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The main external alternative NAD(P)H dehydrogenase of *Neurospora crassa* mitochondria

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Abstract

A DNA sequence homologous to non-proton-pumping NADH dehydrogenase genes was found in the genome of *Neurospora crassa* encoding a polypeptide of 577 amino acid residues, molecular mass of 64,656 Da, with a putative transmembrane domain. Analysis of fungal mitochondria fractionated with digitonin indicates that the protein is located at the outer face of the inner membrane of the organelle (external enzyme). The corresponding gene was inactivated by the generation of repeat-induced point mutations. Mitochondria from the resulting null-mutant nde2 are highly deficient in the oxidation of cytosolic NADH and NADPH. A triple mutant nde1/nde2/ndi1, lacking mitochondrial alternative NAD(P)H dehydrogenases, was obtained, indicating that these proteins are not essential in *N. crassa*. However, crosses between the nde2 mutant strain and complex I-deficient mutants yielded no viable double mutants. Transcription of the *nde-2* gene, as well as of *ndi-1* (internal enzyme), is repressed in the late exponential phase of fungal growth.

Keywords: Mitochondrion; NADH dehydrogenase; Respiratory mutant; Gene expression; *Neurospora crassa*

1. Introduction

The mitochondrial respiratory chain is a main provider of the high levels of energy required for cellular metabolism and this involves the action of NAD(P)H dehydrogenases. Mitochondria are known to contain the type I NADH:ubiquinone oxidoreductase or complex I, as well as several non-proton-pumping NAD(P)H dehydrogenases [1,2]. The proton-pumping complex I is a ubiquitous enzyme that couples the rotenone-sensitive transfer of two electrons from NADH to ubiquinone with the active transport of four protons across the inner mitochondrial membrane [3]. This enzyme contains a large number of polypeptides (about 45 in mammals) of both nuclear and mitochondrial origin, as well as FMN and several iron–sulfur clusters as prosthetic groups, and is relatively conserved in terms of composition, structure and function [4–6]. In contrast, the alternative non-proton-pumping NAD(P)H dehydrogenases are single polypeptide enzymes that oxidize NAD(P)H originating from either the cytosol (external enzymes) or the mitochondrial matrix (internal enzymes) and feed electrons into the respiratory chain in a rotenone-insensitive manner. These enzymes, using FAD or FMN as a cofactor, have been described in bacteria, protozoa, plants and fungi, however varying widely in their number and specificity [2,7–10].

Four alternative NADH dehydrogenases have been described in plants, two of them thought to be internal enzymes, whereas the other two seem to be external [2], but they were not yet fully identified. In *Saccharomyces cerevisiae* mitochondria, which lacks complex I, one internal and two external enzymes have been described [11]. Only a single external NADH dehydrogenase is present, in addition to complex I, in the inner mitochondrial membrane of the fungus *Yarrowia lipolytica* [8]. These and other alternative dehydrogenases have been described extensively and implicated in several physiological phenomena, such as bacterial redox state regulation [12], cold-stress [13] and photosynthetic metabolism [14]. It was also suggested that they work as ‘‘overflow systems’’ that keep reducing equivalents at physiological

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levels and prevent the production of reactive oxygen species [2]. Despite this, we are still far from understanding their specific cell functions and the molecular mechanisms underlying their physiological role.

The filamentous fungus *Neurospora crassa* appears as a fine model organism to study the role of the different mitochondrial NAD(P)H dehydrogenases. It contains both complex I and 3–4 alternative enzymes, indicating a complex situation comparable to that of plants [10]. Although the presence of alternative dehydrogenases has been proposed as early as 1970 [15], only in the past few years have they been identified and cloned. We have characterized a calcium-dependent external NADPH dehydrogenase [16] and, more recently, the internal alternative NADH dehydrogenase [17], respectively, NDE1 and NDI1. In an attempt to further investigate the mitochondrial respiratory chain of *N. crassa*, we concentrated on a second external NAD(P)H dehydrogenase, whose presence was already suggested by previous work [16]. Here, we report the identification and characterization of the external enzyme NDE2 and of *N. crassa* mutant strains devoid of this polypeptide.

2. Materials and methods

2.1. N. crassa manipulations

Growth and handling of *N. crassa* were carried out according to standard procedures [18]. Strains carrying pCSN44 recombinant vectors were selected on plates containing 100 μg/ml hygromycin B [19,20]. The bacterial strains DH5α and M15 were used as hosts for the pCSN44 and pQE31 (Qiagen) plasmids, respectively.

2.2. Mutant isolation

The *nde-2* gene was amplified from *N. crassa* genomic DNA by PCR using primers 5′-ACTCAGCAGATTC*C*TCG*A*G*C*CCA-3′ and 5′-CGAG*CT**C**G**A**G*GGAAATGTTAG-3′. The italicised bases were altered from the *N. crassa* DNA to create an *Xho*I restriction site (underlined). The amplified 1687 bp fragment was cloned in the pGEMT-easy vector (Promega). The resulting plasmid was digested with *Xho*I and the relevant band cloned into pCSN44 treated with the same enzyme, creating plasmid pNDE2-X. This recombinant vector was used for transformation of *N. crassa* 74-OR23-1A spheroplasts [20]. Genomic DNA from transformants was analysed by Southern blotting to identify strains with single-copy integration. One transformant carrying a duplication of the *nde-2* gene was crossed with strain 74-OR8-1a in order to inactivate this gene by RIP [21]. Identification of nde2 mutants among the progeny of the cross was carried out by Western blot analysis of mitochondrial proteins with an antiserum against NDE2.

