The External Calcium-dependent NADPH Dehydrogenase from
Neurospora crassa Mitochondria*

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We have inactivated the nuclear gene coding for a putative NAD(P)H dehydrogenase from the inner membrane of Neurospora crassa mitochondria by repeat-induced point mutations. The respiratory rates of mitochondria from the resulting mutant (nde-1) were measured, using NADH or NADPH as substrates under different assay conditions. The results showed that the mutant lacks an external calcium-dependent NADPH dehydrogenase. The observation of NADH and NADPH oxidation by intact mitochondria from the nde-1 mutant suggests the existence of a second external NAD(P)H dehydrogenase. The topology of the NDE1 protein was further studied by protease accessibility, in vitro import experiments, and in silico analysis of the amino acid sequence. Taken together, it appears that most of the NDE1 protein extends into the intermembrane space in a tightly folded conformation and that it remains anchored to the inner mitochondrial membrane by an N-terminal transmembrane domain.

In nonphotosynthetic eukaryotes, the mitochondrion is the cellular organelle responsible for producing most of the energy required for cellular metabolism. The process of oxidative phosphorylation takes place in the inner mitochondrial membrane, whereby the electrons produced by the oxidation of substrates like NAD(P)H are transported through the electron transport chain to oxygen, coupled to the generation of a transmembrane proton gradient that eventually leads to ATP synthesis (1). In contrast to mammals, the electron transport chains of plants and fungi possess several nonproton-pumping NAD(P)H dehydrogenases for transferring electrons to ubiquinone (2). In the case of potato tubers, four rotenone-insensitive NAD(P)H dehydrogenases have been identified in the inner mitochondrial membrane, two with the catalytic site facing the matrix (3, 4) and two facing the intermembrane space (5). In mitochondria from Saccharomyces cerevisiae, where the proton-pumping complex I is not present, the oxidation of NADH and NADPH is performed exclusively by three nonproton-pumping enzymes, one facing the matrix and two facing the intermembrane space (6–8). In addition, the genome analysis of Synechocystis revealed three open reading frames that may code for such type II NAD(P)H dehydrogenases (9). On the other hand, only one external type II NADH dehydrogenase was reported for the fungus Yarrowia lipolytica (10). Although NAD(P)H dehydrogenases have been studied for a long time, our understanding of protein function at the molecular level is still very incomplete. The cloning of genes encoding several of these rotenone-insensitive NAD(P)H dehydrogenases from mitochondria of different organisms (7, 10–13) provides important tools for further research in this field. These enzymes might constitute a wasteful system acting to prevent the overreduction of the electron transport components and the production of reactive oxygen species, but their exact roles remain unclear.

Both proton-pumping and nonproton-pumping NAD(P)H dehydrogenases have been described in Neurospora crassa mitochondria. In 1970, the presence of at least two different rotenone-insensitive NADH dehydrogenases in the inner membrane was described (14), one on the inner side catalyzing the oxidation of matrix NADH and a second on the outer side accessible to cytosolic NADH and NADPH. It was later reported that the external NAD(P)H activity is partly calcium-dependent (15). The internal rotenone-insensitive NADH dehydrogenase is not linked to the formation of ATP and is very active in the early exponential phase of growth (16). Here we identify NDE1, previously called p64 (11), as an external calcium-dependent NADPH dehydrogenase and provide evidence for the presence of at least a second dehydrogenase; both enzymes oxidize reduced pyridine nucleotides from the cytosol of N. crassa cells.

EXPERIMENTAL PROCEDURES

Preparation of Mitochondria—General manipulation of N. crassa, including crosses, was performed by standard procedures (17, 18). The wild-type strain 74-OR23–1A and the nde-1 mutant were grown in Vogel’s minimal medium. The techniques for the preparation of N. crassa mitochondria and IO-SMP1 either for oxygen electrode measurements (4) or for digitonin and import experiments (19), as well as the isolation of mitochondria from S. cerevisiae wild-type strain u-273–108 (20), have been described.

In Vitro Import into Mitochondria—The cDNA encoding the open reading frame of NDE1 was amplified by polymerase chain reaction using two specific primers and cloned in PCR II-TOPO vector (Invitrogen). Precursor polypeptides were synthesized in vitro with a coupled transcription/translation system (Promega) in the presence of [35S]methionine.

