Type II NAD(P)H:quinone oxidoreductases are single polypeptide proteins widespread in the living world. They bypass the first site of respiratory energy conservation, constituted by the type I NADH dehydrogenases. To investigate substrate specificities and Ca\(^{2+}\) binding properties of seven predicted type II NAD(P)H dehydrogenases of \textit{Arabidopsis thaliana} we have produced them as T7-tagged fusion proteins in \textit{Escherichia coli}. The NDB1 and NDB2 enzymes were found to bind Ca\(^{2+}\), and a single amino acid substitution in the EF hand motif of NDB1 abolished the Ca\(^{2+}\) binding. NDB2 and NDB4 functionally complemented an \textit{E. coli} mutant deficient in endogenous type I and type II NADH dehydrogenases. This demonstrates that these two plant enzymes can substitute for the NADH dehydrogenases in the bacterial respiratory chain. Three NDB-type enzymes displayed distinct catalytic profiles with substrate specificities and Ca\(^{2+}\) stimulation being considerably affected by changes in pH and substrate concentrations. Under physiologically relevant conditions, the NDB1 fusion protein acted as a Ca\(^{2+}\)-dependent NADPH dehydrogenase. NDB2 and NDB4 fusion proteins were NADH-specific, and NDB2 was stimulated by Ca\(^{2+}\). The observed activity profiles of the NDB-type enzymes provide a fundament for understanding the mitochondrial system for direct oxidation of cytosolic NAD(P)H in plants. Our findings also suggest different modes of regulation and metabolic roles for the analyzed \textit{A. thaliana} enzymes.

The type II NAD(P)H DHs usually possess one non-covalently bound FAD, except for in hyperthermophilic Archaea, where FAD is replaced by FMN (2). The peptide sequence of most enzymes contains two well conserved motifs for dinucleotide binding (1). In many organisms the presence of several type II DH isoenzymes increases the catalytic flexibility of respiratory NAD(P)H oxidation. In bacteria and yeast, diverse roles have been implicated for different homologs. For example, a catalytic function in redox balancing was suggested for homologs in yeast (1, 2, 6), whereas a role in redox sensing was proposed for the homologs in the cyanobacterium \textit{Synechocystis} sp (7). In plants, the relative expression of gene homologs varies between tissues (8, 9), during development (10), in response to light (8, 10, 11) and upon several kinds of stress (12–14). The differential gene expression of plant type II NAD(P)H DH homologs points to diverse physiological roles of the enzymes.

In mitochondria of plants and fungi, type II NAD(P)H DHs are attached to the inner and outer surface of the inner membrane (2, 3). External NADH and NADPH oxidation measured in isolated plant mitochondria is generally dependent on Ca\(^{2+}\) (3, 15) with NADH oxidation being less sensitive to inhibition by chelators (16, 17). NADH oxidation has even been observed in the absence of Ca\(^{2+}\) for several plant materials (14, 18, 19). There is strong evidence that there are separate DHs, each relatively specific for external NADH and NADPH oxidation in plants (20–22). However, the absence of specific inhibitors has made it difficult to study the isoenzymes individually in isolated mitochondria. Several proteins showing NAD(P)H oxidation in the presence of artificial electron acceptors have been purified from different plant species (23–26). However, the requirement for artificial quinones, which can affect substrate specificity and Ca\(^{2+}\) dependence (25, 27), has complicated the catalytic characterization of the purified enzymes.

Type II NADH DHs have been catalytically characterized and/or studied by gene inactivation for \textit{Escherichia coli} (28, 29), \textit{Saccharomyces cerevisiae} (30–32), \textit{Yarrowia lipolytica} (33), \textit{Agrobacterium tumefaciens} (34), \textit{Corynebacterium glutamicum} (35), and \textit{Neurospora crassa} (36). \textit{N. crassa} also contains a homolog mainly catalyzing Ca\(^{2+}\)-dependent NADH oxidation, the external NDE1 (37), whereas the external NDE2 and NDE3 of \textit{N. crassa} were described as Ca\(^{2+}\)-independent DHs accepting both NADH and NADPH (38, 39).

Based on sequence homology to type II NAD(P)H DHs in yeast and \textit{E. coli}, two genes, \textit{nda1} and \textit{nda1}, were described in potato, and their gene products were localized to the internal and external side of the inner mitochondrial membrane, respectively (40). Homologs of type II NAD(P)H DHs with high

---

\(^{1}\) This investigation was supported by The Swedish Natural Science Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\(^{2}\) The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2.

\(^{3}\) To whom correspondence should be addressed. Fax: 46-46-2224113; E-mail: Allan.Rasmusson@cob.lu.se.

\(^{4}\) The abbreviations used are: DH, dehydrogenase; Mops, 3-(N-morpholino)propanesulfonic acid; IPTG, isopropyl-\(\beta\)-D-thiogalactopyranoside; DcQ, decylubiquinone; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxy-methyl)propane-1,3-diol.
amino acid sequence similarity to the potato NDA1 and NDB1 proteins are also present in rice and *Arabidopsis thaliana* (8). The seven homologs found in *A. thaliana* group into three families. These are *nda*1-2 and *ndb*1-4, all of which are closely related to fungal homologs, and *ndc1*, which groups together with cyanobacterial homologs upon phylogenetic analysis (8). The N termini of homologs of all three families target green fluorescent protein to mitochondria (8). Intramitochondrial localization studies suggest that NDB1, NDB2, and NDB4 are external enzymes, whereas NDA- and NDC-type proteins are internally located (9). The plant NDB homologs together with NDE1 of *N. crassa*, and all of these proteins contain an insertion with more or less degenerate EF hand motifs for Ca$^{2+}$ binding (8, 37). However, Ca$^{2+}$ binding by the enzymes has not been shown experimentally.

