and further characterized. Using antibodies directed against the recombinant protein, we show that Nda2 is located in chloroplasts. Based on chlorophyll fluorescence measurements, we show that Nda2 reduces plastoquinones using NADH or NADPH as substrates.
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The function of Nda2 is discussed in relation to its possible involvement in chlororespiration, cyclic electron transfer reactions, and H₂ photoproduction.

EXPERIMENTAL PROCEDURES

Algal and Bacterial Strains and Chemicals—C. reinhardtii cells were grown on a Tris-acetate-phosphate medium (TAP) (18). Algal cultures were grown at 25 °C under continuous agitation and low (1 μmol of photons m⁻² s⁻¹) or medium (about 60 μmol of photons m⁻² s⁻¹) light intensity. The wild-type (WT) strain used in this work was isolated as a mt⁺ segregant of a cross between two strains, which are isogenic to the 137c strain. The C. reinhardtii cell wall-deficient mutant strain CW15 was used for the preparation of chloroplast- and mitochondria-enriched fractions. Cloning and production of recombinant Nda2 were performed in the Escherichia coli dH10B strain. The E. coli mutant lacking both NDH-1 and NDH-2 (ANN0222) was generously provided by Prof. Thorsten Friedrich (University of Freiburg, Germany); this mutant was obtained using the AN387 strain as background. Unless specified, E. coli strains were grown on a LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7). In some experiments, the E. coli strains ANNO222 and AN387 were grown on a minimal M9 medium (2 mM MgSO₄, 10 mM CaCl₂, 47 mM Na₂HPO₄, 22 mM KH₂PO₄, 8.5 mM NaCl, 18 mM NH₄Cl, pH 7.2) supplemented with 0.4% mannitol. Unmentioned otherwise, all chemicals were purchased from Sigma.

Cloning of NDA2—Total RNA was isolated from TAP cultures of WT C. reinhardtii (10⁶ to 10⁷ cells ml⁻¹) with the RNeasy® Plant Mini Kit (Qiagen, Courtaboeuf, France). First-strand cDNA synthesis was performed using the Omniscript™ Reverse Transcriptase system (Qiagen, Courtaboeuf, France). cDNAs were amplified by PCR with Turbo Pfu polymerase enzyme (Stratagen, Amsterdam, NL) using the following primers: (F) 5’-ATGAGCCTTGATGGCCAAAAC-3’ and (R) 5’-TCACACTCCGAGATGTCGCG-3’ (supplemental Fig. S1). EcoRI and SmaI sites (in italics below) were, respectively, inserted upstream and downstream of the start and the stop codons, and a (His)₆-tag coding sequence (underlined) was inserted right upstream of the start codon. For this purpose, the following oligonucleotides were used: 5’-CGGAATT-CATGATCATCATCTACATCATGATGGCCAAAC-3’ and 5’-TCCCCGGGCCACACTCCGAGATGTCGCG-3’. The amplified fragment was digested by EcoRI and SmaI, and ligated into a digested pSD80 plasmid (19). The resultant plasmid (pSD80-NDA2) was sequenced and further used to transform E. coli (strain DH10B) by electroporation. Transformed strains resistant to 100 μg ml⁻¹ ampicillin were screened by PCR by using (F) and (R) primers.

Colony-forming Assays—AN387 and ANN0222 E. coli competent cells were chemically transformed with the pSD80-NDA2 plasmid and transformants were selected on LB agar containing ampicillin (100 μg ml⁻¹). Transformation with an empty pSD80 vector was performed as a control. Transformants were grown to mid-exponential phase at 37 °C in LB medium containing ampicillin (100 μg ml⁻¹). Cells were washed once with sterile M9 medium and serially diluted in the M9 medium supplemented with 0.4% mannitol as the sole source of carbon. Diluted cells suspensions were then spotted on agar plates containing 0.4% mannitol, 100 μg ml⁻¹ ampicillin and 0.1 mM IPTG and further incubated at 37 °C for 2 days. As a control, diluted cell suspensions were spotted on LB agar plates containing 100 μg ml⁻¹ ampicillin and 0.1 mM IPTG and incubated at 37 °C overnight.