2.3. Production of antibodies

The recombinant pGEMT-easy vector containing the *nde-2* gene was double digested with *Bam*HI and *Pst*I (*Bam*HI cuts within the *nde-2* gene and *Pst*I cuts in the vector). The resulting 1156-bp fragment was cloned into the pQE31 expression vector leading to the expression, in *Escherichia coli*, of the C-terminal 369 amino acid residues of the NDE2 polypeptide fused to a His-tag. After purification, the recombinant protein was used to generate rabbit polyclonal antisera [22]. Antisera against the mitochondrial processing peptidase, cytochrome *c* heme lyase and the TOM20 receptor were a kind gift of Dr. Walter Neupert from Germany.

2.4. Oxygen consumption

N. crassa mitochondria were prepared as previously described [16]. Using a Clark-type oxygen electrode (Hansatech), respiration was measured polarographically at 25 °C in a total volume of 1 ml. Mitochondrial assays contained 0.5–1 mg of protein, 0.3 M sucrose, 10 mM potassium phosphate, pH 7.2, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM KCl, 4 μM carbonyl cyanide *m*-chlorophenylhydrazone, 0.02% (w/v) BSA, and 20 μM rotenone. Mitochondrial activities at different pH were assayed in a reaction medium containing 20 mM MES, 20 mM MOPS, 20 mM Tris, 0.3 M sucrose, 4 μM carbonyl cyanide *m*-chlorophenylhydrazone, 0.02% (w/v) BSA, and 20 μM rotenone, adjusted to pH 4.7–9.2 with KOH. The reactions were initiated by the addition of either 1 mM NADH or 1 mM NADPH. Calcium and antimycin A were added to final concentrations of 0.1 mM and 0.2 μg/ml, respectively. Mitochondrial integrity was assessed by measuring 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 [16].

2.5. Northern blot analysis

Total RNA was purified from mycelial tissue harvested at both early exponential (12–16 h growth, about 2.5 g/l) and late exponential phases (20–24 h growth, about 13 g/l), as described previously [23]. RNA was treated with RNase free-DNase I (Boeringher) for 20 min at 37 °C in a reaction buffer containing 20 mM Tris, pH 7.6 and 5 mM $MgSO_4$. Then, it was extracted once with phenol/chloroform, precipitated with ethanol and stored at −20 °C. The RNA was resolved by electrophoresis in formaldehyde/formamide denaturing gels [24]. Hybridisations were conducted with probes labelled with [^{32}P]dCTP with a random primer labelling kit (Boehringer). The following probes were employed: a 2.5 kb *nde-2* fragment (see below), a 1.4 kb *Sac*I/*Eco*RV *nuo-9.8* fragment [25], a 1.8 kb *Kpn*I *ndi-1* fragment [17], a 0.8 kb *Eco*RI nuo-21.3c cDNA fragment [26], a 2.1 kb *nde-1* cDNA fragment [16] and a 5.1 kb

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MASTSRALGRLSAPSMGVARLQTQAVSRLLSSAPRRALISESRQVAVTQQ   50
IRRAHTETTPLPEPPKERRRFRKLRWLWRAPLFVSLAGIAYVGWGVYEER  100
NPGPQVEPDPSKKTLVVLGTGWGSVSLLKKLDTEHYNVIVISPRNYFLFT  150
PLLPSCTTGLIEHRSIMEPIRTILRHKKANVKFYEAEASSVDPERKVVRV  200
LDTSEIRGDVVETEIPYDMLVVGVGAENATFGIPGVREHTCFLKEIGDAQ  250
RIRKKIMDCVETAAFKGQSQEEIDRLLHMVVVGGGPTGVEFAGELQDFFE  300
EDIKKLIPDIADRFRVTLIEALPNVLPSFSKQLIEYTESTFKEEKIDIMT  350
KTMVKRVTEKTVEAEISKPDGTREKITLPYGLLVWATGNAVRPVVKDLME  400
RIPAQKDSRRGLAVNEYLVVQGTRDIWAVGDCAVAGYAPTAQVASQEGNF  450
LAGLFNNMARTEVLEQRVRELSGSLNLAPGNAAEISKEIEEHERQLRRIK  500
DIKPFHYSHQGSLAYIGSEKAVADVSWFNGNLASGGSLTFLFWRSAYLSM  550
CFSTRNRLLVINDWVKSKLFGRDVSRE                          577
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Fig. 1. Deduced primary structure of the NDE2 polypeptide. The putative cleavable mitochondrial targeting sequence is italicised, a putative transmembrane domain is in boldface and the invariant three-G residues within conserved dinucleotide-binding motifs are in boldface and underlined.

*Hin*dIII actin gene fragment. The *nde-2* gene fragment was amplified from genomic DNA by PCR, using the oligonucleotide primers 5′-GACCTAGGATCAGTCAGACTCGG-3′ and, as above, 5′-CGAGCTCGAGGGAAATGTTAG-3′. Northern blots were quantified with a phosphoimager screen using the computer-based programs Typhoon Scan and Image Quant.