For plants, a substrate has been identified only for the NDB1 homolog of potato, which is an external Ca$^{2+}$-dependent NADPH DH, as shown by overproduction in tobacco plants (21). Substantial correlative evidence in potato and *A. thaliana* indicates that NDA1 is a matrix-facing NADH DH (9, 10, 13, 41). Defining the substrate and Ca$^{2+}$ specificities of the *A. thaliana* homologs is essential for interpretation of gene expression profiles and for elucidating the physiological roles of these enzymes.

In this study we have analyzed the Ca$^{2+}$ binding properties of the *A. thaliana* type II NAD(P)H DHs and characterized three of the enzymes in terms of substrate specificities and Ca$^{2+}$ stimulation. NDB1, NDB2, and NDB4 were found to be principally able to oxidize both NADH and NADPH. However, the enzymes showed high substrate specificity at physiologically relevant substrate concentrations and pH.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—As templates for gene amplification using PCR, full-length cDNA clones were provided by the Arabidopsis Biological Resource Center (U51324, encoding Atnda1; U12861, encoding Atndb1) and the German Resource Center for Genome Research (U12390, clone MPIZp768207Q2, encoding Atndb4). For Atndb3, an incomplete EST clone 62A4T7, accession number T41616 (42), was used. For Atnda2, Atndb1, and Atndc1, cDNA synthesized using RNA isolated from *A. thaliana* seedlings (14) was used for amplification. Attempts to clone the complete Atndb3 by reverse transcription-PCR were unsuccessful, as previously reported by another group (9).

The following sense primers were adapted for EcoRI and the antisense primers for EagI: Atnda1, 5’ - GGA ATT CAA AGA GGG AGA GAA GCC GGC GAG A-3’ and 5’-ATA TCG GCC GTC AGA TTT GCC TAA TGT C-3’; Atnda2, 5’-GGA ATT CAG AGA AGG AGA GAA GCC GGC AG-3’ and 5’-AGC GCC GTT AGA TAC GCC TAA TGT CAC GA-3’; Atndb1, 5’-GGA ATT CTA GGA TAA GAA GAA GA-3’ and 5’-AGC GCC GGC TGT AGA TGC GCC TGG TAT ACC-3’; Atndb2, 5’-GGA ATT CTA GGA TAA GAA GAA GA-3’ and 5’-TTA CCG CCG TCA GAT GCT ACT GGA ATC TCT A-3’; Atndc1, 5’-GCA TGG AAT TCC TCG ATA ACA AGA GGC CAA-3’ and 5’-TAC GCC CTC GA CAA GAA CCA CCA AAC A-3’. For Atndb3 and Atndb4, a BamHI restriction site was introduced in the sense primer, and an XhoI restriction site was introduced in the antisense primer: Atndb3, 5’-GGA TCC AAG AAA GAG TTT GAT GTT GA-3’ and 5’-CTC GAG ACA TCT TCC CGT TGT TAT GC-3’; Atndb4, 5’-GGA TCC AAC CCA ATA AGG AAG AAG G-3’ and 5’-CTG CAG AGA AGA ATG GCT TAG ATG CTG C-3’.

PCR was used doing the Advantage-HF 2 proofreading PCR kit (Clontech) following the manufacturer’s instructions. Products were cloned with the TOPO TA cloning kit (Invitrogen). Cloned DNA was excised using the restriction sites introduced by the primers and ligated into pET21a (Novagen). The final plasmids were analyzed by DNA sequencing and were found consistent with available cDNAs and genomic annotations. These were: BT005564, Atnda1; AC004680, Atnda2; NM_118962, Atndb1; BT002241, Atndb2; NM_118269, Atndb3; NM_127645, Atndb4. For Atndc1, NM_120955 and the correction (AJ715502) (11) were used. The plasmids were denoted pET-7Atnda1, -T7Atnda2, -T7Atndb1, -T7Atndb2, -T7Atndb3, -T7Atndb4, and -T7Atndc1. All plasmids encode recombinant *A. thaliana* polypeptides truncated at the N-terminal end as compared with the full-length proteins and instead carry an N-terminal T7 tag of 14 or 16 residues. The T7 tag is fused to amino acid residue 69 in NDA1, 67 in NDA2, 45 in NDB1, 54 in NDB2, 153 in NDB3, 59 in NDB4, and 76 in NDC1 (supplemental Fig. 1).

**Mutagenesis of pET-7Atndb1**—A single base pair substitution changing the codon GAC (Asp-387) to GCC (Ala) in NDB1 was introduced using PCR. A forward primer, 5’-ATC CTT CCT GGC TCA CTG-3’, and a reverse mismatch-primer, 5’-TCT TCC ATG GTC AAG GGT CCT GAG TGC GCA TC-3’, containing the new codon and an Ncol recognition site were used for amplification. The products were cloned into a TOPO vector, cut out with HindIII and Ncol, and then inserted into the pET-7Atndb1 cut with the same enzymes to replace the wild type segment. The obtained plasmid was denoted pET-7Atndb1-D387A and confirmed by DNA sequencing of the inserted region.

