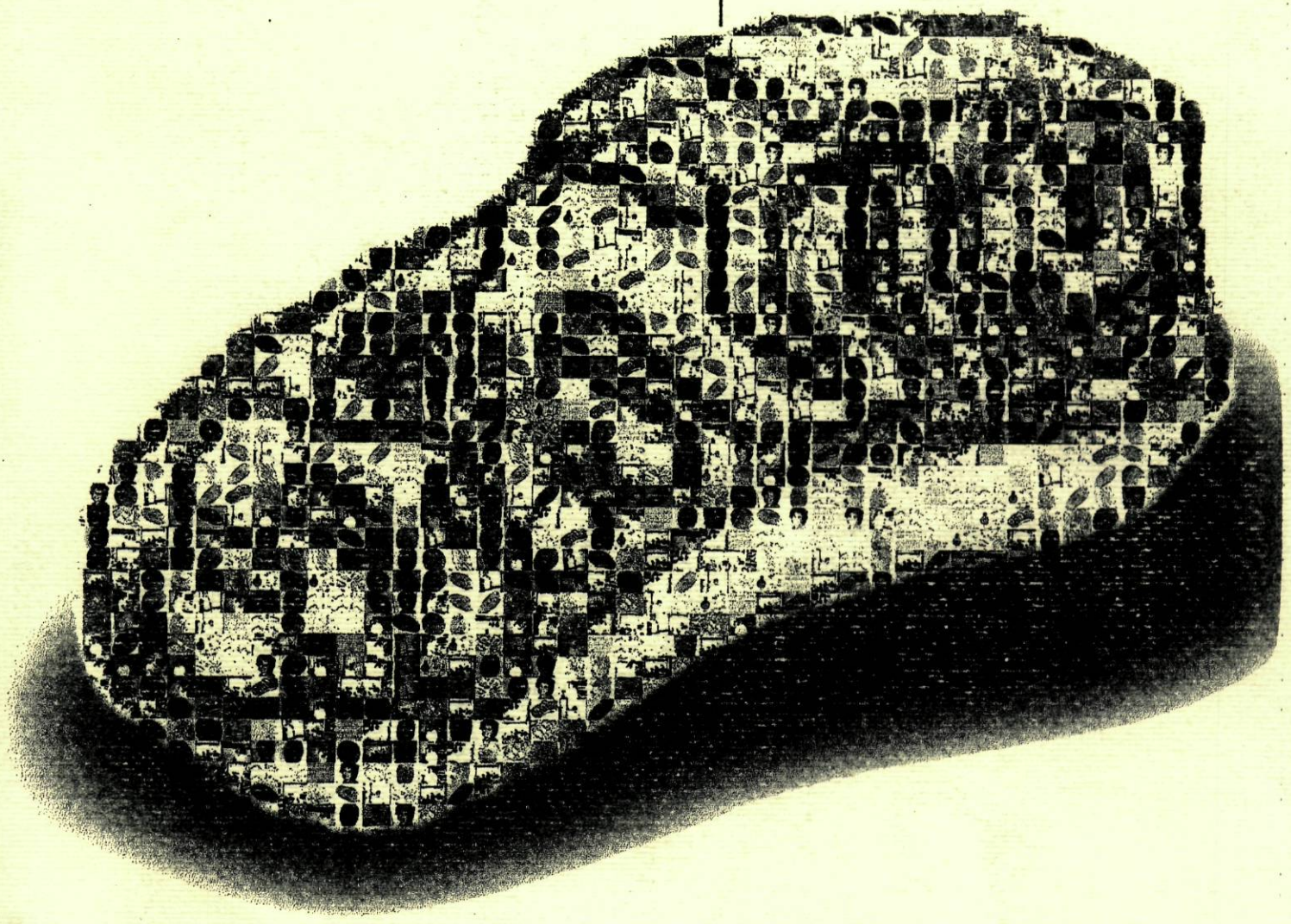
INSTITUTO DE CIÊNCIAS BIOMÉDICAS DE ABEL SALAZAR
UNIVERSIDADE DO PORTO

Characterisation of NAD(P)H Dehydrogenases from Neurospora Mitochondria



Ana Margarida Nunes Portugal Carvalho Melo

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ANA MARGARIDA NUNES PORTUGAL CARVALHO MELO

CHARACTERISATION OF NAD(P)H DEHYDROGENASES FROM NEUROSPORA MITOCHONDRIA

PORTO
2001

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Characterisation of NAD(P)H dehydrogenases from Neurospora mitochondria