2.6. Miscellaneous

Standard protocols were followed for PCR and general cloning procedures [24]. The techniques for digitonin fractionation of mitochondria followed by proteinase K treatment of the organelles [16], protein determination [27], SDS-PAGE [28] and Western blotting [29] have been described previously.

3. Results

3.1. Identification and topology of NDE2

The gene coding for NDE2 was identified in a Blast search of the genomic database of the *Neurospora* Sequencing Project at the Whitehead Institute/MIT Center for Genome Research (www-genome.wi.mit.edu), using the sequence of the NDE1 polypeptide [30] as the query sequence. It codes for a polypeptide of 577 amino acid residues with a predicted molecular mass of 64,656 Da (Fig. 1). Confirming the putative initiation ATG codon, an in-frame stop codon is found 75 nucleotides upstream. The coding sequence is interrupted by two introns of 100 and 64 bp, respectively. The N-terminal 54 amino acids show typical features of a mitochondrial targeting sequence. The protein contains two consensus motifs for the binding of NAD(P)H and FAD, and an N-terminal domain (amino acid residues 76–97) predicted to be transmembranar with high probability. The NDE2 polypeptide sequence displays significant similarity with the alternative NAD(P)H dehydrogenases found in yeast and plant mitochondria (not shown).

To determine the localization of NDE2, we analysed the protease accessibility of the protein upon fractionation of mitochondria with increasing concentrations of digitonin. After proteinase K treatment, the detergent-solubilized organelles were analysed by immunoblotting with antisera against NDE2 and the degradation pattern was compared to that obtained with antisera against the mitochondrial processing peptidase [31], cytochrome *c* heme lyase [32] and the TOM20 receptor [33] as markers for the matrix, intermembrane space and outer mitochondrial membrane, respectively (Fig. 2). Whereas TOM20 is readily digested by proteinase K, NDE2 is resistant to the protease in intact mitochondria. The protein became accessible to the protease only upon solubilization of the outer mitochondrial membrane with 0.2% digitonin. In fact, the pattern of NDE2 parallels that of the intermembrane space cytochrome *c* heme lyase. The integrity of the inner mitochondrial membrane at this digitonin concentration was assessed by the detection of MPP, which disappeared only at higher detergent concentrations. These results provide evidence that NDE2 faces the intermembrane space.

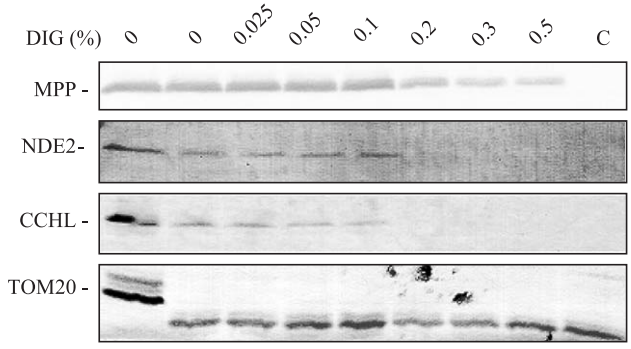

Fig. 2. Protease accessibility upon digitonin solubilization of mitochondria. Mitochondria (100 μg) were solubilized with increasing concentrations of digitonin (DIG), treated with proteinase K (except the first lane), and analysed by Western blotting with antisera against NDE2, the mitochondrial processing peptidase (MPP), the cytochrome *c* heme lyase (CCHL) and the outer membrane protein receptor TOM20. A control experiment with proteinase K in the presence of 1% Triton X-100 is also shown (C).

3.2. The nde2 strain is deficient in NAD(P)H dehydrogenase activities

To further characterize NDE2, a *N. crassa* null-mutant was generated by repeat-induced point mutations in the respective gene. Briefly, the cloned *nde-2* gene was duplicated in the genome of the fungus by transformation and passed through a genetic cross, during which the repeated sequences were inactivated at a certain frequency. Mitochondrial proteins from individual ascospore progeny of the cross were then analysed by Western blotting with antibodies against NDE2. The mutant strain nde2, lacking the NDE2 protein, was identified among 79 spores analysed (Fig. 3).

The linear hyphal extension rates of the nde2 mutant and the wild-type strain are comparable, as evaluated in race tubes with different carbon sources, except for a slight delay of mutant growth in glucose-containing media. When grown in liquid media, the amount of mycelia obtained with the mutant is always slightly less than that obtained with wild-type. Homozygous crosses of nde2 mutant strains are fully fertile, indicating that NDE2 is not essential either for the vegetative or the sexual phase of the life cycle of *Neurospora*. However, we noticed that germinated nde2 spores were less dense than wild-type colonies (Fig. 4). This growth phenotype appears to be due to a lower degree of hyphal branching.

The respiration of nde2 mitochondria was analysed with an oxygen electrode. The rates of oxidation of matrix NADH (generated with pyruvate/malate) by wild-type and nde2 mitochondria were similar and both equally inhibited about 50% by the complex I inhibitor rotenone (not shown). Apparently, there is no special increase in the rates of oxidation of matrix NADH, either by complex I or the alternative NDI1 polypeptide, to compensate for the lack of the external (see below) NDE2 protein. In sharp contrast, a drastic reduction of cytosolic NADH oxidation at pH 7.2 can be observed in intact nde2 mitochondria (Fig. 5A). When a more detailed characterization of exogenous NAD(P)H oxidation was undertaken, we observed that nde2 mitochondria are highly deficient in the oxidation of both NADH and NADPH throughout a large range of pH (Fig. 5B). These results strongly suggest that NDE2 is the main external dehydrogenase responsible for the oxidation of cytosolic NADH and NADPH under physiological conditions.