**Bacterial Strains and Growth Conditions**—For Ca$^{2+}$ binding studies, the pET21 derivatives were transformed into *E. coli* BL21(DE3)/pLysS. Cells were collected from plates and grown in 50 ml of LB containing ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml) at 37˚C and 200 rpm orbital shaking. At an A$_{600}$ of ~0.5, isopropyl-ß-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After 1 h the cells were harvested by centrifugation at 2000 × g for 5 min, washed in 50 mM Tris/Cl pH 8.0, resuspended in 1 ml of high salt medium I (0.5 mM NaCl, 20 mM Tris/Cl, 5 mM imidazole, pH 8.0), and frozen in liquid nitrogen.

For complementation studies and in vitro NAD(P)H oxidation assays, the recombinant genes on plasmids were expressed in the *E. coli* strain MWC008(DE3). The ndh and nuc genes in MWC008 are defective by insertional disruption using kana-mycin and tetracycline resistance markers (43). The gene for T7 RNA polymerase was introduced into the MWC008 chromosome using the ADE3 lysogenization kit (Novagen). In the presence of 0.1–1 mM IPTG negative effects on growth were observed for the MWC008(DE3) strain harboring pET21a (not shown), and therefore, IPTG was not added to the cultures. Low
levels of T7 RNA polymerase are most likely produced in MWC008(DE3) also in the absence of IPTG, and this was sufficient for the transcription of genes cloned in pET21a. The wild type E. coli strain AN387 (44) was used as a control. Antibiotics were used at the following concentrations: 12 μg/ml tetracycline, 50 μg/ml kanamycin, and 100 μg/ml ampicillin. For minimal media, M63 agar (45) was supplemented with 30 mM glucose or mannitol. For liquid cultures, colonies were resuspended from LB plates, and 0.5 or 1 liter of LB medium containing 0.5% glucose and appropriate antibiotics was inoculated to give an A600 of ~0.05. The cultures were grown in 5-liter baffled flasks at 30 °C and 80–90 rpm for 16–18 h. At an mM glucose or mannitol. For liquid cultures, colonies were resuspended from LB plates, and 0.5 or 1 liter of LB medium containing 0.5% glucose and appropriate antibiotics was inoculated to give an A600 of ~0.05. The cultures were grown in 5-liter baffled flasks at 30 °C and 80–90 rpm for 16–18 h. At an A600 of 1.2–1.8, cells were harvested by centrifugation at 7,000 × g for 30 min at 4 °C and washed in 50 mM Tris/Cl, pH 8.0. Cell pellets were stored at −20 °C until used for membrane isolation.

**Protein Extraction**—Extraction of protein for the Ca2+ binding assay was done from transformed BL21(DE3)/pLysS cells frozen in high salt medium I. During thawing of the cells, protease inhibitors were added at the following final concentrations: 1 mM phenylmethylsulfonyl fluoride, 2 μM trans-epoxy-succinyl-leucylamido(4-guanidino)butane, 1 μM pepstatin, and 2 μM leupeptin. The cell suspensions were sonicated for 5 × 5 s and then centrifuged at 100,000 × g for 30 min at 4 °C. Pellets were resuspended in high salt medium I supplemented with 1% (w/v) Triton X-100 and incubated with stirring for 30 min on ice. After centrifugation as above, the pellets constituting insoluble protein fractions were resuspended in high salt medium I containing 6 M urea and incubated as above. Urea-extracted proteins were collected by taking the supernatant after a final centrifugation at 100,000 × g for 30 min at 4 °C.

**Protein Analyses**—Polypeptides were resolved in 8 or 10% SDS-PAGE gels and either stained with Coomassie Brilliant Blue R-250 or wet-transferred to nitrocellulose membranes as described (46). T7-tagged protein was immunodecorated using a T7 monoclonal antibody (Novagen) and horseradish peroxidase-conjugated anti-mouse secondary antibody and detected as described (46). T7 signal intensity was calculated using the Kodak 1D Image Analysis software. Radioactive labeling of electroblotted proteins was carried out as previously described (47) with 1.6 μM 45CaCl2 as the probe and H2O as the washing solution. Radioactive label was detected with phosphor screens using a Personal Molecular Image FX and the image-processing program Quantity One (Bio-Rad). As molecular mass markers in SDS-PAGE, the low molecular weight marker kit (Amer sham Biosciences) and the PageRuler Prestained Protein Ladder (Fermentas) were used. The prestained molecular marker proteins bound 45Ca2+, as previously described (47).

**Membrane Preparation**—MWC008(DE3) cells harboring different plasmids were resuspended to 1/50 of the culture volume in high salt medium II (0.5 M NaCl, 20 mM Tris, 0.1 mM CaCl2, and 1 mM MgCl2, pH 8.0) supplemented with 4 mM MgSO4, 2 μg/ml Dnase I, and 0.1 mM phenylmethylsulfonyl fluoride. The suspension was passed twice through a French press cell operated at 18,000 p.s.i. Unbroken cells and debris were removed by centrifugation at 5000 × g for 10 min. The supernatant was centrifuged at 200,000 × g for 90 min at 4 °C. The obtained pellet containing membranes was homogenized and diluted to 1/50 of the original culture volume in high salt medium II and centrifuged at 200,000 × g for 1 h at 4 °C. The pellet was finally homogenized in 1/50 of the original culture volume of high salt medium II, frozen in liquid nitrogen, and stored at −80 °C. For high EGTA conditions, high salt medium II contained 10 mM EGTA instead of 0.1 mM CaCl2 and additionally 4 mM MgCl2 during washing and resuspension steps. For initial screening purpose, membranes were washed under low salt conditions in 50 mM Tris, pH 8.0, and resuspended in 20 mM Mops, 2.5 mM MgCl2, pH 7.2. Enzyme activities were also tested on fresh preparations to certify that freezing and thawing did not have any major effects on the activities. Membrane protein concentrations were determined using the bicinchoninic acid protein assay (Sigma-Aldrich) with bovine serum albumin as standard.

