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Primary structure and characterisation of a 64 kDa NADH dehydrogenase from the inner membrane of *Neurospora crassa* mitochondria[1]

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Abstract

A cDNA clone encoding a mitochondrial NADH dehydrogenase from *Neurospora crassa* was sequenced. The total DNA sequence encompasses 2570 base pairs and contains an open reading frame of 2019 base pairs coding for a precursor polypeptide of 673 amino acid residues. The protein is encoded by a single-copy gene located to the right side of the centromere in linkage group IV of the fungal genome. The N-terminus of the precursor protein has characteristics of a mitochondrial targeting pre-sequence. The protein displays homology with mitochondrial NADH dehydrogenases from yeast. In contrast to these polypeptides, however, analysis of its primary structure revealed that it contains a well-conserved calcium-binding domain. Rabbit antiserum against the protein expressed in an heterologous system recognises a mitochondrial protein of *N. crassa* with an apparent molecular mass of 64 kDa. Analysis of the fungal mitochondria by swelling, digitonin fractionation and alkaline treatment indicate that the protein is located in the inner membrane of the organelles, possibly facing the matrix side.

Keywords: Mitochondria; Inner membrane; NADH dehydrogenase; cDNA; (*Neurospora crassa*)

1. Introduction

In mitochondria, several enzymes participate in the oxidation of NAD(P)H and reduction of ubiquinone. Complex I of the respiratory chain couples this redox reaction to proton translocation through the inner membrane of the organelles, thus leading to energy conservation. In addition, other non-proton pumping alternative enzymes, depending on the organism, may also be involved. Unlike mammalian mitochondria, plants, baker yeast and fungi mitochondria are able to oxidise NAD(P)H directly from the cytoplasm [1–3]. While the role of alternative NADH dehydrogenases is still unclear, they might act as an overflow mechanism for the oxidation of NAD(P)H under conditions where the level of NAD(P)H is high but the cell has no need for ATP, i.e., under conditions close to state 4 [4]. The existence of these enzymes might be in part responsible for the fact that

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complex I is essential for certain organisms and not for others [5].

In order to understand the biology of NADH oxidation and the specific relevance of the different proteins involved, a thorough characterisation of the participating enzymes is required. In the case of higher plants such as potato tubers, there are four rotenone-insensitive NAD(P)H dehydrogenases in the inner mitochondrial membrane. Two of them are located at the outer surface [6] and the other two at the inner surface of the membrane [4]. Concerning the yeast *Saccharomyces cerevisiae*, three enzymes are known: NDI1 [7] on the inner surface, and NDE1 and NDE2 on the outer surface of the inner mitochondrial membrane [8,9]. With the exception of the latter organism, which lacks complex I, we do not know of any report on the cloning of alternative NADH dehydrogenases from eukaryotes. We started the characterisation of the enzymes present in *N. crassa*. This fungus is a suitable eukaryote that allows genetic manipulation and has been quite well characterised regarding complex I [5,10]. In *N. crassa*, at least three distinct NAD(P)H oxidation activities can be observed in mitochondria, following the determination of respiratory activities with oxygen electrodes. One of these oxidations occurs on the outer side and the other two, which include the activity of complex I, occur in the matrix side of the inner mitochondrial membrane ([11,12]; unpublished results). In this report, we describe the first molecular characterisation of a NAD(P)H dehydrogenase in the inner membrane of *N. crassa* mitochondria.

2. Materials and methods

2.1. N. crassa techniques

The wild-type strain 74-OR23-1A was grown in Vogel's medium [13]. For the preparation of mitochondria, the mycelium was dispersed in isolation medium by 30-s homogenisation in a Krups blender [14] and disrupted by a grind-mill [11] built in the workstation of Institute für Physiologische Chemie, Munich. A crude preparation of the organelles was obtained by differential centrifugation [14]. For gene mapping, the segregation of restriction fragment length polymorphism was analysed in the 38 strains (FGSC nos. 4450–4487) of the Multicent-2 cross kit [15,16]. The probe used was a 2 kb *Eco*RI/*Hin*dIII fragment of the cDNA (see below) labelled with Gene Images (Amersham).