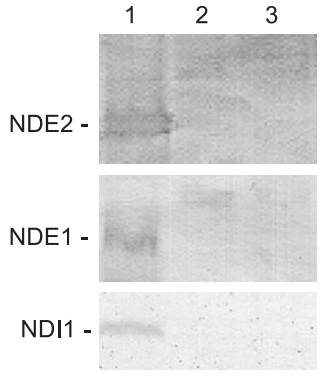

Fig. 3. Identification of nde2 mutant strains. Total mitochondrial proteins (100 μg) from wild-type (lane 1), the single mutants nde2 (lane 2, upper panel), nde1 (lane 2, middle panel) and ndi1 (lane 2, lower panel), and the triple mutant nde1/nde2/ndi1 (lane 3) were analysed by Western blotting with antisera against NDE or NDI proteins, as indicated in the left side.

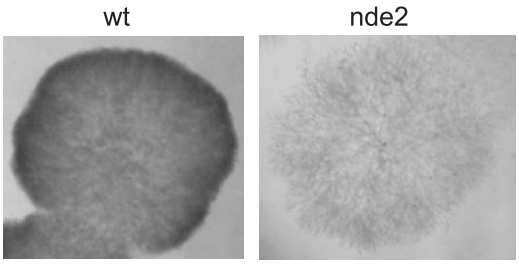

Fig. 4. Germination of wild-type and nde2 spores. The progeny from wild-type and nde2 homozygous crosses were plated on sorbose. The picture shows typical 2–3 mm colonies obtained after incubation of the plates for 2 days.

We found that calcium stimulates the oxidation of cytosolic NADH by nde2 mitochondria (Fig. 5C) and first attributed this effect to stimulation of NDE1. However, this stimulation is also observed in a nde1/nde2 double mutant, though not in the triple mutant nde1/nde2/ndi1 (see below). Thus, it seems that the effect of calcium is due to activity of NDI1. Somehow, the presence of calcium might allow the exogenous NADH to pass the inner mitochondrial membrane and be oxidized by this internal enzyme. Calcium also stimulates cytosolic NADPH activity of nde2 mitochondria but not of the nde1/nde2 double mutant (not shown), confirming NDE1 as an external calcium-dependent NADPH dehydrogenase. As suggested in Ref. [16], it appears that the latter does not use NADH as substrate.

3.3. Gene expression and genetic interactions with other NAD(P)H dehydrogenases

In order to get more insights about the cellular relevance of the alternative NAD(P)H dehydrogenases, we crossed the nde2 mutant with nde1 [16] and ndi1 [17] mutants to obtain double and triple mutants (Fig. 3). The nde1/nde2 and nde1/nde2/ndi1 mutants are perfectly viable. We also crossed the nde2 mutant with several complex I mutants displaying different phenotypes in terms of enzyme assembly and function. The nde2 strain was crossed to nuo21 [34], which carries a functional complex I, and to nuo51 [35], nuo21.3c [20] and nuo20.8 [36], all of which lack a functional enzyme. Viable double mutants were obtained only from the cross between nde2 and nuo21, a mutant with an almost intact and fully functional complex I. Surprisingly, we could not isolate double mutants from crosses between nde2 and any of the complex I mutants that lack a functional enzyme (Table 1). As a control, wild-type recombinants were readily obtained from these crosses.

