The internal alternative NADH dehydrogenase of *Neurospora crassa* mitochondria

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An open reading frame homologous with genes of non-proton-pumping NADH dehydrogenases was identified in the genome of *Neurospora crassa*. The 57 kDa NADH:ubiquinone oxidoreductase acts as internal (alternative) respiratory NADH dehydrogenase (NDI1) in the fungal mitochondria. The precursor polypeptide includes a pre-sequence of 31 amino acids, and the mature enzyme comprises one FAD molecule as a prosthetic group. It catalyses specifically the oxidation of NADH. Western blot analysis of fungal mitochondria fractionated with digitonin indicated that the protein is located at the inner face of the inner membrane of the organelle (internal enzyme). The corresponding gene was inactivated by the generation of repeat-induced point mutations. The respiratory activity of mitochondria from the resulting null-mutant *ndi1* is almost fully inhibited by rotenone, an inhibitor of the proton-pumping complex I, when matrix-generated NADH is used as substrate. Although no effects of the NDI1 defect on vegetative growth and sexual differentiation were observed, the germination of both sexual and asexual ndi1 mutant spores is significantly delayed. Crosses between the ndi1 mutant strain and complex I-deficient mutants yielded no viable double mutants. Our data indicate: (i) that NDI1 represents the sole internal alternative NADH dehydrogenase of *Neurospora* mitochondria; (ii) that NDI1 and complex I are functionally complementary to each other; and (iii) that NDI1 is specially needed during spore germination.

Key words: complex I, mutagenesis, NADH dehydrogenase, respiratory chain.

INTRODUCTION

A main entry point for electrons into the mitochondrial respiratory chain is provided by the NAD(P)H dehydrogenases. These enzymes oxidize NADH and/or NADPH, transferring electrons to ubiquinone. Two types of NAD(P)H dehydrogenases are found in mitochondria: the proton-pumping respiratory complex I and the non-proton-pumping alternative NADH dehydrogenases [1,2]. Complex I represents an assembly of approx. 40 polypeptide subunits and performs the oxidation of matrix NADH, employing FMN and iron–sulphur clusters. It couples the redox reaction with the transfer of protons across the inner membrane, contributing to oxidative phosphorylation. A number of specific inhibitors, like rotenone and piericidin A, are known [3]. Existing in almost all eukaryotes, with the remarkable exception of some yeasts, it is reasonably well conserved in terms of composition, structure and function. Homologous complexes are also present in bacteria, archaea and in plant chloroplasts [4]. In contrast, alternative NADH dehydrogenases are non-proton-pumping, rotenone-resistant single polypeptide enzymes. These use FAD or FMN as a prosthetic group, and work as monomers or homodimers [1,5,6]. The enzymes show high variability in terms of their function and catalytic properties [7,8].

Although none of these proteins had been described in animals, up to four were proposed to exist in the inner membrane of plant and fungal mitochondria [2]. Although the alternative NADH dehydrogenases of plant mitochondria are not yet unequivocally identified, two of them seem to be orientated towards the matrix, oxidizing mitochondrial NADH (internal enzymes), and two others face the intermembrane space, oxidizing cytosolic NADH (external enzymes). *Saccharomyces cerevisiae*, which lacks complex I, was shown to employ one internal and two external NADH dehydrogenases [9], whereas the fungus *Yarrowia lipolytica*, which has complex I, uses only one external NADH dehydrogenase [10]. Similar proteins are present in the cytoplasmic membrane of bacteria, called type II NADH dehydrogenases or NDH2 [1].

It has been suggested that these enzymes work as 'overflow systems' to prevent the over-reduction of electron-transport carriers and the production of reactive oxygen species [2]. Their roles in different cell types are, however, far from being well understood. Inactivation of their genes led to increased glycerol production in yeast [11]; they have been implicated in bacterial regulation [12]; and one plant enzyme was shown to be cold-sensitive [13] and light-regulated [14]. Heterologous alternative NADH dehydrogenases were used in mammalian cells to complement complex I defects: a common cause of mitochondrial disease [15].

The filamentous fungus *Neurospora crassa* has been successfully employed for the study of many biological processes [16], and seems well suited to characterize mitochondrial NAD(P)H dehydrogenases, since it contains several of these types of proteins [17]. The proton-pumping complex I has been studied extensively [8,18,19]. The presence of rotenone-insensitive NADH dehydrogenases, one internal oxidizing matrix NADH and one external using cytosolic NADH and NADPH, has been described over 30 years ago [20]. More recently, we have identified an external calcium-dependent NADPH dehydrogenase, and provided evidence for the presence of at least one additional external dehydrogenase in *N. crassa* mitochondria [21,22]. In the present study, we characterize the internal alternative NADH dehydrogenase of the fungus.

Abbreviations used: Q2/Q6, ubiquinone-2 or -6 respectively.

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MATERIALS AND METHODS

N. crassa and bacterial manipulations

Growth and handling of *N. crassa*, including crosses, were performed according to standard procedures [23]. Linear hyphal extension rates were measured in race tubes [24]. Back-crosses with wild-type were performed to obtain strains with opposite mating types. Strains carrying the pCSN44-derived recombinant vector were selected on plates containing 100 μg/ml hygromycin B [25]. The bacterial strains DH5α and M15 were used as hosts for the pCSN44 and pQE31 (Qiagen) plasmids respectively.