2.2. Cloning and expression of p64

Standard methodology for cloning techniques have been described [17,18]. The cDNA NM1C2 encoding the precursor of the 64 kDa polypeptide [19] was obtained from Dr. Mary-Anne Nelson of the University of New Mexico. Both DNA strands were sequenced with the aid of vector- and cDNA-specific primers. Sequence analysis was performed with computer programs available in the Internet. In order to express p64, the cDNA was amplified by PCR using primer 5′-CCTCTAGGATCCCTCGACCTACAGC-3′ (the underlined bases were modified from the cDNA in order to create a *Bam*HI site) and a vector-specific primer. The PCR product was digested with *Bam*HI and *Kpn*I (which cuts in vector derived sequences), cloned in the expression vector pQE-31 previously treated with the same enzymes and transformed into *Escherichia coli* M15. After induction with IPTG, the bacterial cells express a fusion protein containing the 579 C-terminal amino acid residues of p64. The fusion protein was purified in nickel columns (after Pharmacia protocols) followed by SDS–PAGE [20] and used to immunise rabbits [21].

2.3. Purification, fractionation and analysis of mitochondria

The crude mitochondrial preparations were further purified in Percoll gradients [22]. Hypotonic swelling [23], digitonin treatment for 5 min [24] and alkaline treatment of mitochondria [16] have been described. Proteinase K incubations (50 μg/ml) were carried out for 30 min at 4°C. To disrupt both mitochondrial membranes, the organelles were sonicated 10×7 s in distilled water, using a Microson XL 2000 ultrasonic homogeniser, position 7. The techniques for protein quantification [25], the determination of the activities of adenylate kinase [26] and malate dehydrogenase [27], Western blotting and immunodecoration [21,28] were performed according to the references cited.