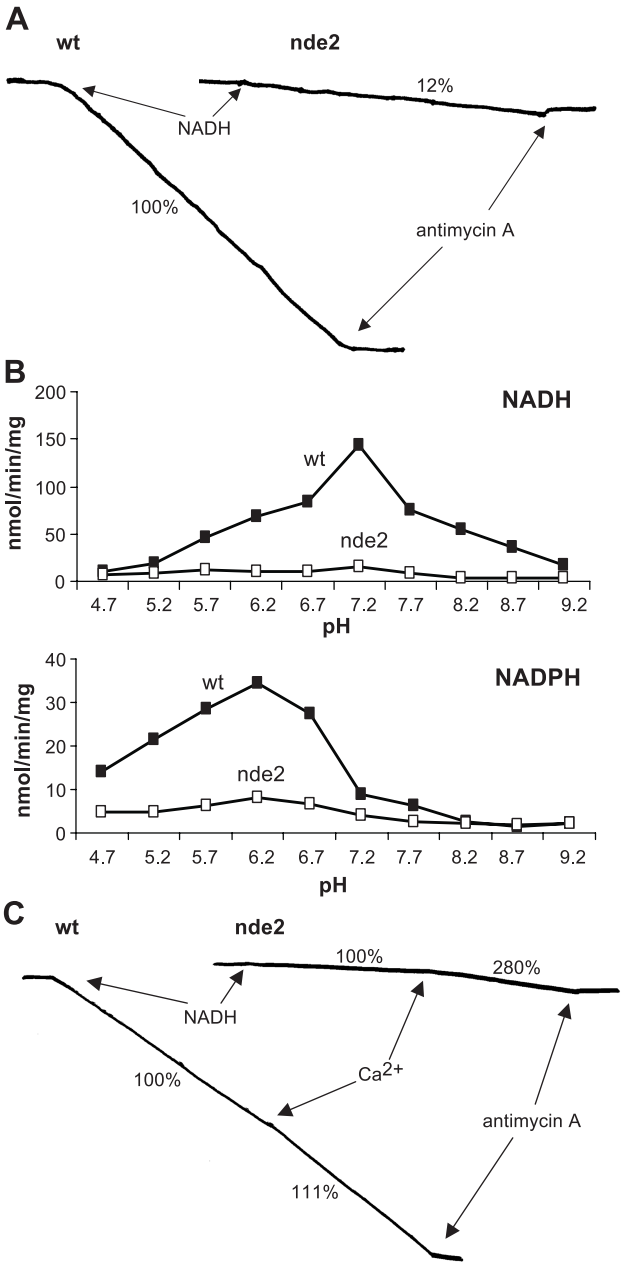

Fig. 5. Oxygen consumption by nde2 mitochondria. (A) Exogenous NADH oxidation at pH 7.2. The initial activities were 143 and 15 nmol of O_2/min per mg of protein for wild-type and nde2 mitochondria, respectively. (B) Exogenous pH-dependent NAD(P)H activities. Each point represents a mean of at least three independent mitochondrial preparations. (C) Calcium effect on exogenous NADH oxidation at pH 6.2. Activities in the absence and presence of calcium were 112 and 124 nmol of O_2/min per mg of protein for wild-type mitochondria, and 10 and 28 nmol of O_2/min per mg of protein for nde2 mitochondria, respectively. In these experiments, latencies of wild-type and nde2 mitochondria were in the range of 84–88% and 87–88% for cytochrome *c* oxidase (outer membrane integrity) and 90–99% and 93–99% for malate dehydrogenase (inner membrane integrity), respectively.

To further investigate whether inactivation of the *nde-2* gene had any effect on expression of the other mitochondrial NAD(P)H dehydrogenases, we compared Northern blots of wild-type and the nde2 mutant. Since the activities of these proteins were claimed to vary with the growth stage [37] (unpublished data), we checked gene expression in fungal cells harvested at both the early and late exponential phases of growth in liquid media. Total RNA was prepared from mycelial tissue of the two strains, grown to early or late exponential phase, and analysed by Northern blotting with different gene probes. In these experiments, the 28S rRNA and the expression of actin were used as controls (Fig. 6). The results obtained with both strains analysed were roughly similar, suggesting that lack of NDE2 has no significant effect on the regulation of genes encoding other mitochondrial dehydrogenases. The transcripts for complex I subunits are present at both growth stages, with a slight decrease from early to late exponential growth. However, this decrease is not so apparent when the values are normalized to the expression of actin. The same applies to the expression

Table 1
Ascospore progeny resulting from crosses between the nde2 strain and complex I mutants

Cross	Progeny				
	nde2	nuo	wt	nde2, nuo	Total
nde2 × nuo21	2	5	2	3	12
nde2 × nuo20.8	13	13	14	0	40
nde2 × nuo51	7	12	7	0	26
nde2 × nuo21.3c	7	15	5	0	27

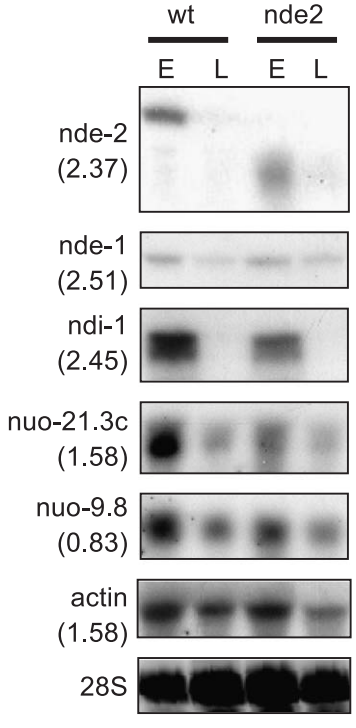

Fig. 6. Gene expression in wild-type and nde2. Total RNA was prepared from strains grown for 12–16 h (E, early exponential phase) or 20–24 h (L, late exponential phase) and analysed by Northern blotting. The gene probes used are indicated in the left side with the size of the respective transcripts in kilobase pairs between brackets. The 28S rRNA (28S) is a direct photograph of the ethidium bromide stained gel.

of the external NADPH dehydrogenase NDE1. In contrast, the genes encoding the alternative NADH dehydrogenases NDE2 and NDI1 are drastically down-regulated from early to late exponential growth, their transcripts are severely reduced and/or absent in late exponential phase. It should be noted that the nde2 mutant produces a truncated transcript of the *nde-2* gene, but the down-regulation of this gene from early to late exponential growth is still maintained.