Generation of antibodies

Plasmid pND-K (see below) was digested with *Xho*I, which cuts in the vector and within the *ndi-1* gene. The resulting 1.3 kb gene fragment was cloned into the *Sal*I site of the pQE31 expression vector. This leads to expression of the C-terminal 448 amino acid residues of NDI1 fused to a His_6 tag. The recombinant protein was induced, purified (following the protocol of Qiagen) and used to generate rabbit polyclonal antisera [26]. Antisera against subunits of complex I were used as obtained previously [27], and antisera against the mitochondrial processing peptidase and cytochrome *c* haem lyase were kindly donated by Dr Walter Neupert (Institute of Physiological Chemistry, University of Munich, Germany).

Mutant isolation

The *ndi-1* gene was obtained from *N. crassa* genomic DNA by PCR. Two primers were designed: the sense primer anneals in the region corresponding to the N-terminus of the mature protein (5′-C*GGTACC*TCCACCGTGAAGTTCAACAGC-3′) and the anti-sense primer anneals 123 bp downstream of the stop codon (5′-GCC*GGTACC*TTCTATTTGATCC-3′). The italicized bases were altered from the *N. crassa* sequence, and the underlined bases indicate *Kpn*I restriction sites. After amplification, the 1751 bp fragment was purified, digested with *Kpn*I and cloned into the pCSN44 vector, creating plasmid pND-K. This recombinant vector was used for transformation of *N. crassa* 74-OR8-1a spheroplasts [28]. Genomic DNA was isolated from transformants [29] and analysed by Southern blotting [30] to identify strains with single copy integration. The *Xho*I gene fragment of 1.3 kb, described above, was labelled with the Gene Images kit (Amersham Biosciences) and used as a probe. One transformant carrying a duplication of the *ndi-1* gene was crossed with strain 74-OR23-1A in order to obtain mutants by repeat-induced point mutations, as described previously [31]. Random progeny from the cross were germinated and their mitochondrial proteins were analysed by Western blotting with the antiserum against NDI1, leading to the identification of mutant ndi1.

Oxygen consumption

Preparation of *N. crassa* mitochondria has been described previously [22]. Respiration was measured polarographically at 25 °C with a Hansatech oxygen electrode in a total volume of 1 ml. Assays contained 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 and 0.02 % (w/v) BSA. Rotenone and antimycin were added to final concentrations of 20 μM and 0.2 μg/ml respectively. The integrity of mitochondria 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 [22].

Protein purification

Mitochondrial membranes of *N. crassa* strain nuo51 were isolated [32] and stirred for 20 min in 20 mM Tris/HCl, pH 7.5, containing 1 mM EDTA, 100 mM NaCl, 0.2 % (v/v) Triton X-100 and 1 mM PMSF at a protein concentration of 7.5 mg/ml on ice. Membranes were sedimented for 60 min at 250 000 ***g***. The supernatant was dialysed against 20 mM Tris/HCl, pH 7.5, containing 1 mM EDTA, 0.05 % (v/v) Triton X-100 and 0.5 mM PMSF, and subjected to anion-exchange chromatography (DEAE-Sepharose; Pharmacia). Proteins were eluted by a salt gradient from 0 to 250 mM NaCl in the dialysis buffer. Fractions with high NADH:ubiquinone-2 (Q2) oxidoreductase activity were pooled and subjected to Blue Sepharose chromatography (Pharmacia). A gradient from 0.1 to 1.5 M NaCl in dialysis buffer was used to elute the proteins. Fractions with high NADH:Q2 oxidoreductase activity were pooled again, concentrated 60-fold by ultrafiltration and loaded on to an Ultrogel AcA 44 (Serva) gel-filtration column in 20 mM Mops/KOH, pH 6.5, containing 100 mM NaCl, 1 mM EDTA and 0.05 % (v/v) Triton X-100. Eluted NADH:Q2 activity was applied to cation-exchange chromatography (Servacel CM-52; Serva) in the same buffer using a gradient from 0.1 to 0.25 M NaCl.

Miscellaneous

Standard protocols were used for PCR and general cloning procedures [30]. PAGE [33], blotting, incubation of blots with antisera and detection of alkaline phosphatase–secondary antibody conjugates [26,28], protein determination [34], digitonin fractionation of mitochondria [35] and alkaline extractions [29] have all been described previously. Flavin was determined fluorimetrically [36], and FAD was identified by reversed-phase chromatography [37]. Redox activities were measured in 20 mM Mops/KOH containing 200 mM KCl, 1 mM EDTA, 5 μM antimycin and 0.05 % Triton X-100. NADH:Q2 activity was routinely determined as NADH oxidation at 340 nm using 100 μM NADH and 100 μM Q2. For Edman degradation, the isolated protein was subjected to SDS/PAGE, blotted on to a PVDF membrane and sequenced on an Applied Biosystems 477A-pulse liquid-protein sequencer [38].