Phylogenetic Analysis—Six C. reinhardtii putative NDH-2 (called NDAX) amino acid sequences were aligned using ClustalW 2.0.9 software (20) with homologous sequences from bacteria, fungi, and plants. Corresponding GenBank™ (GenPept) accession numbers, NCBI RefSeq numbers, or references of the protein sequences used are: E. coli(-ndh): NP_415627; Acidimias ambivalens: CAD33806; Rhodopseudomonas palustris: NP_946455; Halobacterium: NP_279851; Bacillus subtillis: NP_389111; Deinococcus radiodurans: NP_294674; (A. thaliana)At-NDC1: NP_568205; Synecocystis-slr1743: NP_441103; Nostoc-5: NP_488134; Synecocystis-slr1484: NP_442910; Nostoc-6: NP_489251; Nostoc-4: NP_487004; Corynebacterium glutamicum: NP_600682; Agrobacterium tumefaciens: NP_354992; Synecocystis-slr0851: NP_441107; Nostoc-1: NP_485169; Neurospora crassa-NDE2: XP_959908; Saccharomyces cerevisiae-NDE1: NP_013586; S. cerevisiae-NDE1: NP_013865; S. cerevisiae-NDE2: NP_011989; N. crassa-NDC1: NP_013586; Trypanosoma brucei: AAM5239; Oryza sativa-NDA2: NP_001060003; O. sativa-NDA1: NP_001044694; Solanum tuberosum-NDA1: CAB52796; (A. thaliana)At-NDA1: NP_563783; (A. thaliana)At-NDA2: NP_180560; N. crassa-NDE1: CAB41986; (A. thaliana)At-NDB1: NP_567801; S. tuberosum-NDB1: CAB52797; (A. thaliana)At-NDB1: NP_567801; (A. thaliana)At-NDB4: NP_179673; (A. thaliana)At-NDB2: NP_567283; (A. thaliana)At-NDB3: NP_193880; (C. reinhardtii)NDA1: XP_001698901; (C. reinhardtii)NDA2: the sequence used is derived from XP_001703643 and from the cDNA sequenced (supplemental Fig. S1); (C. reinhardtii)NDA3: XP_001702271; (C. reinhardtii)NDA5: XP_001691969; (C. reinhardtii)NDA6: XP_001703055; (C. reinhardtii)NDA7: XP_001703056. Next, the resulting alignment was manually refined using SeaView (21) and regions where homology was doubtful were removed from further analysis. A total of 305 amino acid positions were kept for the phylogenetic analysis (Additional file 2). Phylogenetic analyses were conducted using Neighbor-Joining (NJ), Maximum Likelihood (ML) and Parsimony (Pars) approaches in the Phylogenetic Inference Package PHYLP version 3.67 (22). The PROTDIST program was used for ML analysis and the sequence input order was randomized (20 jumbles). The PROTDIST program was used to create distance matrices. The SEQBOOT and CONSENSE programs were used for bootstrap value calculations on 100 replications and consensus tree reconstructions, respectively. To examine the confidence of nodes, Neighbor-Joining and Parsimony analysis were done using the NEIGHBOR and protpars programs. The phylogenetic tree was drawn with MEGA4 (23).

Production and Purification of His-tagged Nda2—Bacterial cells expressing His-tagged Nda2 were grown at 37 °C in a LB medium supplemented with ampicillin (100 μg ml⁻¹) to an absorbance of 0.5 measured at 600 nm. After induction by 100 μM IPTG and overnight culture at room temperature, bacteria were pelleted by centrifugation and lysed using a French press.
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(16,000 p.s.i.) in buffer A (25 mM imidazole pH 7.5, 20 mM amine-triethanol, 500 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine). The supernatant was recovered by centrifugation (12,000 × g for 20 min) and used for further purification of His-tagged Nda2 by metal-chelating chromatography as previously described (24). After addition of glycerol (50% v/v), the purified enzyme was stored at −20 °C. A rabbit serum was raised against the purified Nda2 protein (Agro-Bio, Villeny, France). The nature of the flavin cofactor of Nda2 was determined by HPLC as described by Ref. 25 after protein denaturation using 10% trichloroacetic acid, FAD, and FMN standards were used as controls and subjected to the same extraction procedure. Chromatographic mobility and absorption spectra of standards were not affected by the extraction procedure, showing that no conversion of FAD into FMN occurred during the experiment.