4. Discussion

In this work, we present the identification and characterization of NDE2, an alternative NAD(P)H dehydrogenase of *N. crassa* mitochondria (Fig. 1). The results obtained with digitonin fractionation of mitochondria (Fig. 2) and analysis of the mitochondrial respiratory activity of an nde2 mutant strain, on exogenously added NAD(P)H substrates (Fig. 5), strongly support the conclusion that NDE2 faces the intermembrane space of the organelles and acts as external NAD(P)H dehydrogenase (oxidizing cytosolic substrates). The protein is probably connected to the inner mitochondrial membrane through a predicted transmembrane domain. We have recently characterized another external calcium-dependent NADPH dehydrogenase and an internal NADH dehydrogenase, NDE1 and NDI1, respectively [16,17], located in the inner membrane of *N. crassa* mitochondria. Thus, we are completing the characterization of the fungal alternative NAD(P)H dehydrogenases. The situation is known in *S. cerevisiae*, where one internal and two external non-proton pumping NADH dehydrogenases are present but complex I is absent [11]. Evidence for the presence of four proteins in plants have been presented, two in each side of the inner mitochondrial membrane [2], but only two were conclusively identified [38]. We are still investigating the presence of a fourth NAD(P)H dehydrogenase in *N. crassa* mitochondria [10].

We found stimulatory effects of calcium on the dehydrogenase activity of nde2 mitochondria upon added NAD(P)H. The calcium effects are not so visible in wild-type mitochondria probably because they are masked by the already high activity of the NDE2 polypeptide. The stimulation of NADPH oxidation is likely due to activity of NDE1, the calcium-dependent NADPH dehydrogenase. We attributed the stimulation of NADH oxidation to activity of the internal NDI1 enzyme, because the effect is maintained in an nde1/nde2 mutant and is abolished in an nde1/nde2/ndi1 mutant. Thus, calcium action might turn the inner mitochondrial membrane permeable to NADH and allow its oxidation by NDI1, since complex I was inhibited by rotenone in our experiments. In fact, calcium was suggested to operate the mitochondrial permeability transition pore in skeletal muscle mitochondria. In this case, pore opening by calcium addition induced a transient stimulation of malate oxidation followed by a progressive inhibition. Respiration could be restored by the addition of NADH that diffused through the pore and was oxidized by complex I in a fully rotenone-sensitive way [39]. In this context, our results may be regarded as evidence that the pore is present in *Neurospora*. A classical Ca^{2+}-dependent permeability transition seems to be absent in *S. cerevisiae* [39,40].

The physiological role of alternative NAD(P)H dehydrogenases remains mostly unclear. We have shown that they are not essential proteins, at least in *N. crassa* grown in laboratory conditions, since a triple mutant lacking all three mitochondrial NAD(P)H dehydrogenases could be isolated (Fig. 3). It was reported that the importance of complex I and alternative enzymes in the respiratory chain of *N. crassa* vary with the growth stage [37] and that alternative enzymes may be advantageous during fast growth when carbon sources are abundant [8,41]. Through the analysis of gene expression, we could now show that the *nde-2* and *ndi-1* NADH dehydrogenase genes are highly down-regulated in late exponential growth of *N. crassa* (Fig. 6). They might be coordinately regulated but differently regulated from the NADPH dehydrogenase gene *nde-1*. We found no significant transcription regulation of complex I genes, despite the fact that the activity of the enzyme increases in the late exponential phase of growth [37]. We have already suggested that NDI1 is involved in the efficiency of germination of both sexual and asexual fungal spores [17], in agreement with findings that there is an increase in NAD(P)H in the initiation of conidial germination, but coupled oxidative phosphorylation only appears a few hours later [42]. The nde2 mutant also displays a phenotype in terms of the initial growth following spore germination (Fig. 4). The nde2 phenotype appears to be related to a few described hypobranching mutants. Colonies developed from nde2 spores are about the same size as wild-type colonies, consistent with findings that branching can be independent of tip extension rate [43]. Thus, despite the fact that they are not essential proteins, we suggest that alternative NADH dehydrogenases may provide an advantage to fungi in the colonization of media.

A striking and puzzling result was the failure to obtain double mutants from crosses between the nde2 strain and mutants lacking a functional complex I (Table 1). This was unexpected since the internal enzyme NDI1 should be able to compensate the lack of complex I in the nde2 mutant background. Since NDE2 does not pump protons [2,8], the problem likely arises from the deficiency in mitochondrial NADH oxidation and suggests that NDE2 complements complex I activity in *N. crassa*. This would imply that *N. crassa* has ways of exchanging matrix/cytosolic NADH. This is in agreement with findings that external NADH oxidation is increased in complex I mutants of *N. crassa* and plants [25,44]. A somehow alternative explanation is that an overall increase in the $NADH/NAD^+$ ratio in the mitochondrial matrix of a double mutant would block the transport of cytoplasmic NADH across the inner mitochondrial membrane and renders it inaccessible to NDI1. It is known that an increase in cytoplasmic NADH redox potential impairs

mitochondrial energy metabolism by inhibiting glucose oxidation [45,46] pointing to the importance of oxidizing cytoplasmic NADH. The activity of a transporter (or shuttle) may be blocked at higher matrix NADH concentrations, yet too low to be efficiently oxidized by NDI1 in the double mutant. In Jerusalem artichoke tubers, for instance, complex I has a 10-fold lower K_m for NADH than NDI1 and its absence can result in an elevation of the matrix NADH concentration [2]. At the moment, we cannot be certain that both complex I and NDE2 are strictly required for fungal vegetative growth. It is possible that the proteins are needed simultaneously during other stages of growth, like spore development, and this would preclude the isolation of double mutants.