RESULTS

Identification and localization of NDI1

To identify genes coding for mitochondrial NAD(P)H dehydrogenases, we performed 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). The previously characterized alternative NADPH dehydrogenase NDE1 [22] was used as the query sequence. Among other homologues, contig 3.8 (assembly version 3) contains the *ndi-1* gene. It encodes a precursor polypeptide of 538 amino acid residues with a predicted mass of 59 922 Da (Figure 1). An in-frame stop codon is present 12 nt before the presumed initiation ATG codon. The coding sequence is interrupted by a single 77 bp intron at position 112 after the translation start codon. The deduced primary structure of the protein includes an N-terminal mitochondrial-targeting sequence not present in the mature enzyme (see below). Two consensus motifs for a dinucleotide-binding $\beta\alpha\beta$-fold are identified as binding sites for the ADP portion of NADH and FAD respectively [3]. Figure 2 displays an alignment of NDI1 with the sequences of alternative NADH dehydrogenases from different organisms. The

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1-   MASITRFARTSSSSLQVSTRSAPLALTARNFSTVKFNNKDRKERVVILGSGWAGYSFAK
61-  DLDPEKYERIFISPRSYFVFTPLLASTAVGTLEFRTVLEPIRRLDYGIGFHQGWAQDID
121- FANKTIRVEANANADSASKAVVPIGQGGQLNQASARGALFDVPYDKLVIACGAYSQTFG
181- IEGVREHANFLRDVGDARRIRLRVLSLFELCAYPKGVDNLTDEDRANLLHFAIVGGGPT
241- GIEYASELHDLIHDDLSKMYPDLLKFVRITVYDVSPKVLPMFDQALSKYAMDAFKRQKI
301- EIRTQHNIERVRPADGKLGSEYGELKLKIKQYGDKEVGAGLVVWSTGLMANPLIKQLAS
361- DKFAVPISPEDRAEARRPKAKLATDARTGGILVDEHFRVRIETQTTDAAKGSEIVPTSS
421- SNSLLRDVFVLGDAAVIESQRTLPKTAQVAAQQATYLAKVLNKANEGVIDVKDAPGFKF
481- RNWGVMTYLGSWKAIHQGPRDELRGWAAWVLWRSAYLAKSMSWRNRFLVPIYWLVSWVF
541- GRGISRF
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Figure 1 Deduced primary structure of the NDI1 precursor polypeptide

The cleavable mitochondrial targeting sequence, as well as invariant three-G residues within conserved dinucleotide-binding motifs, are shown underlined.

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ncNDI1
ncNDE1  MLRTYRVARASGLATAPRTLTLTSTTATRHLFTLPKSLQLRRPEKLSLISQRQLSGRPLP
 stNDB
scNDE1                MIRQSLMKTVWANSSRFSLQSKSGLVKYAKNRSFHAARNLLEDKKV
scNDE2                   MLPRLGFARTARSIHRFKMTQIS---KPFFHSTE---VGKPG
 scNDI
 stNDA

ncNDI1                                MASITRFARTSSSSLQVSTRSAPL-----------
ncNDE1  RTQSRLLNFGYRTAAWFGSSIAFVGLSFVAFFLYDASTYSSHATNQGDITVPKLALNPRR
 stNDB                                MRGFTYLSKVLHSHSSYSKLLVLCSVST----
scNDE1  ILQKVAPTTGVVAKQSFFKRTGKFTLKALLYSALAGTAYVSYSLYRE-------------
scNDE2  PQQKLSKSYTAVFKKWFVRG-----LKLTFYTTLAGTLYVSYELYKE-------------
 scNDI                MLSKNLYSNKRLLTSTNTLVRFASTRSTGVENS---G-----------
 stNDA                   MPWFKNLIKISKTITNQSSSYKSITPLASPLLTQFLQFTK-------

ncNDI1  ---------ALTARNFSTVKFNNKDRKERVVILGSGWAGYSFAKDLDPEKYERIFISPRS
ncNDE1  GGPKNLPILEIFLDDDDSEEKKKHKEKPRLVILGGGWGSVALLKFLNPDDYHVTVVSPAN
 stNDB  GGLLVYAESNVESGKQVVEQNQPESKKKRVVVLGTGWGGTSFLKDVDISSYDVQVVSPRN
scNDE1  --------ANPSTQVPQSDTFPNGSKRKTLVILGSGWGSVSLLKNLDTTLYNVVVVSPRN
scNDE2  --------SNPPKQVPQSTAFANGLKKKELVILGTGWGAISLLKKLDTSLYNVTVVSPRS
 scNDI  --------AGPTSFKTMKVIDPQHSDKPNVLILGSGWGAISFLKHIDTKKYNVSIISPRS
 stNDA  --------QYSTNDHVVGLEATKSDQKPRIVVLGSGWAGCRLMKDIDTNIYDVVCVSPRN

ncNDI1  YFVFTPLLASTAVGTLEFRTVLEPIRRLDYGIGFHQGWAQDIDFANKTIRVEANANADSA
ncNDE1  YFLFTPMLPSATVGTLELNSLVEPIRNI--IDR-VKGH--YIRAAAEDVDFS-SRLVEVS
 stNDB  YFAFTPLLPSVTCGTVEARSIVEPVRNI--IKK-RSGEIQFWEAECLKIDPV-NRTVSCR
scNDE1  YFLFTPLLPSTPVGTIELKSIVEPVRTI--ARR-SHGEVHYYEAEAYDVDPE-NKTIKVK
scNDE2  FFLFTPLLPSTPVGTIEMKSIVEPVRSI--ARR-TPGEVHYIEAEALDVDPK-AKKVMVQ
 scNDI  YFLFTPLLPSAPVGTVDEKSIIEPIVNF--ALK-KKGNVTYYEAEATSINPD-RNTVTIK
 stNDA  HMVFTPLLASTCVGTLEFRSVAEPIGRIQPAVS-TQPASYFFLANCNAIDFD-NHMIECE