Activity Measurements—Assays of NADH or NADPH oxidation were performed by monitoring absorbance decay at 340 nm. NADH or NADPH, electron acceptors, and protein extracts were added successively. Bovine SOD (500 units ml⁻¹) and catalase (1000 units ml⁻¹) were added to the assay medium. Measurements of O₂ uptake by E. coli membranes in the presence of NAD(P)H and measurements of NAD(P)H-triggered chlorophyll fluorescence rise in thylakoid preparations from C. reinhardtii were performed according to Ref. 25.

Protein Separation and Immunoblot Analysis—C. reinhardtii or E. coli protein extracts were loaded on 10% SDS/PAGE gel and transferred to nitrocellulose membrane using a semidry transfer technique when not specified otherwise. A commercial antibody directed against the His tag sequence (Sigma Ref. H-1029) was used to identify the purified protein on 13% SDS/PAGE gels. The Nda2 antiserum was diluted to 1:10,000 and Alexa 680 goat anti-rabbit (Invitrogen, Molecular Probes) was used as a secondary antibody. Immunoblots were scanned by using a LICOR Odyssey system (Li-Cor Biosciences, Lincoln, NE).

Preparation of Chloroplasts and Mitochondria—C. reinhardtii chloroplasts and mitochondria were prepared according to Ref. 26 with a few modifications. Synchronized cells (i.e. grown under a 12-h/12-h light/dark regime) of a cell wall-deficient C. reinhardtii strain (cw15) were grown on TAP medium to middle exponential phase (around 5 × 10⁶ cells ml⁻¹). Cells were harvested at the beginning of the light period, pelleted by centrifugation (2,000 × g for 10 min), and resuspended in buffer BB (50 mM HEPES-KOH, pH 7.5, 0.25 M sorbitol, 10 mM MgCl₂, 2 mM EDTA) supplemented with 1% bovine serum albumin. Cells were lysed using a Yeda press (4.2 bar, 210 s) and briefly centrifuged (760 × g for 2 min). Mitochondria, in the supernatant, were pelleted (10,000 × g for 10 min) and further purified on Percoll gradient (27). The pellet, containing intact or partially broken cells and intact chloroplasts, was resuspended in buffer BB supplemented with 1%
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bovine serum albumin and loaded on a Percoll discontinuous gradient (20, 45, and 65% Percoll). After centrifugation (4,200 × g for 15 min) intact chloroplasts were collected at the 45–65% Percoll interface (28). Intact chloroplasts were washed twice in 5 volumes of buffer BB with brief centrifugation (670 × g for 2 min). Fractionation of chloroplasts was performed as previously described (29).

Two-dimensional Gel Electrophoresis—Two-dimensional gel electrophoresis was performed by using a Zoom Benchtop Proteomics system (Invitrogen) according to the manufacturer’s instructions with few modifications. Intact chloroplast proteins (100 μg) were precipitated twice with acetone (80 and 100% v/v, respectively) and resuspended in R2D2 solubilization buffer (30). Proteins were first focused on a non-linear 3–10 pH gradient strip, then separated on a 4–12% acrylamide gradient gel. The in-gel immunodetection of Nda2 was performed using an Odyssey system (Li-Cor Biosciences, Lincoln, NE) according to the manufacturer’s instructions. After excision, stained polypeptides were sequenced by MS/MS as previously described (31). The homology threshold given by Mascot (p value < 0.05) has been previously described (31) was 19 for the data base search that identified the protein XP_001703643. As the peptide Mascot scores were above this threshold (22 and 55 for spots 1 and 2, respectively), the protein was validated by inhouse software used for protein identification (IRMa, CEA/DSV/irTISV/LEDyP).