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References

[1] Y. Hatefi, The mitochondrial electron transport and oxidative phosphorylation system, Ann. Rev. Biochem. 54 (1985) 1015–1069.
[2] I.M. Moller, Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species, Annu. Rev. Plant Physiol. Plant Mol. Biol. 52 (2001) 561–591.
[3] A.S. Galkin, V.G. Grivennikova, A.D. Vinogradov, $H^+/2e^-$ stoichiometry in NADH–quinone reductase reactions catalyzed by bovine heart submitochondrial particles, FEBS Lett. 451 (1999) 157–161.
[4] J.E. Walker, The NADH:ubiquinone oxidoreductase (complex I) of respiratory chains, Q. Rev. Biophys. 25 (1992) 253–324.
[5] V. Guenebaut, A. Schlitt, H. Weiss, K. Leonard, T. Friedrich, Consistent structure between bacterial and mitochondrial NADH:ubiquinone oxidoreductase (complex I), J. Mol. Biol. 276 (1998) 105–112.
[6] A. Videira, Complex I from the fungus *Neurospora crassa*, Biochim. Biophys. Acta 1364 (1998) 89–100.
[7] T. Yagi, The bacterial energy-transducing NADH–quinone oxidoreductases, Biochim. Biophys. Acta 1141 (1993) 1–17.
[8] S.J. Kerscher, Diversity and origin of alternative NADH: ubiquinone oxidoreductases, Biochim. Biophys. Acta 1459 (2000) 274–283.
[9] J. Fang, D.S. Beattie, Novel FMN-containing rotenone-insensitive NADH dehydrogenase from *Trypanosoma brucei* mitochondria: isolation and characterization, Biochemistry 41 (2002) 3065–3072.
[10] A. Videira, M. Duarte, From NADH to ubiquinone in *Neurospora* mitochondria, Biochim. Biophys. Acta 1555 (2002) 187–191.
[11] K.M. Overkamp, B.M. Bakker, P. Kotter, A. van Tuijl, S. de Vries, J.P. van Dijken, J.T. Pronk, In vivo analysis of the mechanisms for oxidation of cytosolic NADH by *Saccharomyces cerevisiae* mitochondria, J. Bacteriol. 182 (2000) 2823–2830.
[12] C.A. Howitt, P.K. Udall, W.F. Vermaas, Type 2 NADH dehydrogenases in the cyanobacterium *Synechocystis* sp. strain PCC 6803 are involved in regulation rather than respiration, J. Bacteriol. 181 (1999) 3994–4003.
[13] A.S. Svensson, F.I. Johansson, I.M. Moller, A.G. Rasmusson, Cold stress decreases the capacity for respiratory NADH oxidation in potato leaves, FEBS Lett. 517 (2002) 79–82.
[14] A.A. Svensson, A.G. Rasmusson, Light-dependent gene expression for proteins in the respiratory chain of potato leaves, Plant J. 28 (2001) 73–82.
[15] H. Weiss, G. von Jagow, M. Klingenberg, T. Buecher, Characterization of *Neurospora crassa* mitochondria prepared with a grind-mill, Eur. J. Biochem. 14 (1970) 75–82.
[16] A.M. Melo, M. Duarte, I.M. Moller, H. Prokisch, P.L. Dolan, L. Pinto, M.A. Nelson, A. Videira, The external calcium-dependent NADPH dehydrogenase from *Neurospora crassa* mitochondria, J. Biol. Chem. 276 (2001) 3947–3951.
[17] M. Duarte, M. Peters, U. Schulte, A. Videira, The internal alternative NADH dehydrogenase of *Neurospora crassa* mitochondria, Biochem. J. 371 (2003) 1005–1011.
[18] R.H. Davis, F.J. de Serres, Genetic and microbiological research techniques for *Neurospora crassa*, Methods Enzymol. 17A (1970) 79–143.
[19] C. Staben, B. Jensen, M. Singer, J. Pollock, M. Schechtman, J. Kinsey, E.U. Selker, Use of a bacterial hygromycin B resistance gene as a dominant marker in *Neurospora crassa* transformation, Fungal Genet. Newslett. 36 (1989) 79–81.
[20] M. Duarte, A. Videira, Respiratory chain complex I is essential for sexual development in *Neurospora* and binding of iron–sulfur clusters are required for enzyme assembly, Genetics 156 (2000) 607–615.
[21] E.U. Selker, Premeiotic instability of repeated sequences in *Neurospora crassa*, Annu. Rev. Genet. 24 (1990) 579–613.
[22] A. Videira, S. Werner, Assembly kinetics and identification of precursor proteins of complex I from *Neurospora crassa*, Eur. J. Biochem. 181 (1989) 493–502.
[23] V. Sokolovsky, R. Kaldenhoff, M. Ricci, V.E.A. Russo, Fast and reliable miniprep RNA extraction from *Neurospora crassa*, Fungal Genet. Newslett. 37 (1990) 41.
[24] J. Sambrook, D.W. Russell, Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 2001.
[25] I. Marques, M. Duarte, A. Videira, The 9.8 kDa subunit of complex I, related to bacterial $Na(^+)$-translocating NADH dehydrogenases, is required for enzyme assembly and function in *Neurospora crassa*, J. Mol. Biol. 329 (2003) 283–290.
[26] M. Duarte, M. Finel, A. Videira, Primary structure of a ferredoxin-like iron–sulfur subunit of complex I from *Neurospora crassa*, Biochim. Biophys. Acta 1275 (1996) 151–153.
[27] M.M. Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[28] R. Zauner, J. Christner, G. Jung, U. Borchart, W. Machleidt, A. Videira, S. Werner, Identification of the polypeptide encoded by the URF-1 gene of *Neurospora crassa* mtDNA, Eur. J. Biochem. 150 (1985) 447–454.
[29] H. Towbin, T. Staechelin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, Proc. Natl. Acad. Sci. U. S. A. 76 (1979) 4350–4354.
[30] A.M. Melo, M. Duarte, A. Videira, Primary structure and characterisation of a 64 kDa NADH dehydrogenase from the inner membrane of *Neurospora crassa* mitochondria, Biochim. Biophys. Acta 1412 (1999) 282–287.
[31] G. Hawlitschek, H. Schneider, B. Schmidt, M. Tropschug, F.U. Hartl, W. Neupert, Mitochondrial protein import: identification of processing peptidase and of PEP, a processing enhancing protein, Cell 53 (1988) 795–806.
[32] R. Lill, R.A. Stuart, M.E. Drygas, F.E. Nargang, W. Neupert, Import of cytochrome *c* heme lyase into mitochondria: a novel pathway into the intermembrane space, EMBO J. 11 (1992) 449–456.
[33] T. Sollner, G. Griffiths, R. Pfaller, N. Pfanner, W. Neupert, MOM19, an import receptor for mitochondrial precursor proteins, Cell 59 (1989) 1061–1070.
[34] F. Ferreirinha, M. Duarte, A.M. Melo, A. Videira, Effects of disrupt-