ncNDI1  SKAVVPIGQGGQLNQASARGALFDVPYDKLVIACGAYSQTFGIEGVREHANFLRDVGDAR
ncNDE1  Q--KDPRGNE----------VRFYVPYDKLVIAVGSTTNPHGVKG-LENCHFLKDINDAR
 stNDB  SGINDNLAGH----------NDFSLQYDYLVVAVGAQVNTFNTPGVMEHCHFLKEVEDAQ
scNDE1  SSAKNNDY------------DLDLKYDYLVVGVGAQPNTFGTPGVYEYSSFLKEISDAQ
scNDE2  SVSEDEYF-------------VSSLSYDYLVVSVGAKTTTFNIPGVYGNANFLKEIEDAQ
 scNDI  SLSAVSQLYQPENHLGLHQAEPAEIKYDYLISAVGAEPNTFGIPGVTDYGHFLKEIPNSL
 stNDA  TVTEGVETLE---------AWKFNVSYDKLVIASGAHALTFGIKGVNEHATFLREVHHAQ

ncNDI1  RIRLRVLSLFELCAYPKGVDNLTDEDRANLLHFAIVGGGPTGIEYASELHDLIHDDLSKM
ncNDE1  QIRNKIIQNLELSCLP----TTSDEERKRLLSFVVCGGGPTGVEFAAELFDLLNEDLTLH
 stNDB  RIRRTVIDCFEKSVIP----GLSEEERRTNLHFVIVGGGPTGVEFAAELHDYVYEDLVKI
scNDE1  EIRLKIMSSIEKAASL----SPKDPERARLLSFVVVGGGPTGVEFAAELRDYVDQDLRKW
scNDE2  NIRMKLMKTIEQASSF----PVNDPERKRLLTFVVVGGGPTGVEFAAELQDYINQDLRKW
 scNDI  EIRRTFAANLEKANLL----PKGDPERRRLLSIVVVGGGPTGVEAAGELQDYVHQDLRKF
 stNDA  EIRRKLLLNLMLSDVP----GVSEEEKRRLLHCVVVGGGPTGVEFSGELSDFILKDVHQR

ncNDI1  YPDL-LKFVRITVVDVSPKVLPMFDQALSKYAMDAFKRQKIEIRTQHNIERVRPADGKLG
ncNDE1  FPRLLRNEISVHLIQSRDHILNTYDEAVSKYAEDRFSRDQVDVLVNSRVAEVRPESILFT
 stNDB  YPSV-KDFVKITVIQSGDHILNTFDERISSFAEQKFQRDGIEVSTGCRVTSVSDHFINMK
scNDE1  MPEL-SKEIKVTLVEALPNILNMFDKYLVDYAQDLFKEEKIDLRLKTMVKKVDATTITAK
scNDE2  MPDL-SKEMKVILIEALPNILNMFDKTLIKYAEDLFARDEIDLQVNTAVKVVEPTYIRTL
 scNDI  LPAL-AEEVQIHLVEALPIVLNMFEKKLSSYAQSHLENTSIKVHLRTAVAKVEEKQLLAK
 stNDA  YAHV-KDYIHVTLIEANE-ILSSFDDRLRVYATNQLTKSGVRL-VRGLVQHVQPDNIILS

ncNDI1  SEYGELKLKIKQYGDKEVGAGLVVWSTGLMANPLIKQL----ASKDFAVPISPEDRAEAR
ncNDE1  QRGPDGKTTV----TKECPMGFCLWSTGVSQAEFCKRISRQLGPAQTNRHALETDTHLRL
 stNDB  VKS----TGK----HVEVPYGMVVWSTGVGTRPFVKDFMEQVG--QEKRRILATDEWLRV
scNDE1  TGDGD----I----E-NIPYGVLVWATGNAPREVSKNLMTKLEEQDS-RRGLLIDNKLQL
scNDE2  QNGQT----N----T-DIEYGMLVWATGNEPIDFSKTLMSRIPEQTN-RRGLLINDKLEL
 scNDI  TKHEDGKITE----E-TIPYGTLIWATGNKARPVITDLFKKIPEQNSSKRGLAVNDFLQV
 stNDA  DGT-------------NVPYGLLVWSTGVGPSPFVNSL-----DIPKAKGRIGIDEWLRV

ncNDI1  RPKAKLATDARTGGILVDEHFRVRIETQTTDAAKGSEIVPTSSSNSLL--RDVFVLGDAA
ncNDE1  NGTPLGDVYAIGDCSTIQNNVADHIITFLRNLAWKHGKDPESLELHFSDWRDVAQQIKKR
 stNDB  KGC--SNVYALGDCASVDQHKVMEDISTIFEAADKDDSGTLSVE----EFRDVLEDIIIR
scNDE1  LGAK-GSIFAIGDC----------------------------------------------
scNDE2  LGSE-NSIYAIGDC----------------------------------------------
 scNDI  KGS--NNIFAIGD----------------------------------------------
 stNDA  PSV--QDVYSIGDC----------------------------------------------

ncNDI1  ---------------------------------------------VIESQRTLPKTAQVAAQ
ncNDE1  FPQATAHLKR-----LDKLFEEYDKDQNGTLDFGELRELLKQIDSKLTS-LPATAQRAHQ
 stNDB  YPQVDLYLKNKHLLEAKDLFRDSEGNEREEVDIEGFKLALSHVDSQMKS-LPATAQVAAQ
scNDE1  ----------------------------------------------TFHPGLFPTAQVAHQ
scNDE2  ----------------------------------------------TAHTGFFPTAQVAHQ
 scNDI  ----------------------------------------------NAFAGLPPTAQVAHQ
 stNDA  ----------------------------------------------SGFLESTGRQVLPA