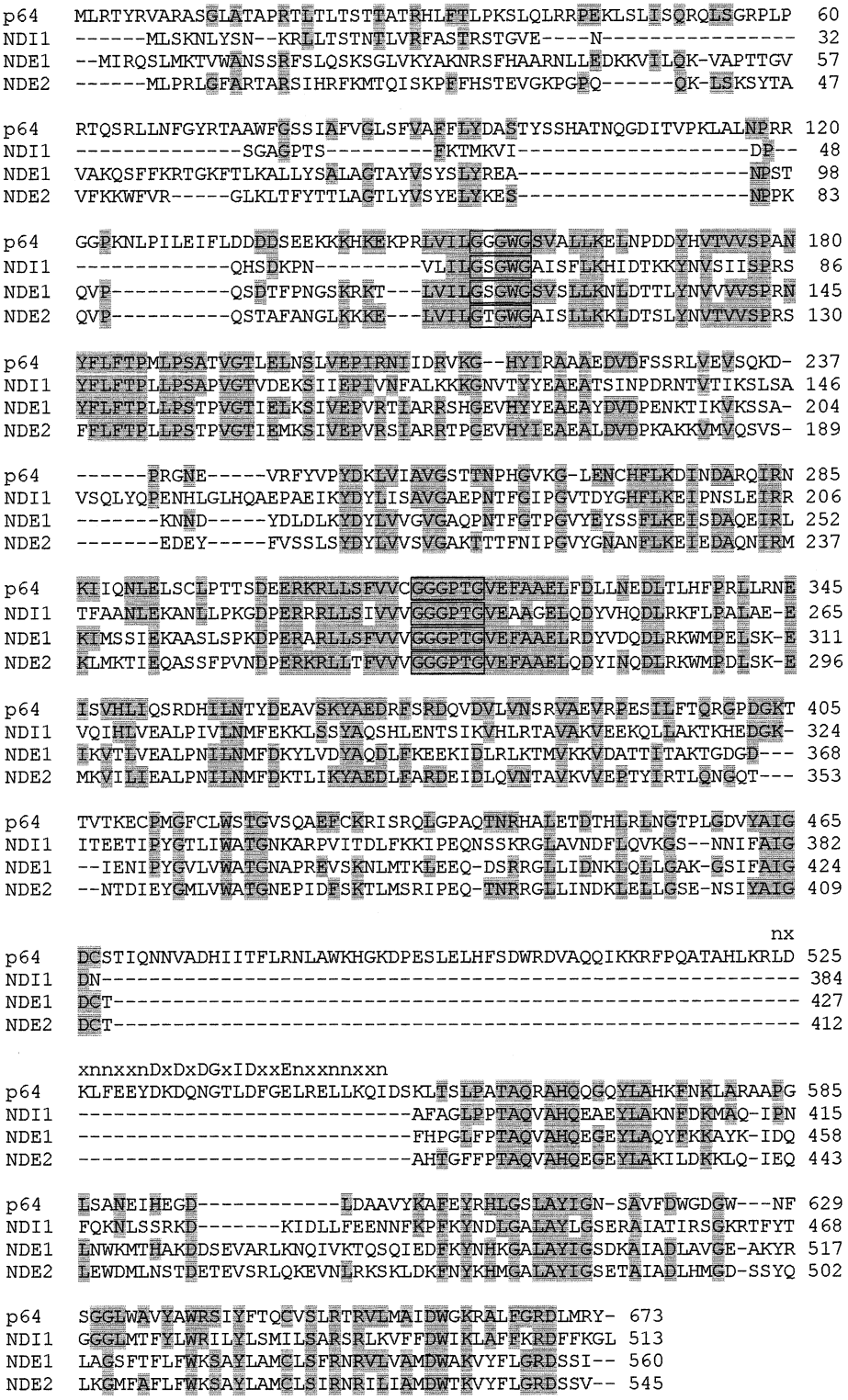

Fig. 1. A Clustal alignment of the amino acid sequences of the 64 kDa protein from *N. crassa* with the NDI1, NDE1 and NDE2 proteins from *S. cerevisiae*. Amino acid residues identical to p64 are shadowed. The boxed sequences depict three invariant G residues within NADH-binding motives. A consensus sequence for calcium binding is shown above the sequence of p64.

3. Results and discussion

3.1. Gene characterisation

The partially sequenced NM1C2 cDNA clone was isolated from a mycelial *N. crassa* library [19]. Complete sequence of both DNA strands of the 2570 bp cDNA revealed that it contains an open reading frame of 2019 bp encoding a polypeptide chain of 673 amino acid residues with a molecular mass of 75 891 Da. Several in-frame stop codons are found upstream of the presumed first ATG codon indicating that, in fact, it represents the initiation codon (not shown). The N-terminus of the deduced polypeptide sequence has characteristics of a mitochondrial targeting pre-sequence. Up to 2–3 domains of the protein are predicted to be transmembraneous, depending on the computer program used for analysis. Thus, both issues require further experimental evidence.

The deduced primary structure of the protein, called p64 (see above), is similar to prokaryote and eukaryote NADH dehydrogenases. Fig. 1 depicts an alignment of the protein sequence with those of NDI1 [7], NDE1 and NDE2 enzymes of *S. cerevisiae* [8,9]. The proteins are well conserved and contain two sequence motifs for the binding of the ADP portion of NADH [29]. Namely, among other conserved amino acid residues in the surroundings, three invariant glycine residues separated by 1–2 amino acids can be observed within regions that potentially form β-sheet and α-helix structures, respectively. Compared with the yeast sequences, the *N. crassa* polypeptide is slightly extended in the N-terminal region and also contains an internal extension of 80–90 amino acid residues. Interestingly, this internal extension presents a perfectly well-conserved consensus sequence for the binding of calcium ([30]; J. Cox, personal communication), which is missing in the yeast as well as in the sequences of other known NADH dehydrogenases. We suggest that the fungal protein might be regulated by calcium and it is therefore unique in this respect. Calcium-dependent NAD(P)H oxidising activities are known in plant mitochondria [2].