ing the 21 kDa subunit of complex I from *Neurospora crassa*, Biochem. J. 342 (1999) 551–554.
[35] W. Fecke, V.D. Sled, T. Ohnishi, H. Weiss, Disruption of the gene encoding the NADH-binding subunit of NADH: ubiquinone oxidoreductase in *Neurospora crassa*. Formation of a partially assembled enzyme without FMN and the iron–sulphur cluster N-3, Eur. J. Biochem. 220 (1994) 551–558.
[36] M.V. da Silva, P.C. Alves, M. Duarte, N. Mota, A. Lobo-da-Cunha, T.A. Harkness, F.E. Nargang, A. Videira, Disruption of the nuclear gene encoding the 20.8-kDa subunit of NADH: ubiquinone reductase of *Neurospora* mitochondria, Mol. Gen. Genet. 252 (1996) 177–183.
[37] J.P. Schwitzguebel, J.M. Palmer, Properties of mitochondria as a function of the growth stages of *Neurospora crassa*, J. Bacteriol. 149 (1982) 612–619.
[38] A.G. Rasmusson, S. Svensson, V. Knoop, L. Grohmann, A. Brennicke, Homologues of yeast and bacterial rotenone-insensitive NADH dehydrogenases in higher eukaryotes: two enzymes are present in potato mitochondria, Plant J. 20 (1999) 79–87.
[39] E. Fontaine, O. Eriksson, F. Ichas, P. Bernardi, Regulation of the permeability transition pore in skeletal muscle mitochondria. Modulation by electron flow through the respiratory chain complex I, J. Biol. Chem. 273 (1998) 12662–12668.
[40] D.W. Jung, P.C. Bradshaw, D.R. Pfeiffer, Properties of a cyclosporin-insensitive permeability transition pore in yeast mitochondria, J. Biol. Chem. 272 (1997) 21104–21112.
[41] I.L. Pahlman, C. Larsson, N. Averet, O. Bunoust, S. Boubekeur, L. Gustafsson, M. Rigoulet, Kinetic regulation of the mitochondrial glycerol-3-phosphate dehydrogenase by the external NADH dehydrogenase in *Saccharomyces cerevisiae*, J. Biol. Chem. 277 (2002) 27991–27995.
[42] J.C. Schmit, S. Brody, Biochemical genetics of *Neurospora crassa* conidial germination, Bacteriol. Rev. 40 (1976) 1–41.
[43] M.K. Watters, A.J. Griffiths, Tests of a cellular model for constant branch distribution in the filamentous fungus *Neurospora crassa*, Appl. Environ. Microbiol. 67 (2001) 1788–1792.
[44] M. Sabar, R. de Paepe, Y. de Kouchkovsky, Complex I impairment, respiratory compensations, and photosynthetic decrease in nuclear and mitochondrial male sterile mutants of *Nicotiana sylvestris*, Plant Physiol. 124 (2000) 1239–1250.
[45] J.T. Barron, L. Gu, J.E. Parrillo, Malate-aspartate shuttle, cytoplasmic NADH redox potential, and energetics in vascular smooth muscle, J. Mol. Cell Cardiol. 30 (1998) 1571–1579.
[46] K. Eto, Y. Tsubamoto, Y. Terauchi, T. Sugiyama, T. Kishimoto, N. Yamauchi, N. Yamauchi, N. Kubota, S. Murayama, T. Aizawa, Y. Aizawa, S. Aizawa, H. Kasai, Y. Yazaki, T. Kadowaki, Role of NADH shuttle system in glucose-induced activation of mitochondrial metabolism and insulin secretion, Science 283 (1999) 981–985.