ncNDI1  QATYLAKVLNKAN----------EGV----------IDVKDAPGFKFRNWGVMTYL
ncNDE1  QGQYLAHKFNKLARAAPGLSANEIHEGD----------LDAAVYKAFEYRHLGSLAYI
 stNDB  QGTYLARCLNRWDQCKSNP------EGP-------RRFKSSGRHEFLPFEYRHLGQFAPL
scNDE1  EGEYLAQYFKKAYK-IDQLNWKMTHAKDDSEVARLKNQIVKTQSQIEDFKYNHKGALAYI
scNDE2  EGEYLAKILDKKLQ-IEQLEWDMLNSTDETEVSRLQKEVNLRKSKLDKFNYKHMGALAYI
 scNDI  EAEYLAKNFDKMAQ-IPNFQKNLSSRKD-------KIDLLFEENNFKPFKYNDLGALAYL
 stNDA  ----LAQVAERQGKYLASLLNKVGKQGGGHA------NCAQNINLGDPFVYKHLGSMATI

ncNDI1  GSWKAIHQ---GPRDE---LRGWAAWVLWRSAYLAKSMSWRNRFLVPIYWLVSWVF-GRG
ncNDE1  GNS-AVFDW-GDGWNFS---GGLWAVYAWRSIYFTQCVSLRTRVLMAIDWGKRALF-GRD
 stNDB  GGDQAAAELP-GDWVS----MGHSTQWLWYSVYASKQVSWRTRYLVVGDWVRRYIF-GRD
scNDE1  GSDKAIADLAVGEA--KYRLAGSFTFLFWKSAYLAMCLSFRNRVLVAMDWAKVY-FLGRD
scNDE2  GSETAIADLHMGDS--SYQLKGMFAFLFWKSAYLAMCLSIRNRILIAMDWTKVY-FLGRD
 scNDI  GSERAIATIRSGKRTF-YTGGGLMTFYLWRILYLSMILSARSRLKVFFDWIKLAFF-KRD
 stNDA  GRYKALVDLRESKEAKGVSLAGFTSFFVWRSAYLTRVVSWRNKIYVLINWLTTLVF-GRD

ncNDI1  ISRF  538
ncNDE1  LMRY  673
 stNDB  SSRI  577
scNDE1  SSI   560
scNDE2  SSV   545
 scNDI  FFKGL 513
 stNDA  ISRI  495
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Figure 2 Alignment of sequences of alternative NADH dehydrogenases

Identical amino acid residues present in at least four of the proteins from *N. crassa* (nc), *S. cerevisiae* (sc) and *Solanum tuberosum* (st) are shown on a grey background. Amino acid regions containing the three-G residues within conserved dinucleotide-binding motifs are shown underlined. Accession numbers of the sequences shown are NCU00153.1, CAB41986, CAB52797, CAA87359, CAA98651, S26704 and CAB52796 for ncNDI1, ncNDE1, stNDB, scNDE1, scNDE2, scNDI and stNDA respectively.

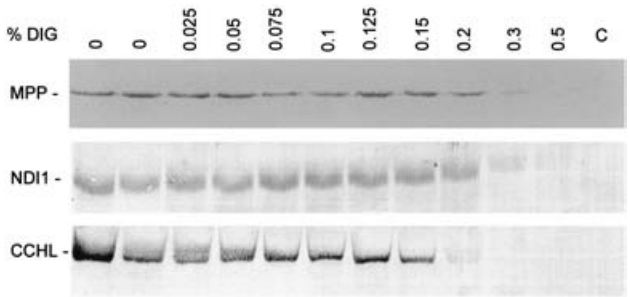

Figure 3 Protease accessibility upon digitonin solubilization of mitochondria

Mitochondria (100 μg) were solubilized with different concentrations of digitonin (DIG), treated with proteinase K (except the first lane) and analysed by Western blotting with antisera against NDI1, the mitochondrial processing peptidase (MPP) and the cytochrome *c* haem lyase (CCHL). The control experiment (C) was performed in the presence of Triton X-100.

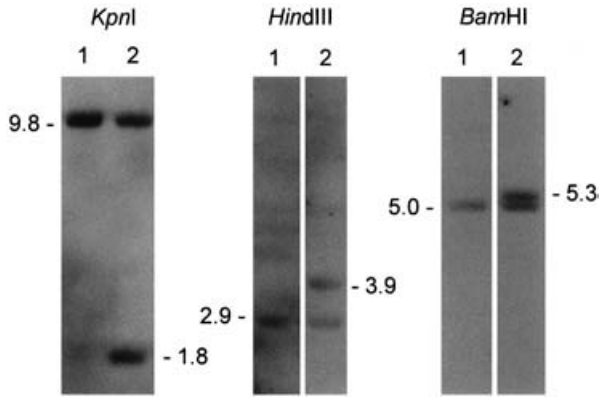

Figure 4 Single-copy transformant of the *ndi-1* gene

Genomic DNA from wild-type *Neurospora* (lanes 1) and transformant T10 (lanes 2) was digested with the indicated restriction enzymes and analysed by Southern blotting using the *ndi-1* gene as a probe. The sizes of the detected bands are indicated (in kb).

sequences are highly conserved around the nucleotide-binding motifs. Insertions that correspond to calcium-binding motifs of NDE1 [21] and NDB [14] are evident.