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1 The abbreviations used are: IO-SMP, inside-out submitochondrial particles; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.
thionine (21) and imported into N. crassa or S. cerevisiae mitochondria. Import reactions were performed in a medium containing 0.5 M sorbitol, 80 mM KCl, 50 mM Hepes, pH 7.2, 3% (w/v) bovine serum albumin, 10 mM Mg(CH₃COO)₂, 2 mM potassium phosphate, 2.5 mM EDTA, 1 mM MnCl₂, 2 mM NADH, 2 mM ATP, 1% (w/v) ethanol, 2 mM creatine phosphate, 0.1 mg/ml creatine kinase, 1% (w/v) of rabbit reticulocytes with the relevant radiolabeled precursors, and 0.2 mg/ml mitochondria for 20 min at 25 °C. To abolish the membrane potential, 0.1 mM valinomycin was added, and creatine phosphate and creatine kinase were omitted. After import, mitochondria were incubated with 30 µg/ml trypsin on ice for 15 min, and the protease activity was stopped by the addition of 800 µg/ml soybean trypsin inhibitor (22). Swelling of yeast mitochondria was achieved in media without sorbitol.

Disruption of nde-1—The cDNA encoding NDE1 (11) was excised from pBluescript by digestion at flanking PvuII restriction sites, inserted in the EcoRV restriction site of the pCSN44 vector, and transformed into N. crassa spheroplasts (18). Individual transformants were selected on hygromycin B (Sigma) plates (200 µg/ml) and purified by several asexual transfers in Vogel's minimal medium plus 150 µg/ml hygromycin B. A single copy transformant was identified by Southern blot analysis of digested genomic DNA (23, 24) and crossed with the cDNA clones obtained from N. crassa cDNA libraries (30). The probes used were NM1C2, encoding restriction fragments from four point mutations (25, 26). Detection of nde-1 mutants among the progeny of the cross was carried out by the analysis of Western blots (27) of mitochondrial proteins with an antiserum against NDE1 (11).

Northern Blot Analysis—To analyze gene expression, N. crassa mRNA was isolated from conidial (germinating asexual spores), mycelial (branching hyphae), or perithecial (fruiting body) tissues (28). Northern blots (29) were hybridized with random primer-labeled restriction fragments from four N. crassa cDNA clones obtained from three cDNA libraries (30). The probes used were NMC12, encoding NDE1, and SCS15, SM1P6, and SPS10, which encode the 12.3-, 17.8-, and 21-kDa subunits, respectively, of the mitochondrial complex I.

Oxygen Consumption—Respiration was measured polarographically at 25 °C with a Hansatech oxygen electrode in a total volume of 1 ml. Assays with mitochondria and IO-SMP contained 0.3–0.5 mg of protein, 0.3 mM succinate, 10 mM potassium phosphate, pH 7.2, 5 mM MgCl₂, 1 mM EGTA, 10 mM KCl, 4 mM carbonyl cyanide m-chlorophenylhydrazone, and 0.02% (w/v) bovine serum albumin. For the pH experiments, the reaction medium contained 20 mM MES, 20 mM Tris, 20 mM MOPS, 0.3 mM succinate, 0.1 mM CaCl₂, 4 mM carbonyl cyanide m-chlorophenylhydrazone, and 0.02% (w/v) bovine serum albumin adjusted to pH 4.7–9.2 with KOH. Calcium depletion was achieved with 1 mM EGTA. The assays were initiated by the addition of either 1 mM NADH or 1 mM NADPH. Rotenone and antimycin A were added to final concentrations of 20 µM and 0.2 µg/ml, respectively. Integrity of mitochondria and sidedness of IO-SMP were assessed by the activities of cytochrome-c oxidase (EC 1.9.3.1) and malate dehydrogenase (EC 1.1.1.37) in the absence and presence of Triton X-100 (31).

Miscellaneous—Standard procedures were used for cloning, agarose gel electrophoresis and Southern blotting (32, 33), polyacrylamide gel electrophoresis (34), protein determination (35), and the development of rabbit antisera (36). Digitonin solubilization followed by proteinase K treatment (37) and Na₂CO₃ extraction (24) of mitochondrial proteins have been described.