Dissertation for Obtaining a Philosophy Doctor Degree in Biomedical Sciences, Submitted to the Instituto de Ciências Biomédicas de Abel Salazar, University of Porto. The works were developed at the Laboratory of Molecular Genetics from the Instituto de Ciências Biomédicas de Abel Salazar and at the Laboratory of Molecular Genetics and Biogenesis of the Mitochondrion from the Instituto de Biologia Molecular e Celular.

Supervisor: Professor Arnaldo António de Moura Silvestre Videira
University of Porto, Portugal

Co-supervisor: Professor Ian Max Møller
Lund University, Sweden and Risø National Laboratory, Denmark

Porto, January 2001

A Deus e a todos os que Lhe dão vida, nesta história.

"Permanecemos na praia do conhecimento e olhamos lá para longe, para o mar de mistério, e pronunciamos o Seu nome. O Seu nome não é apreensível por todos os credos e todas as teorias da ciência. Ele é, de facto, a "essência temida que fica para além da lógica". A ciência prolonga a praia ao longo da qual somos capazes de perceber o mistério, mas não esgota o mistério. À medida que o conhecimento se torna mais profundo, o mesmo acontece com o espanto."

Chet Raymo

Acknowledgments

I am greatly indebted to:

Aires, Albertina, Alexandre, Ana, Ana, Ana, Ana, Ana, André, Ângelo, Anita, António, António Alberto, **Arnaldo**, Arrabaça, Augusta, Augusta, Augustinha, Bete, Beto, Bruno, Christian, Clara, Daniel, David, Dejana, Duarte, ***Elisa***, Fátima, Fátima, ***Fernando***, Fernando, Fernando, Filipa, Filipa, Francisca, Gonçalo, Guida, Heiko, Helena, Holger, Inês, Inês, ***Isabel***, Isabel, Isabel, Jaime, Janeca, Joaquim, Joaquim, Joana, Joana, João, João, João, João, João, João, Joca, Jorge, Jorge, Julio, Laura, Lena, Leonor, Lu, Luís, Maluxa, Manela, Manela, **Margarida**, Maria, Mariana, Marta, Matilde, **Max**, Miguel, Miguel, Miguel, Miguel, Miguel, Mónica, Natália, Nela, Neupert, Nuno, Nuno, Paula, Paulo, Pedro, Pedro, Quéqué, Quintanilha, Rita, Rita, Rita, Rosário, Rui, Rui, Sara, Sara, Sérgio, Silva, Susana, Susan, Tadeu, Teresa, TiJoão, Tó, Tó, Tom, Toni, ***Victor***, Xana, Xico, Zé, Zé, Zé, Zé, Zé.

When you are around everything gets funnier and easier! Thanks a lot!

The Portuguese Foundation for Science and Technology funded this work.

Luís skilfully drew figure 1, 2 and 4.

Miguel generously created the front page.

Dr Corália Vicente gave me a precious help on the statistical analysis of the NDE1 activity data.

Hassan translated and Cláudia and revised the Résumé.

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List of publications resulting from the doctorate work

This thesis is based on the following publications, from which tables and figures were used with the permission of the editors.

I **Melo, A. M. P.**, Duarte, M., and Videira A. (1999) "Primary structure and characterisation of a 64 kDa NADH dehydrogenase from the inner membrane of *Neurospora crassa* mitochondria" *Biochim. Biophys. Acta* 1412, 282-287.

II Almeida, T., Duarte, M., **Melo, A. M. P.**, and Videira A. (1999) "The 24 kDa iron-sulfur subunit of complex I is required for enzyme activity" *Eur. J. Biochem.* 265, 86-92.

III Ferreirinha, F., Duarte, M., **Melo, A. M. P.**, and Videira A. (1999) "Effects of disrupting a 21 kDa subunit of complex I from *Neurospora crassa*" *Biochem. J.* 342, 551-554.