The mature NDI1 protein with an apparent molecular mass of 57 kDa was identified by Western blot analysis with specific polyclonal antisera (see Figure 3) in mitochondrial membrane preparations, obtained by sonication of the organelles followed by high-speed centrifugation. The protein is fully extracted by alkaline treatment of mitochondria, as is, for example, the 30.4 kDa subunit of complex I [39], suggesting that it is not an intrinsic membrane protein (results not shown). To obtain a more detailed localization of NDI1, we performed digitonin fractionations of mitochondria. After treatment with proteinase K, the fractions were analysed by immunoblotting with antisera against NDI1 (Figure 3). The protein is resistant to the protease both in intact organelles and upon opening of the outer mitochondrial membrane with low concentrations of digitonin, as can be seen from the degradation pattern of cytochrome *c* haem lyase, an intermembrane space protein marker [40]. NDI1 behaves similarly to mitochondrial processing peptidase, a matrix protein marker [41], suggesting that it becomes accessible to the protease only when both mitochondrial membranes are solubilized by detergent. Taken together, these results indicate that NDI1 is located in the inner mitochondrial membrane, facing the matrix side.

The NDI1 protein was isolated from mitochondrial membranes of *N. crassa* mutant nuo51 [32]. This mutant lacks an active proton-pumping complex I, and thus provides mitochondria with reduced background NADH dehydrogenase activity. Indicative for NDI1, we determined the NADH:Q2 redox activity [42], which is lower, but more specific, than NADH:ferricyanide activity. Although NDI1 contributes only a small fraction to the high NADH:ferricyanide redox activity of *N. crassa* mitochondria, it appears to be the major enzyme providing NADH:Q2 redox activity in nuo51 mitochondria. NDI1 was extracted from the membranes by a low concentration of Triton X-100, leaving most integral membrane proteins insoluble. The enzyme was isolated by a range of chromatographic procedures, including anion-exchange chromatography, Blue Sepharose chromatography, gel-filtration and cation-exchange chromatography. The final preparation showed a single band of 57 kDa on SDS/PAGE. N-terminal sequencing yielded the sequence STVKFNNKDRKERVVILG (this sequence includes parts of both exons, confirming the intron). Thus the first 31 amino acid residues of the precursor polypeptide are removed after import into mitochondria, yielding a mature protein of 56611 Da. Corresponding to this molecular mass, the isolated protein contained 0.9 mol of FAD/mol of protein.

The isolated enzyme is highly specific for the electron donor NADH. Deamino-NADH, as well as NADPH, yielded more-than-100-fold-lower rates. A K_m value of 56 μM was determined for NADH using Q2 as electron acceptor. Affinities for deamino-NADH and NADPH were not determined exactly, because of the very low activity with these substrates. The enzyme can utilize various electron acceptors. Highest activities were obtained with ferricyanide, yielding a maximal catalytic-centre activity of 800 s^{-1}. Quinones Q2 and ubiquinone-6 (Q6) yielded 3-fold- and 20-fold-lower rates respectively. Enzymic properties were routinely assayed using Q2 as the electron acceptor, because of its higher specificity compared with ferricyanide.

The ndi1 mutant lacks an internal NADH dehydrogenase activity

To investigate further the specific role of NDI1, we disrupted the corresponding gene by repeat-induced point mutations ('RIPing'). This unusual phenomenon introduces GC → AT transition mutations with a certain frequency in repeated sequences in *Neurospora*, when they pass through a genetic cross, leading to gene inactivation [43]. Thus we duplicated the *ndi-1* gene in *N. crassa* by transforming fungal spheroplasts with plasmid pND-K. To identify the single-copy transformant T10, genomic DNA from 12 strains was analysed by Southern blotting using *ndi-1* gene sequences as the probe. Figure 4 shows that digestion of wild-type DNA with *Kpn*I results in the detection of a 9.8 kb band. An extra band of 1.8 kb is detected when DNA from T10 is treated with the same enzyme, indicating that the fragment introduced in the genome is intact. Treatment of wild-type DNA with *Hin*dIII or *Bam*HI results in the detection of single bands of 2.9 or 5.0 kb respectively. The same treatments of T10 DNA result in the detection of single extra bands of 3.9 or 5.3 kb respectively. These results indicate that a single chromosomal gene codes for NDI1 in *N. crassa* and that strain T10 has an ectopic duplicated copy of the 1.8 kb *Kpn*I fragment. The T10 transformant was crossed with a wild-type strain and random ascospore progeny were germinated and used for the isolation of mitochondria. The mitochondrial proteins were analysed by Western blotting with antiserum against NDI1. A mutant strain lacking the NDI1 protein was identified (Figure 5) among 98 spores analysed. The identified mutant was

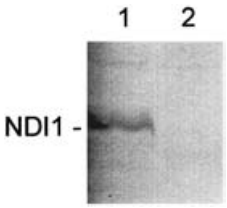

Figure 5 Mutant strain lacking the NDI1 protein

Total mitochondrial proteins (100 μg) from wild-type (lane 1) and the ndi1 mutant (lane 2) were analysed by Western blotting with an antiserum against the NDI1 protein.