RESULTS

Inactivation of nde-1—To investigate the specific role of NDE1, we disrupted the corresponding gene by the generation of repeat-induced point mutations, an unusual phenomenon that causes methylation and GC to AT transition mutations of repeat-induced point mutations, an unusual phenomenon

![Image](https://via.placeholder.com/150)

**Fig. 1. Identification of nde-1 mutants.** Total mitochondrial proteins (100 µg) from wild type (lane 1), the nde-1 mutant (lane 2), and the double mutant nde-1 nuo51 (lane 3) were analyzed by Western blotting with an antiserum against the NDE1 protein and a mixture of antisera against the 51-, 30.4-, 20.8-, and 12.3-kDa subunits of complex I. (43), respectively, which display various phenotypes in terms of complex I assembly and function. Fig. 1, lane 3 depicts the analysis of the double mutant nde-1 nuo51, which expresses a nonfunctional complex I. We would expect these double mutants to be nonviable due to severe deficiency in the oxidation of matrix NADH, because only complex I and one alternative dehydrogenase were described in Neurospora (14). Thus, these results represented the first indication that NDE1 was using cytosolic NAD(P)⁺ as a substrate.

Thus, despite the fact that complex I is required for sexual development (44), neither complex I nor NDE1 is essential for Neurospora vegetative growth under standard conditions. We analyzed the expression of the corresponding genes throughout the life cycle of the fungus and performed a Northern blot analysis of mRNA from conidia (germinating asexual spores), mycelia (branching hyphae), or perithecia (fruiting bodies) using cDNAs encoding NDE1 and complex I subunits as probes. The results demonstrated that all transcripts, and probably the encoded proteins, are expressed constitutively throughout the Neurospora life cycle (data not shown). This finding is corroborated by the identification of the various cDNA clones in three different cDNA libraries, representing both vegetative and sexual stages of development (30).

Characterization of NDE1 Activity—To characterize the activity of NDE1, mitochondria and IO-SMP were prepared from wild type and the nde-1 mutant and tested for different activities under various conditions. The oxidation rates of NADH and NADPH were followed polarographically with an oxygen electrode. The rotenone-insensitive oxidation rates for both substrates at pH 7.2 were similar in IO-SMP from both strains. In addition, when intact mitochondria were assayed with NADH at pH 7.2 no significant difference was found between the two strains. However, NDE1-defective mitochondria showed very reduced oxidation activity relative to the wild-type organelles when NADPH was used as substrate. All activities were fully inhibited by antimycin A (data not shown).

Therefore, we carried out a detailed characterization of exogenous NAD(P)⁺ oxidation by mitochondria from both strains by following the oxidation rates of NADH and NADPH in the pH range from 4.7 to 9.2, in the presence or absence of calcium (Fig. 2). The data were examined by the statistical analysis of variance-covariance (ANCOVA), using as covariate the value of pH (45). There was no significant difference in the pattern of NADH oxidation between mitochondria from the two strains

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2 M. A. Nelson, unpublished results.
throughout the pH range, neither in the presence ($p = 0.29$) nor in the absence of calcium ($p = 0.089$). NADH oxidation by both strains was unaffected by calcium at acidic pH, whereas decreased activity in the absence of calcium was observed under alkaline conditions (Fig. 2, a and b). The differences were not statistically significant either in wild type ($p = 0.62$) or in nde-1 ($p = 0.89$) in the pH range from 4.7 to 7.4 but were significant both in wild type ($p < 0.001$) and nde-1 ($p < 0.01$) when the pH range from 7.7 to 9.2 was considered. In contrast, there was a clear difference in NADPH oxidation. In the presence of calcium, wild-type mitochondria oxidized NADPH from pH 4.7 to 8.3, whereas mitochondria from the nde-1 mutant had no activity above pH 7.2. Under calcium depletion conditions, a drastic reduction in NADPH oxidation rate was observed in wild-type mitochondria. At pH 8, this activity was totally dependent on calcium (Fig. 2c). The statistical analysis revealed that calcium had a highly significant effect on wild-type activity throughout the pH range ($p < 0.001$). In contrast, the oxidation of NADPH by mitochondria from the nde-1 mutant was not significantly affected by calcium (Fig. 2d), as confirmed by the statistical analysis ($p = 0.63$). These results clearly indicate that NDE1 is the external calcium-dependent NADPH dehydrogenase.