IV **Melo A. M. P.**, Duarte, M., Møller, I. M., Prokisch, H., Dolan, P., Pinto, L., Nelson, M., and Videira, A. (2001) "The external calcium-dependent NADPH dehydrogenase from *Neurospora crassa* mitochondria" *J. Biol. Chem.* (*in press*).

Abbreviations

ACP	acyl carrier protein
APS	ammonium persulfate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
bp	base pairs
BSA	bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CCHL	Cytochrome *c* heme lyase
CHAPS	3-[(3-cholamidopropyl)-dimethylammonium]-1-propanesulfonate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytosine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddTTP	dideoxythymidine triphosphate
DMSO	dimethyl sulphoxide
DNAse	deoxyribonuclease
DNA	deoxyribonucleic acid
DPI	diphenyleneiodonium
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetate
EGTA	ethyleneglycol-bis-aminoethylether tetraacetate
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
IPTG	isopropyl β-D-thiogalactopyranoside
IO-SMP	inside-out submitochondrial particles

kDa	kilo Dalton
LB	luria broth
MES	2-[N-Morpholino]ethanesulfonic acid
min	minutes
MOPS	N-Morpholinopropanesulfonic acid
MPP	matrix processing peptidase
MRI	molecular resonance imaging
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
OD	optical density
PCR	polymerase chain reaction
PEG	polyethyleneglycol
PMSF	phenylmethylsulphonylfluoride
RNAse	ribonuclease
RNAsin	ribonuclease inhibitor
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TEMED	N,N,N',N'-tetramethylethylenediamine
TES	2-([2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino)ethanesulfonic acid
Tris	tris[hydroxymethyl]aminomethane

Resumo

Em sistemas eucarióticos não fotossintéticos, a mitocôndria é o organelo responsável pela síntese de ATP, uma molécula muito energética usada na biossíntese e manutenção celulares, bem como na tomada de iões. É, também, na mitocôndria que ocorre a síntese de intermediários para várias reacções biossintéticas.

O NADH é o maior dador de electrões à cadeia transportadora de electrões mitocondrial e, consequentemente, da sua oxidação resulta a maior parte da energia produzida no organelo. Dois tipos de enzimas levam a cabo a transferência de electrões do NADH para a ubiquinona: as que acoplam a transferência de electrões à translocação de protões, através da membrana interna mitocondrial, e as que realizam a mesma transferência sem transdução de energia. No primeiro caso, temos o complexo I (NADH:ubiquinona oxidorredutase), sensível à rotenona. Trata-se de uma enzima multimérica ancorada à membrana interna mitocondrial, com um domínio matricial, presente na maioria dos sistemas eucarióticos. O segundo grupo integra as NAD(P)H desidrogenases alternativas, resistentes à rotenona, que contribuem para a formação do potencial de membrana apenas através dos complexos III e IV. O fungo *Neurospora crassa*, o organismo escolhido para realizar este projecto, contém os dois tipos de NAD(P)H desidrogenases nas suas mitocôndrias.

O complexo I de dois mutantes da enzima foi caracterizado no que se refere à sua actividade de NADH: ubiquinona oxidorredutase. Estes mutantes não têm as subunidades de 24 e 21 kDa e são chamados de nuo24 e nuo21, respectivamente. Determinou-se o consumo de oxigénio por mitocôndrias e partículas submitocondriais invertidas dos mutantes do complexo I nuo24 e nuo21 a oxidar, respectivamente, malato/piruvato e NADH. A oxidação sensível à rotenona observada no nuo21 foi semelhante à do tipo selvagem, enquanto o nuo24 não apresentou qualquer oxidação sensível ao inibidor. Assim, em contraste com a subunidade de 21 kDa, a subunidade de 24 kDa é indispensável para a actividade do complexo I. O facto de mitocôndrias do nuo24 apresentarem actividade NADH: ubiquinona oxidorredutase confirma a existência de outras enzimas responsáveis pela

oxidação do NADH matricial, para além do complexo I, em mitocôndrias de Neurospora.