RESULTS

Six C. reinhardtii Genes Encode for Putative Type II NADH Dehydrogenases—A data base screening of the C. reinhardtii nuclear genome based on sequence homologies with A. thaliana NDH2 previously revealed the presence of seven gene models (NDa1 to NDa7) encoding putative type II NADH dehydrogenases (17). Among the 7 gene models initially annotated in the JGI version 2.0 of the C. reinhardtii genome, one (NDa4) was suppressed in JGI version 3.0, NDa2 and NDa4 being proposed to encode the same protein (Nda2). The six C. reinhardtii Nda amino acid sequences were compared with homologous sequences from bacteria, fungi, and plants by performing a phylogenetic analysis (Fig. 1). Plant type II NAD(P)H dehydrogenases have been previously reported to group in three distinct subgroups (NDA, NDB, NDC), based on homologies with cyanobacterial and fungal sequences (15). According to our phylogenetic analysis, the C. reinhardtii Nda2 and Nda3 appear to be, respectively, members of the plant NDB and yeast NDE/NDI groups, respectively. On the other hand, the C. reinhardtii Nda1 sequence was relatively close to plants NDA, although these sequences did not form a cladistic group (Fig. 1). Nda5, and possibly Nda6 and Nda7, appear to belong to the plant NDC group, which contains plant and cyanobacterial proteins. Nda2 was chosen in the present study for further characterization. Based on the motif prediction tool MOTIF_SCAN, the Nda2 sequence is predicted to contain a conserved nucleotide-binding motif attributed to NAD(P)H binding (Fig. 2). Although no nucleotide-binding motif was predicted in this region, the 79–110 sequence aligned with predicted FAD-binding domains of several type II NAD(P)H dehydrogenases protein sequences (see supplemental Fig. S2). Although the NAD(P)H-binding motif contains the GXXGXXG sequence of the ADP-binding fingerprint (32), the putative flavin-binding motif contains a related sequence in which the third G residue is replaced by an A residue. Note that the presence of an A residue in place of the third G has been reported in other cases, for instance in the FAD-binding motif of the Arabidopsis NADPH-dependent thioredoxin reductase (33). The Glu residue at the end of the second βαβ-fold domain of Nda2 indicates a likely substrate preference for NADH versus NADPH (34).

Nda2 Complements Growth of an E. coli Mutant Lacking NDH-1 and NDH-2—To determine whether NDA2 encodes a functional protein type II NAD(P)H dehydrogenase, it was expressed in an E. coli mutant strain (ANN022), lacking both NDH-1 and NDH-2 (Fig. 3). This deleted strain grows normally on rich (LB) medium, but is unable to grow on minimal medium (M9) supplemented with mannitol as the sole source of carbon (25). The ANN022 strain harboring the pSD80-NDa2 construct was able of growth on a minimal medium in the absence of IPTG, but not in the presence of the inducer (Fig. 3A). Such a behavior, which was previously observed in the case of the A. tumefaciens NDH-2 (25), was attributed to a promoter leakage in the absence of IPTG, and to the fact that Nda2 could be toxic at a high expression level. This interpretation is substantiated by the significant protein expression observed in the
absence of IPTG (Fig. 3B) and by the growth inhibition observed when Nda2 was expressed in the control strain (AN387), after IPTG induction on a LB rich medium. We conclude from these experiments that at a low expression level, Nda2 is able to complement growth of E. coli lacking both NDH-1 and NDH-2 by interacting with the bacterial respiratory chain, while at a high expression level Nda2 is toxic for bacterial cells.

**Purification and Characterization of a Recombinant Nda2**—Following induction by IPTG and cell disruption, Nda2 was recovered in two fractions, a soluble yellow-colored protein fraction (containing the flavin cofactor) and a non-colored insoluble protein fraction, most likely composed of non-folded protein present as inclusion bodies. Recombinant Nda2 was purified from the soluble pool by Ni²⁺ affinity chromatography (Fig. 4A). Eluted fractions E1 and E2 harbored a yellow color indicating the presence of a flavin cofactor (37). A single polypeptide of ~61 kDa was eluted by 200 mM imidazole and was recognized by an antiserum raised against a polyHis sequence (Fig. 4B). The absorption spectrum of Nda2 (Fig. 4C) was characterized by two peaks (at 373.5 and 446.5 nm) typical of flavoproteins (38). The stoichiometric ratio of the flavin cofactor was estimated around 1.3 mol per mol of protein. Release of the flavin cofactor was induced by trichloroacetic acid denaturation, thus showing that it is noncovalently bound to the protein. The flavin cofactor, determined by reverse-phase HPLC and fluorescence detection, was shown to be FMN (Fig. 4D). Note that the presence of FMN as a cofactor is a rather unusual feature among members of the NDH-2 family, which contain FAD in most cases (12).