**Enzyme Assays**—O2 consumption of isolated membranes was measured at 25 °C using an O2 electrode (Rank Brothers, Cambridge, UK). NAD(P)H oxidation of bacterial membranes was measured at room temperature (21 °C) using an Aminco DW2a or an Olis DW2 Conversion dual wavelength spectrophotometer at 340–400 nm. NAD(P)H and succinate oxidation was measured in medium A (20 mM Mops/KOH, 2.5 mM MgCl2, 0.5 mM EGTA, pH 7.2). Additions and substrate concentrations were as indicated in the figure legends. When using EGTA at 10 mM, the concentration of Mops in medium A was increased to 100 mM, and CaCl2 was added to 10.5 mM in the assay. Enzyme activities over a range of different pH values were measured in medium B, designed to maintain constant ionic strength. To obtain medium B, solutions of 20 mM BisTris in 20 mM HCl were mixed with 20 mM triethanolamine in 20 mM KCl to give a certain pH. Medium B additionally contained 2.5 mM MgCl2 and 0.5 mM EGTA. For measurement of NAD(P)H oxidation with decylubiquinone (DCQ) as electron acceptor, 40 μM DCQ, 1 mM KCN, and 120 μM NAD(P)H were added. Student’s t tests were performed using Excel (Microsoft).

## RESULTS

**Production of A. thaliana Type II NAD(P)H DHs as T7-tagged Fusion Proteins in E. coli**—Plasmids were constructed to contain cDNAs for seven A. thaliana type II NAD(P)H DHs and denoted pET-7TAtnda1, -7TAtnda2, -7TAtndb1, -7TAtndb2, -7TAtndb3, -7TAtndb4, and -7TAtndc1. Gene amplification was done from full-length sequences, except for the lowly expressed NDB3 (8, 9), which was derived from a partial cDNA clone. It has been reported for yeast NDI1 that only the mature, but not the full-length protein, can be functionally produced with a T7 tag in E. coli (32). Also, when the potato NAD1 polypeptide is imported into mitochondria, N-terminal processing has been observed (40). Therefore, the inserts derived from the six full-length A. thaliana cDNAs were designed to lack the parts coding for unconserved N termini and to start at a position aligning to the E. coli NDI2-2 start codon. All plasmids were constructed to encode fusion proteins with a T7 tag at the N-terminal end (supplemental Fig. 1).

For investigation of Ca2+ binding properties, the different A. thaliana NAD(P)H DHs were produced in E. coli BL21(DE3)/pLysS transformed with the engineered plant genes cloned in pET21a. Gene expression was induced by the addition of IPTG.
to the growth medium. In total cell lysates the NDB- and NDC-type proteins were visible upon SDS-PAGE analysis and Coomassie staining (not shown). All fusion proteins were visibly enriched in urea extracts of insoluble cell material (Fig. 1A). This fraction was, therefore, used for Ca\textsuperscript{2+} binding analysis.

**FIGURE 1. Production of *A. thaliana* type II NAD(P)H DHs in *E. coli* BL21(DE3)/pLysS and Ca\textsuperscript{2+} binding analysis of the proteins.** Fusion gene expression was induced by the addition of IPTG to cultures of BL21(DE3)/pLysS/pET-T7 (A1), -T7Atndb1 (B1), -T7Atndb2 (A2), -T7Atndb3 (B3), -T7Atndb4 (B4), and -T7Atndc1 (C1). A, SDS-PAGE and Coomassie-staining of urea-extracted insoluble cell proteins. B, Western analysis of urea-extracted protein using an antibody against the T7 tag. No signal was detected with total cell protein from BL21(DE3)/pLysS/pET21a (not shown). C, binding of \textsuperscript{45}Ca\textsuperscript{2+} to *A. thaliana* type II NAD(P)H DHs. Urea-extracted proteins were resolved by SDS-PAGE, electroblotted, and analyzed by \textsuperscript{45}Ca\textsuperscript{2+} overlay. M denotes the prestained molecular marker. Lanes 1, 2, and 3 were loaded with 0.1, 0.25, and 0.5 \mu g of 17-kDa calmodulin from bovine brain (Sigma-Aldrich), respectively. In all three panels the samples loaded correspond to the following volumes of *E. coli* culture: A1, 600 \mu l; B1, 300 \mu l; A2, 600 \mu l; B2, 300 \mu l; B3, 300 \mu l; B4, 412.5 \mu l; C1, 200 \mu l. The masses for unstained (A) and prestained (B and C) molecular markers are denoted in kDa. Arrows denote the positions of *A. thaliana* fusion proteins. Representative results from one of two separate experiments, with independent bacterial transformations, are shown.