Um clone parcialmente sequenciado, codificante de uma hipotética NAD(P)H desidrogenase, foi obtido do *Fungal Genetics Stock Center* e totalmente sequenciado nas duas cadeias. O clone contém 2570 pares de bases e uma região codificante de 2019 pares de bases. Estes codificam uma proteína de 673 aminoácidos com uma massa molecular aparente de 64 kDa, de acordo com electroforese em gel desnaturante de poliacrilamida. Os primeiros 74 aminoácidos da cadeia polipeptídica representam uma possível pré-sequência mitocondrial. A estrutura primária da proteína de 64 kDa apresenta cerca de 35 % de identidade com NADH desidrogenases, de outros organismos, também resistentes à rotenona. Uma sequência de consenso para um domínio de ligação ao cálcio está presente na estrutura primária da proteína de 64 kDa, o que sugere que o catião tem um papel na regulação da actividade da enzima.

A região C-terminal da hipotética NAD(P)H desidrogenase foi expressa em *Escherichia coli*, como proteína de fusão, purificada e utilizada para imunizar coelhos, visando a produção de anticorpos policlonais. O gene *nde1* (que codifica a proteína de 64 kDa ou NDE1) foi inactivado pelo processo de indução repetida de mutações pontuais (RIP). Esferoplastos de Neurospora foram transformados com um vector transportando uma cópia do cDNA do *nde1*, e a estirpe resultante foi cruzada com a estirpe do tipo selvagem. Prepararam-se mitocôndrias da progenia do cruzamento e os mutantes nde1 foram seleccionados com anticorpos contra a NDE1. Cruzamentos homozigóticos do mutante nde1 originaram descendência, indicando que a proteína não é necessária ao desenvolvimento sexual. Vários mutantes deficientes em subunidades do complexo I foram cruzados com o mutante nde1, observando-se mutantes duplos em todos os cruzamentos. Isto sugere que o produto do gene *nde1* não está envolvido na oxidação do NADH matricial, ou, se este envolvimento se verificar, existem outras vias para assegurar o processo em mitocôndrias de *N. crassa*.

O subfraccionamento de mitocôndrias, com concentrações progressivas de digitonina, localizou a NDE1 na membrana interna mitocondrial. Experiências semelhantes, em que as fracções resultantes da incubação com digitonina foram

tratadas com proteases, mostraram que esta tem domínios expostos ao espaço intermembranar. A região codificante da NDE1 foi usada num sistema de tradução/transcrição para a síntese *in vitro* de um precursor. Este foi importado para mitocôndrias de Neurospora e levedura, e processado na presença de potencial de membrana. O precursor processado ficou acessível à tripsina em mitoplastos de levedura, indicando que, tal como *in vivo*, a proteína processada e importada apresenta domínios no espaço intermembranar.

Prepararam-se mitocôndrias e partículas submitocondriais invertidas das estirpes do tipo selvagem e do mutante nde1 e procedeu-se à caracterização da oxidação de NADH e NADPH. Relativamente às partículas submitocondriais, não há diferenças entre as duas estirpes. Este resultado sugere que as NAD(P)H desidrogenases alternativas internas não foram afectas pela inactivação do *nde1*. A comparação da oxidação de NADH por mitocôndrias das duas estirpes também revelou características semelhantes. Pelo contrário, enquanto mitocôndrias do tipo selvagem oxidam NADPH a pH fisiológico, mitocôndrias do mutante nde1 não apresentam esta actividade, o que indica que a NDE1 é uma NADPH desidrogenase externa. Uma caracterização mais detalhada da actividade da NDE1 demonstrou que a oxidação de NADPH, por esta enzima, varia em função do pH e é estimulada pela presença de cálcio. Apesar da ausência de actividade da NDE1, mitocôndrias do mutante nde1 oxidam NADPH e NADH exógenos na zona acídica e ao longo da, escala de pH, respectivamente. Este facto evidencia a existência de uma segunda NAD(P)H desidrogenase resistente à rotenona, a NDE2. A NDE1 e a NDE2 contribuem para o "turnover" do NAD(P)H citosólico, em células de *N. crassa*.