The capacity of purified Nda2 to oxidize NADH and NADPH was measured by monitoring absorbance decay at 340 nm in presence of ubiquinone (UQ) or Q₀ as electron acceptors. A significant activity was repetitively observed in the presence of decyl-ubiquinone or decyl-plastoquinone, but likely due to the relative insolubility of this quinone in the conditions of the experiments, measurements were variable from one experiment to the other (supplemental Fig. S3). For this reason, the activity characterization was performed in the presence of Q₀ (Fig. 5 and Table 1). Nda2 was found able to significantly oxidize NADH at pH ranging from 5.5 to 9.0 with an optimum at neutral pH (Fig. 5A). Significant, but much lower NADPH oxidation rates were observed at acidic pH from 5.5 to 6.0, while the activity at neutral pH remained very low (Fig. 5B). Such a pattern of activity has already been described for other NADH-dependent NDH-2 enzymes (35, 39, 40). Catalytic properties of Nda2 were determined at optimal pH by using NADH or NADPH as electron donors and Q₀ as an acceptor (Table 1). The $K_{\text{mNADH}}$ ($K_m$ referring to the Michaelis-Menten constant, following subscript indicating substrate) value was about 19-fold lower than the $K_{\text{mNADPH}}$ value, reflecting a clear preference of Nda2 for NADH versus NADPH. Catalytic efficiencies, determined by the ratio $k_{cat}/K_m$, was about 130-fold higher for NADH than for NADPH. In absence of acceptors, NDH-2 enzymes have been reported to directly interact with O₂ (25, 41). By measuring O₂ consumption in the presence of NADH or NADPH, we found that Nda2 is able to directly reduce O₂. $V_{\text{max}}$ values were respectively 30-fold and 35-fold lower than in presence of Q₀, respectively, for NADH and NADPH (data not shown).

**Chloroplast Localization of Nda2**—To determine Nda2 cellular localization, algal cells were fractionated to obtain enriched fractions containing mitochondria or chloroplast. For this purpose, we used a cell wall-less Chlamydomonas mutant.
(CW15), which allows efficient organelle preparation (42). Each fraction was subjected to immunodetection using antibodies against a PSI subunit (PSAD) and against a mitochondrial ATPase subunit (\(H_9252\)), to respectively probe for the presence of chloroplasts and mitochondria. Respective distributions of PSAD and ATPase (Fig. 6, B and C) indicate that chloroplasts were efficiently separated from mitochondria, although some unavoidable cross contamination was observed (26, 42). Interestingly, the signal detected by the Nda2 antibody (Fig. 6A) was predominantly present in the chloroplast fraction (about 25-fold stronger in chloroplast than in mitochondrial fractions). Upon chloroplast fractionation, Nda2 was mainly recovered in thylakoid membranes and was not detected in stromal or envelope fractions (Fig. 6, D and E). To confirm the nature of the protein detected with the Nda2 antibody, we performed two-dimensional gel electrophoresis followed by immunodetection (Fig. 7). Two faint spots with similar molecular weight but different pI were detected. Corresponding gel samples were excised and subjected to sequencing by mass spectrometry. A single 14-amino acid peptide corresponding to Nda2 was detected in both spots (Fig. 2). This confirms the nature of the protein recognized by the antibody raised against Nda2 and further indicates that Nda2 is subjected to post-translational modifications. Note that phosphorylated forms of Nda2 were recently identified in the phosphoproteome of the \(C.\ reinhardtii\) eyespot (43), a single Nda2 peptide carrying three phosphorylation sites (Fig. 2). This strongly suggests that the two protein forms observed in Fig. 7 result from the existence of

![Image of Ni²⁺ affinity purification and characterization of recombinant Nda2.](image)

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**FIGURE 4.** Ni²⁺ affinity purification and characterization of recombinant Nda2. S, soluble protein fraction; NF, non-fixed fraction, E1 and E2, eluted fractions from Ni²⁺-affinity chromatography using 200 mM imidazole. A, Coomassie Brilliant Blue staining of a 10% SDS/PAGE. B, Immunoblot analysis using an anti-His antiserum. C, UV-visible spectrum of purified Nda2 (3 μM protein concentration). D, HPLC separation and fluorometric analysis of the flavin cofactor. Dashed line, cofactor extracted from purified recombinant Nda2; light gray line, FMN standard; dark gray line, FAD standard.