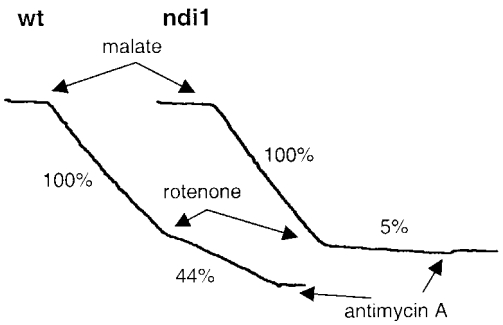

Figure 6 Matrix NADH oxidation by wild-type and ndi1 mitochondria

The initial activities were 69 and 51 nmol of O_2/min per mg of protein for wild-type and mutant mitochondria respectively. Latencies of wild-type and ndi1 mitochondria were 93% and 92% for cytochrome *c* oxidase (outer membrane integrity) and 96% and 93% for malate dehydrogenase (inner membrane integrity) respectively.

Table 1 Ascospore progeny resulting from genetic crosses between the ndi1 strain and different complex I mutants

	Progeny				
Cross	ndi1	nuo	wt	ndi1, nuo	Total
ndi1 × nuo21	13	3	6	3	25
ndi1 × nuo51	48	10	12	0	70
ndi1 × nuo21.3c	24	9	3	0	36

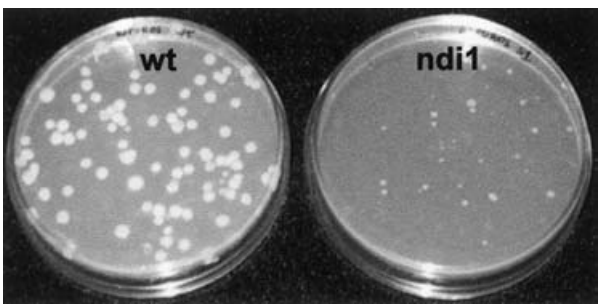

Figure 7 Delayed germination of ndi1 mutant spores

Wild-type and ndi1 ascospore progeny (resulting from wt × wt or ndi1 × ndi1 crosses respectively) were plated on sorbose plates and incubated. The picture was taken after 3 days.

hygromycin-resistant, due to the ectopically integrated plasmid pND-K. The position of the integrated plasmid is unknown, and thus could affect an unknown gene at the integration site. To avoid this uncertainty, we back-crossed the mutant with wild-type and isolated a hygromycin-sensitive ndi1 mutant.

Respiration of mitochondria from the ndi1 mutant was analysed with an oxygen electrode. The oxidation rates of NADH and NADPH in ndi1-intact mitochondria were similar to those of the wild-type strain (results not shown). These activities arise from enzymes located in the outer face of the inner mitochondrial membrane and thus acting on cytosolic substrates (external enzymes). A marked difference between activities of wild-type and mutant mitochondria was determined concerning the oxidation of matrix NADH. The oxygen uptake of wild-type mitochondria supplied with pyruvate/malate, which causes formation of NADH in the matrix, can be inhibited by 50% with the complex I inhibitor rotenone (Figure 6). The remaining rotenone-resistant activity can be attributed to one (or possibly more) internal alternative NADH dehydrogenase(s) [44]. In contrast, 95% of the same activity of mutant mitochondria is inhibited by rotenone (Figure 6). All of the tested respiratory activities are completely blocked by antimycin in both wild-type and ndi1 strains. These results strongly suggest that NDI1 represents the sole internal alternative NADH dehydrogenase responsible for the rotenone-insensitive oxidation of matrix NADH in *Neurospora* mitochondria.

NDI1 plays a role in spore germination

To test the above hypothesis further, we tried to generate double mutants between ndi1 and different complex I mutants displaying various phenotypes in terms of enzyme assembly and function. Therefore we crossed the ndi1 strain with mutants nuo51 [32] and nuo21.3c [28], both of which lack a functional enzyme, and with mutant nuo21 [45], which carries a functional complex. Mitochondria from ascospore progeny of the three crosses were analysed for the presence of the relevant proteins by immunoblotting, and the results are shown in Table 1. As expected, double mutants seem to be viable only when some complex I is present to compensate for the NDI1 deficiency. No double mutant was obtained in the crosses between ndi1 and nuo51 or nuo21.3c, whereas double mutants were isolated from the cross between ndi1 and nuo21. As a positive control, wild-type recombinants were recovered from all crosses. These results support the idea that only one alternative NADH dehydrogenase faces the mitochondrial matrix in *N. crassa* mitochondria. Apparently, a complete deficiency in the oxidation of matrix NADH prevents the viability of double mutants, at least under the conditions used.

The NDI1 dehydrogenase is not essential for vegetative or sexual development in *N. crassa*. In standard media, the growth and ability to produce conidia of the ndi1 mutant and wild-type are comparable. In addition, the linear hyphal extension rates were measured in race tubes, varying the carbon source and temperature conditions. No appreciable growth defects were observed in the mutant, except for a slightly longer lag phase when acetate was employed as the carbon source (results not shown). Homozygous crosses between ndi1 mutants are fully fertile. However, ascospores (Figure 7), as well as conidia from ndi1, germinate much more slowly than wild-type spores. It seems that NDI1 plays a key role in the initial steps of germination of both sexual and asexual spores of the fungus, and its absence significantly delays the process.