Como se pode inferir do presente trabalho, a mitocôndria de *N. crassa* tem, pelo menos, três NAD(P)H desidrogenases insensíveis à rotenona. Até ao presente, o gene que codifica a NDE1 foi o único a ser clonado, sequenciado e identificado. Num futuro próximo, deverá ser possível identificar os outros dois genes, inactivá-los e caracterizar a actividade das suas mitocôndrias, possibilitando a clarificação do papel fisiológico das NAD(P)H desidrogenases resistentes à rotenona. A produção de uma estirpe de Neurospora sem NAD(P)H desidrogenases resistentes à rotenona providenciará modelos para o estudo do complexo I selvagem e de complexos I

deficientes em subunidades desta enzima. Deste modo, poder-se-á estabelecer a importância de cada uma das subunidades da proteína na sua actividade.

Resumé

Chez les eucaryotes qui n'utilisent pas la photosynthèse, la mitochondrie est l'organelle responsable de la synthèse d'ATP, une molécule très énergétique, utilisées dans la biosynthèse, l'entretien des cellules et l'acquisition d'ions. La mitochondrie est aussi le lieu de synthèse d'intermédiaires des différents reactions biosynthétiques.

La NADH est le principal donneur d'électrons dans la chaîne mitochondriale de transfert d'électrons. Il existe deux types d'enzymes qui permettent le transfert d'électrons du NADH à l'ubiquinone: les enzymes qui couplent le transfert d'électrons à la translocation de protons à travers la membrane interne de la mitochondrie; et les enzymes qui permettent le même transfert mais sans transduction d'énergie. Dans le premier type d'enzymes, il y a le complexe I (NADH:ubiquinone oxydoréductase) qui est sensible au rotenone. C'est une enzyme multimerique ancrée à la membrane interne mitochondriale, avec un domaine matriciel, qui existe chez la majorité d'eucaryotes. Chez le second groupe se trouvent les NAD(P)H déshydrogénases résistant au rotenone. Dans ce cas, seulement les complexes III et IV contribuent pour la formation du potentiel de la membrane. Le champignon *Neurospora crassa* a servi de modèle d'études afin de réaliser ce travail, étant donné que les deux types de NAD(P)H déshydrogénases sont présentes dans cet organisme.

Deux mutants ont servi pour la caractérisation de l'activité de NADH:ubiquinone oxydoréductase du complexe I. Ces mutants sont dépourvus des sous-unités de 24 et 21 kDa du complexe I et sont désignés nuo24 et nuo21, respectivement. La consommation d'oxygène par les mitochondries et particules sous-mitochondriales inversées chez les mutants nuo24 et nuo21, oxydant respectivement le malate/piruvate e la NADH, a été comparée avec la souche sauvage. L'oxydation sensible au rotenone observée dans nuo21 était comparable à celle de la souche sauvage, alors que l'oxydation dans nuo24 n'a montré aucune sensibilité à l'inhibiteur. Au contraire de la sous-unité de 21 kDa, la sous-unité de 24 kDa est indispensable à l'activité du complexe I.

Grâce au *Fungal Genetics Stock Center*, il nous a été possible d'obtenir un clone de cDNA (NM1C2) partiellement séquencé, en codant une hypothétique NAD(P)H déshydrogénase. Les deux chaînes de ce clone ont été entièrement séquencées. La région codante, de 2019 pbs, code pour une protéine de 673 aminoacides et de poids moléculaire d'environ 64 kDa selon son électrophorèse sur gel dénaturant de polyacrilamide. Les premiers 74 aminoacides de la chaîne polipeptidique représentent une possible pré-séquence mitochondriale. L'analyse de sa structure primaire a révélé environ 35% d'identité avec la NADH déshydrogénase d'autres organismes, et une séquence consensus d'un domaine de liaison au calcium en suggérant un rôle du calcium dans la regulation de l'activité de cet enzyme.

L'expression de la région C-terminale de la possible NAD(P)H déshydrogénase a été induite chez *Escherichia coli*, et la protéine de fusion a été purifiée en vue de production d'anticorps polyclonaux chez le lapin. Le gêne *nde1* (qui codifie la protéine de 64 kDa ou NDE1) a été inactivé au moyen d'induction répétée de mutations ponctuelles (RIP). Un vecteur, en transportant une copie du cDNA de nde1, a transformé les spheroplastes de Neurospora, la souche résultante a été croisée avec une souche sauvage. Mitochondries de la descendence du croisement ont été préparées et analisées, par Western blot en utilisant des anticorps anti-NDE1, pour la sélection des mutants nde1. Les croisements homozygotiques du mutant nde1 ont produit une descendance, ce qui indique que la protéine n'est pas nécessaire pour le développement sexuel. Différents mutants des sous-unités du complexe I ont été croisés avec nde1, ayant donné tous des doubles mutants. Ce qui sugére que la proteíne de 64-kDa ne s'occupe pas de la oxidation du NADH matriciel, ou qu'il y a d'autres routes pour faire cette oxidation dans les mitochondries de Neurospora.