Strain	A 1	A 4	B 6	B 7	C 1	C 4	D 5	D 7	E 1	E 3	E 5	E 7	F 1	F 3	G 1	G 4	H 5	H 7	I 6	I 8
RF	M	O	M	M	M	O	O	M	M	O	O	M	O	M	O	M	O	O	O	M

Strain	J 1	J 4	K 1	K 4	L 1	L 4	M 5	M 8	N 2	N 3	O 2	O 4	P 1	P 4	Q 2	Q 4	R 1	R 4
RF	O	O	O	M	O	M	O	M	M	O	M	O	O	M	M	M	M	O

Fig. 2. Mapping of the gene coding for p64. Segregation pattern of a *Sal*I restriction fragment length polymorphism among the 38 strains of the Multicent-2 cross kit. Restriction fragments (RF) of the Mauriceville type (M) and of the Oak Ridge type (O) are indicated.

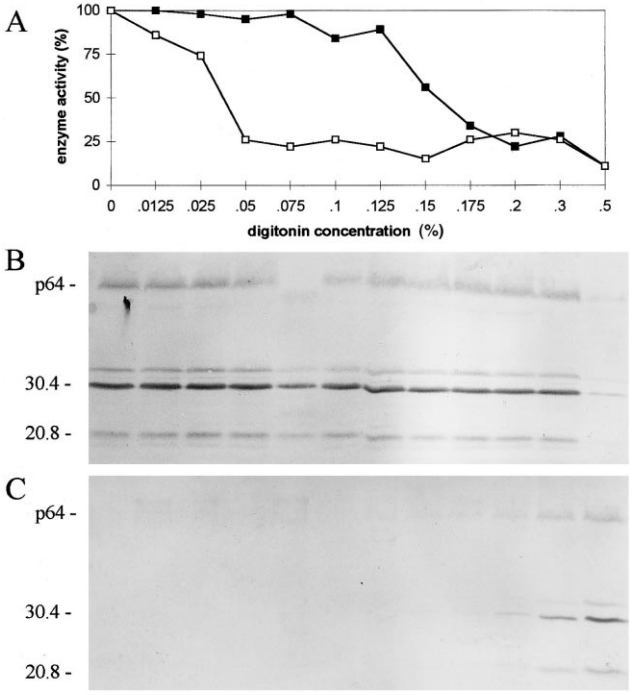

Fig. 3. Digitonin fractionation of mitochondria. Aliquots of a mitochondrial preparation were treated with increasing amounts of digitonin, as indicated, and resolved by centrifugation into supernatant and pellet. The activities of malate dehydrogenase (■) and adenylate kinase (□) were determined in the pellet fraction (A). Both pellet (B) and supernatant (C) were resolved by SDS–PAGE and analysed by Western blotting with a mixture of antisera against p64 and against the 30.4 and 20.8 kDa subunits of complex I.

In order to determine the chromosomal location of the gene encoding p64, we performed a Southern blot analysis of the 38 strains of the Multicent-2 cross kit, which represent the progeny of a cross between two highly polymorphic strains [15]. The segregation of polymorphic restriction fragments among those strains is characteristic of their position in the genome. We found a polymorphism with the restriction enzyme *Sal*I and the pattern of segregation of this polymorphism is shown in Fig. 2. A comparison with the segregation of genes with

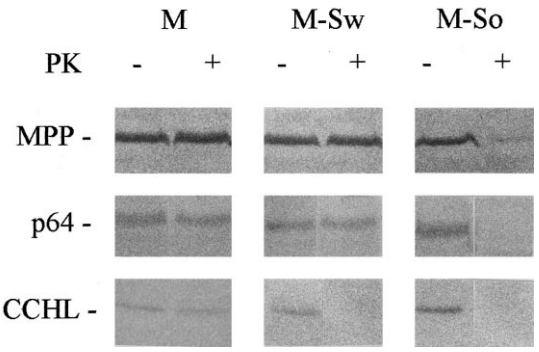

Fig. 4. Treatment of mitochondrial fractions with proteinase K. Isolated mitochondria (M), swelled mitochondria (M-Sw) and sonicated mitochondria (M-So) were incubated in the absence (−) or presence (+) of proteinase K (PK). The material was then resolved by SDS–PAGE and analysed by Western blotting with antisera against the mitochondrial processing peptidase (MPP), the 64 kDa protein (p64) and cytochrome *c* heme lyase (CCHL).

known chromosomal localisation [31] revealed that the gene encoding p64 is located close to *Fsr-4*, in the right side of the centromere of linkage group IV. These results also indicated that p64 is encoded by a single-copy gene in *N. crassa*.