**FIGURE 5.** Effect of pH on the Nda2 activity measured in the presence of NADH (A) or NADPH (B). Experimental results are the means of triplicate measurements performed on three independent protein preparations.

**TABLE 1**

Kinetic parameters of NADH and NADPH oxidation by Nda2

|                  | NADH       | NADPH      |
|------------------|------------|------------|
| \(K_m\) (μM)     | 10.9 ± 0.7 | 206.4 ± 72 |
| \(V_{max}\) (μmol/min·mg⁻¹) | 138.5 ± 32.8 | 20.9 ± 2.6 |
| \(k_{cat}\) (s⁻¹)   | 139 ± 33  | 21 ± 2.6   |

Kinetic parameters were measured at optimal pH (pH 7.0 for NADH and pH 6.0 for NADPH), using Q₈, (200 μM) as an acceptor, in the presence of SOD (500 units/ml) and catalase (1000 units/ml) in the reaction medium. Enzyme activity was measured by monitoring NADH consumption expressed as μmol of NAD(P)H/min·mg⁻¹ protein. Average values and error bars were calculated from 9 measurements (triplicate measurements were performed on three independent protein preparations).
phosphorylated forms of the enzyme. Based on washing experiments of thylakoid membranes using different NaCl, urea, or CHAPS concentrations, we conclude that part of the Nda2 pool is peripheral (released by a NaCl treatment), while another pool is more tightly bound, requiring CHAPS treatment to be released (supplemental Fig. S4).

Interaction of Nda2 with Bacterial and Thylakoid Membranes—We then tested the ability of Nda2 to interact with the respiratory chain of bacterial membranes by measuring O2 uptake rates in membranes prepared from the ANN0222 strain expressing Nda2 (Fig. 8A). When using membranes of the ANN0222 strain lacking both NDH-1 and NDH-2 as a control, no significant O2 uptake was detected upon addition of saturating concentrations (2 mM) of NADH or NADPH. However, significant rates of NADH oxidation were observed when the same experiment was performed using bacterial membranes prepared from the ANN0222 strain expressing Nda2. Note that in such conditions, about 30% of the protein produced was soluble (supplemental Fig. S5). Quite interestingly, while purified Nda2 shows a 20-fold higher $V_{\text{max}}$ for NADH than for NADPH (Table 1), maximal activity measured on bacterial membranes expressing Nda2 was about half of that for NADH, thus indicating that the affinity of the protein for its substrates may change when it is interacting with membranes.

When recombinant Nda2 was added in vitro to bacterial membranes of the ANN0222 strain, a low activity but significant activity was measured (supplemental Table S1). We conclude that interaction of Nda2 with bacterial membranes, although not very efficient in vitro, is efficient enough in vivo to complement growth of the ANN0222 mutant strain. We then evaluated the capacity of Nda2 to interact with the photosynthetic electron transport chain by performing chlorophyll fluorescence measurements in C. reinhardtii thylakoids (Fig. 8, B and C). When placed in anoxia, addition of NADH or NADPH to C. reinhardtii thylakoids results in a significant increase in chlorophyll fluorescence level. This phenomenon was previously attributed to the activity of an endogenous type II NAD(P)H dehydrogenase in C. reinhardtii thylakoids (5). When the recombinant Nda2 was added to the thylakoid membrane preparation, a stimulation of the fluorescence increase was observed upon NADH addition, this effect being dependent on the protein concentration (Fig. 8B). A similar effect was observed upon NADPH addition, the effect observed in the presence of NADPH being about half of that measured in the presence of NADH (Fig. 8C). This is another indication that
the relative affinities of Nda2 for its substrates (NADH versus NADPH) are modified when the enzyme interacts with membrane systems. We conclude from these experiments that Nda2 is able to interact with photosynthetic electron transport chains by transferring electrons from NADH or NADPH to plastoquinones.