Le sous-fractionnement de mitochondries, avec des concentrations progressives de digitonine, a permit de localiser NDE1 dans la membrane interne mitochondriale; le traitement des fractions résultantes avec des protéases a montré que cette protéine possède des domaines exposés à l'espace intermembranaire. La région codant de NDE1 a été utilisée dans la synthèse *in vitro* d'un précurseur qui a été importé vers les mitochondries de Neurospora et levure, et ce processus a été effectué en présence du potentiel de membrane. Après le processus, le précurseur est devenu accessible à

la trypsine dans les mitoplastes de levure, en indiquant que , comme *in vivo*, la protéine processé et importé présente des domaines à l'éspace intermembranaire.

Des mitochondries et particules sous-mitochondriales inversées des souches sauvage et nde1 ont été préparées et l'oxydation de NADH et de NADPH a été caractérisée. En ce qui concerne les particules sous-mitochondriales, il n'y a pas de différence quant à l'oxydation des deux substrats par les deux souches, suggérant que les NAD(P)H déshydrgénases alternatives internes n'ont pas été affectées par l'inactivation de NDE1. L'analyse comparative de l'oxydation de NADH par les mitochondries des deux souches a révélé aussi un comportement similaire. Cependant, alors que les mitochondries de type sauvage oxydent la NADPH à pH physiologique, les mitochondries de nde1 n'ont pas montré cette activité, ce que indique que la NDE1 est une NADPH déshydrogénase externe. Une caractérisation plus poussée de l'activité de NDE1 a démontré que l'oxydation de NADPH par cette enzyme varie en fonction du pH et est stimulée en présence du calcium. Malgré l'absence d'activité de NDE1, les mitochondries de nde1 oxydent NADPH et NADH exogènes, au niveau de la région acidique et le long de l'échelle de pH, respectivement. Ce fait montre l'existence d'une deuxième NAD(P)H déshydrogénase alternative, la NDE2. La NDE1 et la NDE2 contribuent pour le «turnover» de la NAD(P)H cytosolique, dans les cellules de *N. crassa*.

Ce travail montre que la mitochondrie de *N. crassa* possède au moins trois NAD(P)H déshydrogénases insensibles au rotenone. Jusqu'à présent, le gêne codant pour la NDE1 a été le premier à être cloné, séquencé et identifié. Dans un futur très proche, il devrait être possible d'identifier et puis inactiver les deux autres gênes afin de caractériser l'activité de leurs mitochondries. Ceci aiderait à éclaircir le rôle physiologique des NAD(P)H deshydrogénases résistantes au rotenone. La production d'une souche de Neurospora dépourvus des NAD(P)H déshydrogénases résistantes au rotenone nous donnera des modelles pour l'étude du complex I sauvage et des complexes I defficientes en sous-unités de cette enzyme. Ansi, sera possible d'établissir l'importance de chaque sous-unité de la protéine en son activité.

Summary

In non-photosynthetic eukaryotic systems the mitochondrion is the organelle responsible for the synthesis of ATP, a very energetic molecule that is used in cellular biosynthesis and maintenance as well as in ion uptake. It is also in the mitochondrion that the synthesis of many intermediates for several biosynthetic reactions takes place.

NADH is the major electron donor to the mitochondrial electron transport chain. There are two distinct types of enzymes carrying out the electron transfer from NADH to ubiquinone: those that couple the electron transfer to proton translocation across the inner mitochondrial membrane, and the others that perform the transfer of electrons without energy transduction. In the first group is the rotenone-sensitive complex I, or NADH:ubiquinone oxidoreductase, a multimeric enzyme anchored to the inner mitochondrial membrane, with a matrix domain, that is present in most eukaryotic systems. The second group of enzymes comprises the alternative NAD(P)H dehydrogenases, also known as rotenone-insensitive NAD(P)H dehydrogenases. In this case, only complexes III and IV contribute to the generation of a membrane potential. The fungus *Neurospora crassa*, the organism chosen to develop this project, contains both types of NAD(P)H dehydrogenases in its mitochondria.