3.2. Protein localisation

We expressed the 579 C-terminal amino acids of the *N. crassa* NADH dehydrogenase in an heterologous system and used it to immunise rabbits. The resulting antiserum recognises a protein band with an apparent molecular mass of 64 kDa in Western blots of total mitochondrial proteins from the fungus (e.g., Fig. 3). We should add that the protein can also be clearly detected in mitochondrial membrane preparations, obtained by sonication followed by centrifugation of the organelles (not shown). To achieve a more precise localisation within the organelles, purified mitochondria were incubated with increasing concentrations of digitonin in order to obtain a sequential opening of the intermembrane space and matrix compartments. This can be judged by the sequential release of the respective marker enzymes, adenylate kinase and malate dehydrogenase (Fig. 3A). In these experiments, the pattern of release of the 64 kDa protein parallels that of the 20.8 kDa [18] and the 30.4 kDa [32] subunits of respiratory chain complex I, an inner membrane component, indicating that p64 is located in the inner mitochondrial membrane (Fig. 3B,C).

The topology of p64 was further investigated by protease treatment of intact mitochondria and mitochondria subjected to hypotonic swelling, which destroys the integrity of the outer membrane only. The mitochondrial processing peptidase of the mitochondrial matrix [33] and cytochrome *c* heme lyase of the intermembrane space [34] were used as controls in these assays (Fig. 4). It can be observed that p64 is resistant to externally added proteinase K both in intact mitochondria (Fig. 4A) and in the mitoplasts resulting from hypotonic swelling of the organelles (Fig. 4B). This is not due to natural resistant of p64 to proteinase K, because it can be digested when the protease is added to mitochondria previously disrupted by sonication (Fig. 4C). Thus, it appears that p64, or most of it, is placed in the matrix side of the inner mitochondrial membrane. However, the protein is predicted to be transmembraneous (for example, residues 76 to 94 and residues 632 to 650) and it is possible that portions of the protein are located as well on the cytoplasmic face of the membrane in a protease-resistant manner.

In order to characterise the type of interactions of p64 with the inner mitochondrial membrane, an alkaline extraction of mitochondria was carried out (Fig. 5). In the conditions employed, the 30.4 kDa subunit of the peripheral arm of complex I can be solubilized while the 20.8 kDa subunit of the membrane arm of the enzyme remains in the membrane

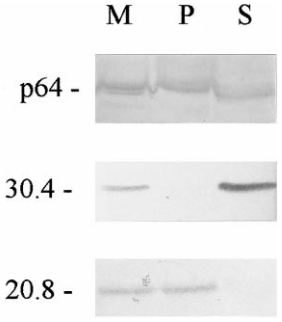

Fig. 5. Alkaline extraction of mitochondrial proteins. Mitochondria were incubated in alkaline conditions and resolved by centrifugation into a supernatant and a pellet fractions. The mitochondria before alkali treatment (M), the pellet (P) and supernatant (S) were resolved by SDS–PAGE and analysed by Western blotting with a mixture of antisera against p64 and against the 30.4 and 20.8 kDa subunits of complex I.

pellet. In the case of p64, roughly half of the protein remains in the pellet, suggesting that it is an integral membrane protein. We are tempted to speculate that two populations of the protein exist, since part of it can be extracted by the alkaline treatment. In conclusion, we described the characterisation of a novel NADH dehydrogenase located in the inner membrane of *N. crassa* mitochondria, possibly facing the matrix space. The availability of the respective gene will allow genetic manipulation and further research on the relevance of different proteins for the oxidation of NADH by mitochondria.

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