The seven fusion proteins and C-terminal-truncated products were detected by Western analysis using an antibody against the N-terminal T7 tag (Fig. 1B). After initial analyses, the gels were loaded with unequal amounts of *E. coli* extracts to achieve similar T7-tag Western band intensities for the different plant proteins. The standardized gels and blots are the ones shown in Fig. 1. The apparent sizes of the fusion proteins were consistent with those calculated from the cDNA sequences.

**FIGURE 2. Ca\textsuperscript{2+}-binding analysis of NDB1 and NDB1-D387A mutant protein.** Total cellular proteins corresponding to 120 \mu l culture volume were separated by SDS-PAGE and Ca\textsuperscript{2+} binding was analyzed as in Fig. 1C. Lane 1, BL21(DE3)/pLysS/pET-T7Atndb1; lane 2, BL21(DE3)/pLysS/pET-T7Atndb1-D387A; lane 3, BL21(DE3)/pLysS/pET-T7Atndb1-D387A. Panel A shows the phosphorimage of the \textsuperscript{45}Ca\textsuperscript{2+} overlay analysis. Panel B shows the polypeptide pattern after SDS-PAGE and staining with Coomassie. Arrows denote the position of the *A. thaliana* NDB1 and NDB1-D387A proteins. Molecular mass standards are denoted as for Fig. 1. Representative results are shown, as for Fig. 1.
Plant Type II NADH and NADPH Dehydrogenases

Functional Complementation of an E. coli Strain Deficient in Type I and Type II NADH DHs by NDB2 and NDB4—The functionality of the A. thaliana fusion proteins in the bacterial system was tested in vivo under conditions where the limiting factor for growth is the capacity to reoxidize NADH. E. coli strain MWC008 is deficient in both type I and type II respiratory NADH DHs. The strain is, therefore, unable to grow with mannitol as the only carbon source (43). MWC008 was equipped with the gene for T7 RNA polymerase and then transformed with the various pET21a derivatives containing genes for the different A. thaliana NAD(P)H DHs. Wild type E. coli grew to a higher cell density than MWC008(De3) containing the different plasmids on all media (Fig. 3). All strains grew on minimal medium supplemented with mannitol (M63 + mannitol) or glucose (M63 + glucose) and on rich medium (LB) with or without glucose. Representative results are shown as for Fig. 1.

Substrate Concentration Affects Apparent Specificity and Ca\(^{2+}\) Dependence of NDB-type Enzymes—Lack of complementation of E. coli MWC008(De3) for growth does not exclude in vitro activity of the A. thaliana fusion proteins. For example, the amount of enzyme protein produced might be too low for sufficient complementation, or the enzyme could be inactive in E. coli cells due to a lack of cofactors. To screen for type II NAD(P)H DH activities and to study enzyme properties, membranes were isolated from MWC008(De3) containing the different plasmids after growth in LB plus glucose and in the absence of IPTG. Western blot analyses of isolated membrane fractions showed single bands of expected sizes for the NDB1, NDB2, NDB4, and NDB1-D387A fusion proteins (not shown). Quantification of the T7-tag signal indicated 5–10 times higher concentrations of NDB2 and NDB4 antigens as compared with NDB1 and NDB1-D387A, which showed similar signal intensities. No antigen signal was seen for NDA1, and only very faint signals were detected for NDA2 and NDC1 (not shown).

NAD(P)H oxidation with O\(_2\) as final electron acceptor was detected in isolated membranes containing NDB1, NDB2, NDB4, and NDB1-D387A proteins, and each of them displayed distinct catalytic profiles (Fig. 4). At high nucleotide substrate concentrations (0.8 mm), NDB1 oxidized both NADPH and NADH, and the activities were highly dependent on Ca\(^{2+}\) (Fig. 4A). The NDB1-D387A protein showed Ca\(^{2+}\)-independent NADPH oxidation similar to NDB1 but without the Ca\(^{2+}\)-dependent component. Virtually no NADH oxidation activity was detected for NDB1-D387A either in absence or presence of Ca\(^{2+}\) (Fig. 4A). NDB2 showed a clear preference for NADH over NADPH. Ca\(^{2+}\) had no significant effect on the steady-state rate with NADH but induced a low rate of NADPH oxidation. The NDB4 fusion protein was found to oxidize NADH and to a lesser extent NADPH, and both activities were unaffected by Ca\(^{2+}\) (Fig. 4A). No NAD(P)H oxidation was detected in membranes of MWC008(De3)/pET21a under any condition (not shown). Membrane preparations had a succinate oxidase activity similar to vector control (38 ± 9 nmol O\(_2\) min\(^{-1}\) mg\(^{-1}\)) irrespective of plasmid expressed by the cells (not shown).

To investigate if the substrate concentration affects the specificity profiles of the NDB-type enzymes, activities in the membrane preparations were also measured using 10× lower NAD(P)H concentrations (80 \(\mu\)M). Under these conditions, NDB1 oxidized NADPH at about 5-fold higher rates than NADH and in a completely Ca\(^{2+}\)-dependent manner (Fig. 4B). NADPH oxidation by NDB1 under low substrate conditions reached 60% of the activity measured at high substrate concentration, as calculated by converting rates of NAD(P)H oxidation into O\(_2\) consumption using a factor of two. For the NDB1-D387A mutant most of the Ca\(^{2+}\)-independent NADPH oxidation seen at higher substrate concentration was absent at the lower substrate level. NDB2 oxidized exclusively NADH at low nucleotide concentrations. The steady-state rate was stimulated by Ca\(^{2+}\) with a statistically significant difference in a paired t test at \(p < 0.01\). Also NDB4 displayed a strong specificity for NADH under low substrate concentrations (Fig. 4B). It can be concluded that the A. thaliana NDB-type enzymes bound to the E. coli membranes are highly specific to single nucleotide substrates at an NADH or NADPH concentration of 80 \(\mu\)M.