DISCUSSION

In the present work, we report on the identification and characterization of a type II NAD(P)H dehydrogenase in the chloroplast compartment of *C. reinhardtii*. While localizations of other putative *C. reinhardtii* Ndas have not been established yet, Nda2 is clearly localized in the chloroplasts (Fig. 6). Until now, most of the type II NAD(P)H dehydrogenases described so far have been shown, or proposed, to be located either in mitochondrial membranes of eukaryotic cells or in plasmic membranes of prokaryotes. Among the six genes encoding putative type II NAD(P)H dehydrogenases in the *A. thaliana* genome, it was initially concluded that at least five of them (the two NDAs, two of the four NDBs and the NDC) are targeted to mitochondria (15). A recent study concluded, however, that the *A. thaliana* type II NAD(P)H dehydrogenase belonging to the NDC family (NDC1 At5g08740) is targeted to both mitochondria and chloroplasts (44). Interestingly, two independent proteomic studies performed in *Arabidopsis* identified the same protein in plastoglobules, chloroplast substructures involved in lipid storage and tocopherol biosynthesis (45, 46). It was proposed that this enzyme may catalyze electron transfer reactions from or to quinones present within plastoglobules (45). In *C. reinhardtii*, a recent study of the phosphoproteome eye spot reported the presence of phosphorylated forms of Nda2 in this primitive visual system. Although the presence of Nda2 was considered as resulting from a contamination with mitochondrial material (43), and although the *Arabidopsis* protein (At5g08740), which belongs to the plant NDC group is rather distant from Nda2 (Fig. 1), the fact that a NDH-2 is present in plastoglobules (which are structurally related to the eyespot), may indicate a common function of NDH-2s in these two related structures.

A striking feature of Nda2 is that it contains noncovalently bound FMN as a flavin cofactor, most type II NAD(P)H dehydrogenase described so far containing FAD. In Archaea, the existence of a covalently bound flavin has reported in type II NAD(P)H dehydrogenases from *A. ambivalens* (47) and *Sulfolobus metallicus* (48). Based on 31P-NMR spectroscopy analysis, it was concluded that these enzymes contain FMN as a cofactor (47, 48), although in the case of *A. ambivalens* the presence of FMN was not confirmed in a further study (49). Until now, the only biochemical study reporting the presence of FMN as a NDH-2 cofactor was performed on *T. brucei* (50). The fact that Nda2 contains FMN as a cofactor may appear paradoxical considering the presence of a relatively well-conserved ADP-binding motif in the putative flavin-binding domain. Indeed, it has been considered that FAD and FMN-binding motifs may differ on a structural basis, some FMN-binding structural domains being similar to flavodoxins and differing from described FAD-binding sites (51). However, a comparison between ferredoxin reductase and phthalate dioxygenase of *C. reinhardtii* thylakoids.