**Topology of NDE1**—Several approaches were employed to obtain a more detailed characterization of the topology of NDE1. Fig. 3 displays the accessibility of the protein to protease K upon the fractionation of mitochondria with increasing concentrations of digitonin. The opening of mitochondrial membranes was monitored by the use of antisera against the mitochondrial processing peptidase (46), the ADP/ATP carrier (47), cytochrome c heme-lyase (48), and TOM20 (49) as markers for the matrix, inner membrane, intermembrane space, and outer membrane, respectively. TOM20, with domains facing the cytosol, was readily digested by protease K. Total solubilization of the outer mitochondrial membrane was achieved at a digitonin concentration of 0.2% as attested by the disappearance of cytochrome c heme-lyase. The inner mitochondrial membrane was solubilized at the highest digitonin concentration (2%). The behavior of NDE1 parallels that of cytochrome c heme-lyase; when the outer membrane was opened at 0.2% digitonin concentration, its exposure to protease K resulted in two resistant fragments. The larger fragment of 57 kDa remained membrane-bound as shown in Fig. 3, right panel. In this experiment, a mitochondrial sample incubated with 0.3% digitonin and protease K was incubated with Na2CO3 and resolved into pellet and supernatant to discriminate between intrinsic and extrinsic membrane proteins. The mitochondrial processing peptidase and the ADP/ATP carrier were used as controls.

Preliminary experiments of in vitro import of NDE1 into mitochondria were performed. In parallel experiments, the precursor of subunit F1β of ATPase (50) was used as control. The precursor of NDE1 was synthesized in vitro and imported into Neurospora mitochondria with and without a membrane potential. Import and processing to the mature form was only achieved in the former case (data not shown), a typical result for an inner membrane protein (51). Because it is not possible to swell Neurospora mitochondria, the NDE1 precursor was also successfully imported into and processed in yeast mitochondria, as deduced by the resistance of the mature form of the protein to added trypsin, again only in the presence of a membrane potential (Fig. 4). In addition, and in contrast to F1β, mature NDE1 was made accessible to the protease in the
its effect on NADPH oxidation in the two strains. Wild-type mitochondria displayed strong calcium sensitivity, and the presence of the cation was absolutely required for activity at physiological pH. Because oxidation of NADPH by nde-1 mitochondria was not affected by calcium, we demonstrated experimentally that the NADPH oxidation activity of the NDE1 protein is calcium-dependent. Previous work has documented a calcium stimulation of NADP/H oxidase activities in Neurospora (15) and plant (53) mitochondria, although the mechanism by which calcium stimulates activity is not yet clear. It was suggested that this divalent cation has an important role in avoiding electrostatic repulsion between the phosphate groups of NADPH and the catalytic site of the enzyme localized in a net negatively charged environment like the outer surface of the inner mitochondrial membrane (54). It is also likely that calcium binding induces conformational changes that increase the enzyme-substrate affinity.

Following external NADH and NADPH oxidation from acidic to alkaline conditions (pH 4.7–9.2), two distinct activities were identified in N. crassa mitochondria. One corresponds to NDE1, which oxidizes NADPH and is calcium-dependent. The other, which remains active in mutant nde-1 mitochondria, can be attributed to an NDE2 enzyme that oxidizes NADH throughout the pH range and is also able to oxidize NADPH at acidic pH. The inability of the putative NDE2 to oxidize NADPH at pH higher than 7.2 may explain the need for a separate enzyme, NDE1, to regenerate the cytosolic NAD(P)⁺ pool required in several biosynthetic pathways. N. crassa also contains at least one internal rotenone-insensitive NADH dehydrogenase (14). The presence of these nonproton-pumping alternative NAD(P)H dehydrogenases varies between different organisms. They might be involved in situations of NAD(P)H stress, but their specific role is unclear. In addition to metabolic functions (7, 8, 10), a regulatory role in response to the redox state of the plastoquinone pool has been suggested in cyanobacteria (9).

This is the first time that the gene for a mitochondrial NADH dehydrogenase has been identified and that evidence for at least two external NAD(P)H dehydrogenases in N. crassa mitochondria has been obtained. The cloning and disruption of the additional rotenone-insensitive NADH dehydrogenases will help to clarify their function(s) in mitochondrial and cellular metabolism.

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