The enzyme activity of NDB4 was found to be sensitive to the membrane isolation procedure. Washing and resuspending the membrane fraction of MWC008(De3)/pET-T7ndb4 at low salt reduced O\(_2\) consumption in membranes by about 80% as compared with high salt conditions (not shown). Neither NDB1 nor NDB2 was affected by the low salt conditions during preparation. Immunodetection revealed similar amounts of NDB4 protein in membrane fractions prepared under low and high salt.
conditions (not shown). Therefore, it is likely that the absence of ions during membrane isolation inactivated NDB4.

**Effect of pH on Substrate Specificity and Ca\(^{2+}\) Dependence of NDB-type Proteins**—Oxidation of 0.8 mM NAD(P)H by membranes containing NDB-type fusion proteins was measured as O\(_2\) consumption over a physiologically relevant pH range (6.8–7.8) in medium B. Activities at pH 7.2 in this buffer (Fig. 5A) were similar to those observed in medium A (Fig. 4A). This excludes any significant buffer-specific effects on the enzyme activities. The NDB1 enzyme activities were considerably affected by pH. At pH 6.8 and 7.2, the maximal oxidation rates for NADH and NADPH by NDB1 were similar. The Ca\(^{2+}\) dependence of NADPH oxidation was 80% at pH 7.2 but only 25% at pH 6.8 (Fig. 5A). A somewhat lower Ca\(^{2+}\) dependence at pH 6.8 was also seen for NADH oxidation by NDB1. At pH 7.5, oxidation of NADPH was 3-fold higher than of NADH, thereby substantially increasing the NADPH specificity of NDB1 (Fig. 5A). At pH 7.8, the enzyme was completely NADPH-specific, albeit less active. In the NDB1-D387A mutant, the Ca\(^{2+}\)-dependent rates seen for NDB1 were abolished, but not the Ca\(^{2+}\)-independent rates (Fig. 5A).

NADH oxidation by NDB2 and NDB4 was little affected by pH over the measured range, but a Ca\(^{2+}\)-stimulation was evident for NDB2 at pH 7.5 and 7.8. The difference was significant at *p* < 0.05 in a paired *t* test of the unnormalized data. For both NDB2 and NDB4, the observed NADPH oxidation activity was negligible at pH 7.8 but increased at lower pH (not shown), which indicates a low NADPH oxidase activity.

Intact plant mitochondria oxidize NADPH directly to the quinone analog DcQ with high Ca\(^{2+}\) dependence (14, 21). Isolated *E. coli* membranes containing the different NDB-type enzymes were used to investigate if NAD(P)H oxidation rates to DCQ are consistent with those to O\(_2\). Using DCQ, possible restrictions in the bacterial respiratory chain, such as insufficient terminal oxidase capacity or low quinone availability, are circumvented. The pH curves for NDB1 obtained by oxidation of 120 μM NAD(P)H to DCQ (Fig. 5B) are highly similar to those seen with 0.8 mM NAD(P)H measured to O\(_2\) (Fig. 5A). The only difference was a higher ratio of NADPH:NADH oxidation at pH 7.2 for NDB1 in the DCQ assay. Membranes containing NDB2 or NDB4 fusion protein displayed similar pH profiles for NADH oxidation with both terminal electron acceptors. However, NADPH oxidation was virtually absent with DCQ for both enzymes. The steady-state NADH to DCQ activity by NDB2 was stimulated by...
investigated NDB-type enzymes accepted DcQ efficiently, as the rates for the main substrates were in all cases 1.5–2 times higher than with O₂ as final electron acceptor.

**Ca²⁺ and pH Affect NDB2 Initial Catalytic Rates**—Membranes containing NDB1 and NDB4 proteins generally displayed immediate linear rates of O₂ consumption after the addition of NAD(P)H. However, for NDB2, the addition of NADH in the absence of Ca²⁺ always resulted in a lag phase before the linear oxidation rate was reached (Table 1). The lag phase lasted several minutes at higher pH, whereas at lower pH the lag phase was shorter and less pronounced (supplemental Fig. 2). The addition of Ca²⁺ before NADH resulted in that the maximum rate was reached from start. Also, complete oxidation of a small amount of NADH (20 μM) before starting the measurement (by the addition of 0.8 mM NADH) shortened the lag time considerably (not shown). To investigate if the lag phase may be caused by mobilization of residual Ca²⁺ bound to E. coli membranes, the sample was preincubated with 10 mM EGTA in the reaction mixture for 1 h at 4 °C before the assay was performed. Also, membranes were isolated with 10 mM EGTA present in all buffers. However, a similar lag phase for NADH oxidation was observed in both cases (not shown). The results, thus, show that Ca²⁺ has a distinct stimulating effect on NADH oxidation by NDB2, which is especially pronounced during the pre-steady state.