**FIGURE 8.** Nda2 interacts with respiratory and photosynthetic electron transport chains. A, effect of NADH or NADPH addition on O2 uptake rates measured in *E. coli* membrane fractions prepared from the ANN0222 strain (Control) or from the ANN0222/NDA2 strain (+Nda2). 10-μl membrane extracts (containing a total protein amount of 40 μg in control and 64 μg in +Nda2) were added in a Clark electrode vessel to 1 ml of measuring buffer. B, effect of Nda2 on non-photochemical reduction of plastoquinones measured by the chlorophyll fluorescence rise in anoxia in response to addition of NADH 200 μM to a *C. reinhardtii* thylakoid suspension (50 μg Chl·ml–1). C, stimulation by Nda2 of chlorophyll fluorescence rise triggered by NADH (black curve) and by NADPH (gray curve). These two curves were obtained by calculating the difference between fluorescence-rise kinetics observed in the presence of 5 μg of Nda2 and those observed in control thylakoids.
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genase reductase provided an example of enzymes employing the same domain topology to bind both FMN and FAD, most of the selectivity being attributed to residues involved in phosphate binding (52). Although it does not harbor the AMP moiety of FAD, FMN contains the isoalloxazine ring, ribitol, and pyrophosphate. From sequence structure analysis of FAD-containing proteins, it was proposed that the isoalloxazine ring is involved in catalytic function, the pyrophosphate moiety in molecular recognition, and that the adenine ring stabilizes cofactor binding (53). We may therefore speculate that the FAD-binding motif of Nda2 has lost its ability to bind FAD, due to mutations in distant residues involved in the stabilization of the adenine ring, while keeping the ability to bind the FMN moiety. The stabilization of the FMN cofactor may have required specific mutations such as, for instance, mutations in residues involved in phosphate binding. Whether the presence of FMN (instead of FAD) as a prosthetic group confers particular properties is not really established. It has been proposed that, in contrast to FAD-containing enzymes, which mediate two-electron transfer reactions, FMN-containing enzymes, which mediate one-electron transfer reactions, would be particularly prone to reactive oxygen species production (50). The toxicity observed in growth of bacterial cells expressing high Nda2 levels may reflect the formation of reactive oxygen species by this FMN-containing enzyme, although a direct toxicity of the inclusion bodies formed under these conditions cannot be excluded. Interestingly, putative Ca\(^{2+}\)-binding EF-hand domains are predicted in the Nda2 sequence. A recent study carried out on the seven recombinant type II NAD(P)H dehydrogenases of Arabidopsis thaliana demonstrated that two enzymes, NDB1 and NDB2, effectively bind Ca\(^{2+}\) when produced in E. coli (36). Whether Nda2 is able to bind Ca\(^{2+}\) and whether Ca\(^{2+}\) has a regulatory function on the enzymatic activity of Nda2 remains to be elucidated.

The presence of a functional type II dehydrogenase in chloroplasts questions the physiological role of such a typical respiratory enzyme in a compartment that is devoted to photosynthesis. In higher plant chloroplasts, the presence of a functional NDH-1 complex catalyzing non-photochemical reduction of PQs from stromal electron donors has been demonstrated (1, 31). This complex has been shown to function in close association to photosynthesis by participating in cyclic electron transfer around PSI and chlororespiration (2, 3). Based on the absence of plastidial ndh genes in the Chlamydomonas plastid genome, it has been proposed that this algal species, like other unicellular algae and some gymnosperms, would not harbor a plastidial NDH-1 type complex (2). The absence of genes encoding subunits of the chloroplast NDH-1 complex was confirmed by the recent sequencing of the Chlamydomonas nuclear genome. Despite the absence of a plastidial NDH-1 complex, a significant non-photochemical reduction of PQ has been reported in Chlamydomonas thylakoids (4, 5, 54). Moreover, both chlororespiration and cyclic electron flow activity around PSI have been reported in the algal species (6, 7, 55, 56). Based on a pharmacological approach, Mus et al. (5) concluded that a type II NAD(P)H dehydrogenase would be involved in the NAD(P)H-dependent non-photochemical reduction of PQ in C. reinhardtii thylakoids. In bacterial species lacking NDH-1, type II dehydrogenases have been reported to sustain respiratory activity by allowing NADH turnover (12). Based on the localization of Nda2 in thylakoid membranes, we propose that in the absence of a functional NDH-1, green algae would use a type II enzyme to support this activity.

In addition to its role in chlororespiration and cyclic electron flow around PSI, non-photochemical reduction of PQ is involved in hydrogen production. The green alga C. reinhardtii is able to produce hydrogen in the light thanks to the presence of an iron hydrogenase coupled to the photosynthetic electron transfer chain (57–59). Two main pathways (a PSII-dependent and a PSII-independent pathway) have been proposed to contribute to the hydrogen production process (58, 60). The PSII-independent pathway, which transforms the reducing power generated by anaerobic starch catabolism into hydrogen through a PSI-dependent process, involves non-photochemical reduction of PQ. This electron transport reaction, which has been previously identified as the limiting step of the PSII-independent hydrogen production process (10), represents an interesting target toward improving hydrogen production by microalgae. Whether phosphorylations of Nda2, recently evidenced in C. reinhardtii eye spot fractions (43), are related to changes in enzyme activity and involved in the regulation of photosynthetic electron transport activity will require additional work to be elucidated.

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