DISCUSSION

Mitochondrial NAD(P)H dehydrogenases are key enzymes of cellular metabolism, as attested by the fact that deleting or inactivating their genes can be a lethal event in unrelated organisms [46,47], and protein mutations are involved in mitochondrial disease [48]. Mammals only possess the proton-pumping complex I. At the other extreme, plants seem to contain up to four alternative enzymes in addition to complex I. Some yeasts, like *S. cerevisiae*, only have three non-proton-pumping alternative NADH dehydrogenases. In this respect, the respiratory chain of *N. crassa* appears to be quite interesting, because it combines the capabilities of humans and *S. cerevisiae*, and may be as complex as those of plants, where calcium regulates enzyme activity [2]. The existence of internal and external alternative NADH dehydrogenases in the inner membrane of *N. crassa* mitochondria was evidenced long ago [20]. Recently, we have characterized an external NADPH dehydrogenase, NDE1, that is activated by calcium, in accordance with a predicted EF-hand motif in its primary structure. We also provided evidence for the presence of at least a second external dehydrogenase, NDE2 [22].

Searching the fungal genome with the NDE1 sequence led us to the identification of three additional homologous coding sequences [17]. One of them does not display obvious characteristics of a mitochondrial protein, and it is not yet clear if it is targeted to the organelle. Another one seems to be located externally in the inner membrane of mitochondria, and probably represents the predicted NDE2 protein (Patrícia Carneiro, personal communication; M. Duarte and A. Videira, unpublished work). The third putative protein was characterized in the present work as the internal NADH dehydrogenase called NDI1. In conclusion, the inner mitochondrial membrane of *N. crassa* contains one internal and two external alternative NADH dehydrogenases. A fourth cellular enzyme of this type remains to be located.

We have obtained convincing evidence that we have characterized the internal NADH dehydrogenase responsible for the rotenone-insensitive oxidation of matrix NADH. Its location and function was concluded from an analysis of the behaviour of the protein upon protease treatment of mitochondria fractionated with increasing concentrations of digitonin, and from the fact that the respiratory activity of ndi1 mutant mitochondria is almost completely inhibited by rotenone. This inhibition, combined with the failure to obtain double mutants in crosses between the ndi1 strain and non-functional complex I mutants, strongly suggests that there is a single internal alternative NADH dehydrogenase. Following the procedure used for isolation of the internal rotenone-insensitive NADH dehydrogenase of *S. cerevisiae* [42], we isolated the mature enzyme from mitochondria. The protein is synthesized as a precursor polypeptide containing a mitochondrial pre-sequence of 31 amino acid residues. As for most homologues [1,2], it contains FAD as the cofactor. Typical of internal alternative NADH dehydrogenases in fungi, it specifically oxidizes NADH, whereas NADPH is used poorly as substrate. Its affinity for NADH is more than 10-fold lower than that of complex I, supporting the assumption that it functions as an overflow valve for excess NADH [2].

Our results also show that complex I and NDI1 have complementary functions in *N. crassa*, as has been found to be the case for other organisms as well. For instance, the NDI1 of *S. cerevisiae* complemented complex I defects in mammalian cells [15], complex I disruption in *Paracoccus denitrificans* required the expression of the alternative NADH dehydrogenase of *Escherichia coli* [46], and complex I disruption in *Y. lipolytica* was only possible when the single external alternative NADH dehydrogenase of this organism was targeted to the matrix side of the inner mitochondrial membrane [47]. The fact that complex I [8] or ndi1 mutants alone are viable in *N. crassa*, but that double mutants cannot be obtained, clearly suggests that the enzymes are complementing each other. It also suggests that these enzymes are the main proteins dealing with mitochondrial NADH oxidation and shuttles to exchange cytosolic/matrix NADH are either not present or not efficient in *Neurospora*. To our knowledge, *N. crassa* is the first eukaryote identified in which, though separately, both complex I and the internal alternative enzyme have been inactivated.

On the other hand, it is also becoming clear that complex I and the alternative enzymes play their own specific roles within the cell, a conjecture that might have been anticipated from the variety in the number and location of these proteins in different organisms. In this respect, it was quite interesting to find that NDI1 is involved in the efficiency of germination of both sexual and asexual fungal spores. When the protein is inactivated by gene disruption, germination is significantly delayed. Whether this occurs by failure to oxidize excess NADH or by a direct involvement of NDI1 in some specific biochemical process remains to be investigated. Following a fire, *Neurospora* is usually the first fungus to appear on burned vegetation (David Perkins, personal communication), and thus requires a highly efficient germination. The importance of NDI1 in spore germination is in agreement with the 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 [49].

The (proton-pumping) complex I is crucial for sexual development in *Neurospora* [28,50] and in other organisms [51,52], but its absence does not significantly influence growth rate or spore germination in the fungus [17,19]. In contrast, NDI1 is not essential for either the sexual or vegetative phases of the fungal life cycle, but is important for the rates of spore germination. These results indicate that, besides their capacity for complementing each other, proton-pumping and non-proton-pumping NADH dehydrogenases have specific cellular functions. It has already been shown that the importance of the alternative enzyme and of complex I in the respiratory chain of *N. crassa* varies from the exponential to the stationary phase of growth respectively [53]. It has been suggested that the higher P/O ratio achieved with complex I may be important when the growth rate is reduced, but using the alternative enzymes may be advantageous during fast growth when carbon sources are abundant [7,54]. Taken as a whole, our knowledge of the function of these enzymes is still limited, and a detailed characterization of the specific alternative NADH dehydrogenases group of *N. crassa* will contribute to our understanding of the biology of NADH utilization in mitochondria.

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