**DISCUSSION**

From the perspective of enzyme function in vivo, substrate and acceptor specificities must be placed in their metabolic context. External mitochondrial NAD(P)H DHs are directed toward the intermembrane space, which is metabolically and ionically connected to the cytosol via porin channels (48). The pH of the plant cytosol is estimated to be 7.2–7.5 (49) but can decrease by 0.3–0.6 units upon light changes or hypoxia (50–53). The estimated concentration of free Ca²⁺ in the plant cytosol is below 1 μM (15, 49) but can
Plant Type II NADH and NADPH Dehydrogenases

| Conditions | Initial rate | Linear rate | Maximum rate |
|------------|--------------|-------------|--------------|
| pH 7.2     | 52 ± 10%     | 89 ± 4      | 425 ± 79     |
| pH 7.2 + CaCl₂ | 100 ± 0     | 100 ± 0     | 436 ± 97     |
| pH 6.8     | 85 ± 3%      | 100 ± 1     | 423 ± 47     |
| pH 7.8     | 37 ± 5%      | 82 ± 1      | 347 ± 57     |

| a | The initial rate was significantly lower than the linear rate in unpaired t tests at p < 0.05. |
| b | CaCl₂ was present from the start. |
| c | The initial rate was significantly lower than the linear rate in unpaired t tests at p < 0.01. |

The presence of a completely Ca²⁺-independent homolog is consistent with observations that Ca²⁺-dependence can vary markedly between tissues in a plant. Mitochondria from fresh sugar beet roots show a strongly Ca²⁺-dependent NADH oxidation, whereas the rates in mitochondria from cold-stored roots are mainly Ca²⁺-independent (19). The observed substrate specificities of NDB1, NDB2, and NDB4 in A. thaliana are usually low (8, 9) but can increase up to 10-fold in response to different stress treatments (12). External Ca²⁺-independent NADH oxidation has been measured in mitochondria from A. thaliana seedlings but was stable upon up-regulation of ndb4 transcripts (14). The reason for this is presently unclear.

Like NDB4, NDB2 complemented the E. coli double mutant, and the activity of the enzyme was virtually specific for NADH (Figs. 4 and 5). The steady-state oxidation rates were stimulated by Ca²⁺, especially at low substrate concentrations or higher pH. The activity lag phase observed in the absence of Ca²⁺ (Table 1) further emphasizes that NDB2 is affected by Ca²⁺. It also confirms that lower pH decreases the Ca²⁺ requirement of NDB2. This is consistent with a lower sensitivity to chelators observed for external NADH oxidation by plant mitochondria at lower pH (59). The complete oxidation of a small amount of NADH also shortened the lag phase, a phenomenon previously observed in Jerusalem artichoke mitochondria (60). Thus, NADH oxidation by NDB2 in the E. coli membranes displays characteristics of external NADH oxidation by plant mitochondria. The lag phase before reaching full activation may reflect a state transition phase for NDB2.

A correlation of up-regulated ndb2 transcript levels and an increase in external Ca²⁺-dependent NADH oxidation of mitochondria has been observed in A. thaliana (14). In the present study Ca²⁺ affected the NDB2 activity and bound to the enzyme but not to NDB3 or NDB4. Thus, NDB2 is most likely the mitochondrial Ca²⁺-dependent external NADH DH, unique to plants.

NDB1 is a Ca²⁺-dependent NADPH DH when analyzed as a fusion protein in E. coli membranes under low substrate conditions (Fig. 4B). This is in line with a previous characterization of potato NDB1 overproduced in transgenic tobacco (21), where, however, a background of NADH oxidation could have masked a low NADH oxidation rate by NDB1. At high substrate concentrations, A. thaliana NDB1 also oxidized NADH in a fully Ca²⁺-dependent manner (Fig. 4). Thus, Ca²⁺-dependent NADH oxidation measured in purified plant mitochondria at pH 7.2 (15) could be due to both NDB2- and NDB1-type enzymes. The higher NADPH:NADH oxidation ratio for NDB1 at low substrate concentrations indicates that NADH oxidation is a low affinity component. Similar characteristics, however, reversed for the substrates are found for AtuNDBH-2 of A. tumefaciens (34). Considering that cytosolic concentrations of total NADH and NADPH can be up to 55 and 150 μM, respectively (58), NDB1 most likely acts as an NADPH-specific enzyme in vivo.

NDB1 transcript is present in several tissues of A. thaliana (8, 9), and the levels in both potato and A. thaliana remain remarkably stable under different conditions (10–14). NDB1 substrate specificity and Ca²⁺ dependence were highly influenced by small pH changes around neutral (Fig. 5). This could indicate that NDB1 is regulated at activity level in vivo. NDB1 might be active only for short periods of time during signal-induced Ca²⁺ oscillations (61) or during conditions that decrease cytosolic pH (49).

The observed substrate specificities of NDB1, NDB2, and NDB4 test previous hypotheses based on sequence similarity in the putative NAD(P)H binding region of eukaryotic and bacterial NAD(P)H DHs (1, 21). In the NADH-specific type II DHs from bacteria and fungi as well as in the NADH-specific NDB2 and NDB4, the second β sheet of the βββ sheet motif for dinucleotide binding (62) ends with a Glu which can form a hydrogen bond to the adenine ribose moiety of NAD(H) while possibly rejecting the phosphate group of NADPH. The N. crassa NDE1 and the NDB1 of potato and A. thaliana, which are all NADPH-specific (21, 37) (Fig. 4), contain an uncharged Gln at this position, which could facilitate binding of the NADP(H) molecule. The presented results for three NDB-type enzymes are consistent with this hypothesis and lend further support to the importance of the terminal residues of the nucleotide binding motif (21). The effects by substrate concentration and pH, however, demonstrate that specificities are not absolute and may involve...
The Ca\(^{2+}\) effects on enzyme activities of NDB1 and NDB2 were consistent with their ability to bind Ca\(^{2+}\) ions in a 45Ca\(^{2+}\) overlay assay (Fig. 1). An insertion containing EF hands and EF hand-like motifs is present in NDE1 of N. cressa and NDB-type homologs of A. thaliana, potato, and rice (3, 21, 37) (Fig. 6). The N-terminal EF hand in plants and the C-terminal EF hand in N. crassa conform with the classical EF hand sequence, whereas the other EF hand-like sequences are degenerate (1, 21). Six critical positions for co-ordination of the Ca\(^{2+}\) ion have been identified in canonical EF hand sequences (64) (Fig. 2). Underlined residues indicate the canonical Ca\(^{2+}\) binding motif, based on an analysis of 165 unique EF hand sequences (64). The residues highlighted in gray are in α-helices as predicted by the SOPMA secondary structure prediction software (71). Amino acid numbers are denoted to the right.

There are indications that potato NDA- and NDB-type proteins reside as high molecular mass forms in the inner mitochondrial membrane (66). In yeast, all three type II NADH DHs are part of a supramolecular complex with other DHs and citric acid cycle enzymes (67). It was also shown that external NADH oxidation via NDE1 and NDE2 inhibits the mitochondrial glycerol 3-phosphate DH in the same complex under high NADH concentrations (68). The data described here show that the analyzed plant NDB-type enzymes are independent of other proteins for activity. However, it remains possible that other mitochondrial proteins modulate the activities, as in yeast. This may explain the relatively small Ca\(^{2+}\)-effect on NDB2 in E. coli membranes, which partly contrasts the strong Ca\(^{2+}\)-dependence of external NADH oxidation often seen in isolated mitochondria (3, 69).

We report here a qualitative enzymatic characterization of the external A. thaliana type II NAD(P)H DHs NDB1, NDB2, and NDB4 produced with an N-terminal T7 tag in E. coli. The enzymes resided in isolated membranes and reduced the quinones of the bacterial respiratory chain. In previous investigations, NAD(P)H oxidation by membrane-bound DHs in plants has always been measured in materials (e.g. isolated mitochondria) containing a mix of enzyme homologs. Both Ca\(^{2+}\) and pH were known to affect NAD(P)H oxidation by type II DHs with species-specific variations (3, 70), but the responses of individual homologs to these parameters have not been studied. Our results on the individual enzymes clearly demonstrate that plant mitochondria contain at least three separate external DHs specific for NADH and NADPH and with different Ca\(^{2+}\)-dependences.

**Acknowledgments**—Drs. R. B. Gennis, University of Illinois, and T. Friedrich, Heinrich-Heine Universität, Düsseldorf, Germany are gratefully acknowledged for donation of bacterial strains. Fredrik Norberg and Zandra Hagman are acknowledged for excellent technical assistance. The Atndb4 clone was generated at MPI-Z (Koeln-Vogelsang) by M. Jakoby and B. Weisshaar.

**REFERENCES**

1. Kerscher, S. J. (2000) Biochim. Biophys. Acta 1459, 274–283
2. Melo, A. M., Bandeiras, T. M., and Teixeira, M. (2004) Microbiol. Mol. Biol. Rev. 68, 603–616
3. Rasmussen, A. G., Soole, K. L., and Elthon, T. E. (2004) Annu. Rev. Plant Biol. 55, 23–39
4. Rasmussen, A. G., Heiser, V., Zabaleta, E., Brennicke, A., and Grohmann, L. (1998) Biochim. Biophys. Acta 1364, 101–111
5. Friedrich, T., Steinmüller, K., and Weiss, H. (1995) FEBS Lett. 367, 107–111
Plant Type II NADH and NADPH Dehydrogenases

6. Overkamp, K. M., Bakker, B. M., Kötter, P., van Tuyl, A., de Vries, S., van Dijken, J. P., and Pronk, J. T. (2000) *J. Bacteriol.* **182**, 2823–2830

7. Howitt, C. A., Udall, P. K., and Vermaas, W. F. (1999) *J. Bacteriol.* **181**, 3994–4003

8. Michalecka, A. M., Svensson, Å. S., Johansson, F. I., Agius, S. C., Johanson, U., Brennicke, A., Binder, S., and Rasmusson, A. G. (2003) *Plant Physiol.* **133**, 642–652

9. Elhafiez, D., Murcha, M. W., Clifton, R., Soole, K. L., Day, D. A., and Whelan, J. (2006) *Plant Cell Physiol.* **47**, 43–54

10. Svensson, Å. S., and Rasmusson, A. G. (2001) *Plant Physiol.* **273**, 3625–3637

11. Michalecka, A. M., Svensson, Å. S., Johansson, F. I., Agius, S. C., Johanson, U., Brennicke, A., Binder, S., and Rasmusson, A. G. (2003) *FEBS Lett.* **517**, 79–82

12. Escobar, M. A., Franklin, S. M., Svensson, Å. S., Salter, M. G., Whitelam, G. C., and Rasmusson, A. G. (2004) *FEBS Lett.* **579–585

13. Svensson, Å. S., Johansson, F. I., Møller, I. M., and Rasmusson, A. G. (2002) *Biochim. Biophys. Acta* **1556**, 160–166