Studies were performed in order to characterise the complex I from two mutants with respect to its NADH:ubiquinone oxidoreductase activity. They lack the subunits of 24 and 21 kDa and are called nuo24 and nuo21, respectively. The oxygen uptake by mitochondria and IO-SMP oxidising malate/pyruvate and NADH, respectively, from nuo24 and nuo21 was measured. The rotenone-sensitive oxidation of substrates by nuo21 and wild type was similar, while nuo24 did not present any rotenone-sensitive activity. Thus, in contrast to the 21-kDa subunit, the subunit of 24 kDa is required for complex I activity. The fact that nuo24 mitochondria can still oxidise NADH dehydrogenase confirms the presence of other enzymes, beyond complex I, responsible for the oxidation of matrix NADH in Neurospora mitochondria.

A partially sequenced clone, encoding a putative rotenone-insensitive NAD(P)H dehydrogenase, was obtained from the Fungal Genetics Stock Center and sequenced fully in both strands. It has 2570 bp and contains an open reading frame of 2019 bp. The encoded protein consists of 673 amino acid residues, with an apparent molecular mass of 64 kDa, as determined by SDS-PAGE. The first 74 amino acid residues of the polypeptide constitute a putative mitochondrial-targeting sequence. When compared with rotenone-insensitive NADH dehydrogenases from other organisms, the primary structure of the Neurospora protein displays around 35 % identity. A consensus sequence for a calcium-binding domain is also present in the primary structure of the 64-kDa protein, what suggested that this cation might have a role in the regulation of the enzyme.

The C-terminal region of the putative NAD(P)H dehydrogenase was expressed in *Escherichia coli* as a fusion protein, purified, and used to immunise rabbits in order to produce polyclonal antibodies. *nde1* (the gene encoding the 64-kDa protein or NDE1) was inactivated by repeat-induced point mutations. Neurospora spheroplasts were transformed with a vector carrying a copy of the *nde1* cDNA and the resulting strain was crossed with the wild type strain. Mitochondria from the progeny were prepared and antibodies against NDE1 polypeptides were used to select nde1 mutants. The homozygous crosses of nde1 mutant yielded progeny indicating that the protein is not essential for sexual development. Several mutants deficient in complex I subunits were crossed with the nde1 mutant producing double mutants. This suggested that either the *nde1*-encoded protein is not involved in the oxidation of matrix NADH or, if it is involved, there are other ways to carry out this activity in the matrix of *N. crassa* mitochondria.

Subfractionation of mitochondria with increasing concentrations of digitonin localised the protein to the inner membrane of the mitochondrion. Similar experiments were performed, where the digitonin-solubilized fractions were submitted to protease treatment, showing that the protein has domains facing the intermembrane space. The open reading frame of NDE1 was used as template for *in vitro* synthesis of a precursor that was imported into Neurospora and yeast mitochondria and processed in the presence of a membrane potential. The processed

precursor was accessible to trypsin in yeast mitoplasts indicating that, as verified *in vivo*, the processed and imported protein has intermembrane facing domains.

Mitochondria and IO-SMP from wild type and nde1 mutant strains were prepared and NADH and NADPH oxidation was characterised. The oxidation of NADH and NADPH by IO-SMP from both wild type and nde1 mutant strains shows a similar behaviour, indicating that the internal alternative NAD(P)H dehydrogenases were not affected by the inactivation of *nde1*. The pattern of NADH oxidation by wild type mitochondria and mitochondria from the nde1 mutant is similar. In contrast, while wild type mitochondria oxidise NADPH at physiologic pH, in nde1 mutant mitochondria this activity is absent, showing that NDE1 is an external NADPH dehydrogenase. Further characterisation of NDE1 activity showed that NADPH oxidation by the enzyme varies as a function of the pH and is stimulated by the presence of calcium. In spite of lacking NDE1 activity, nde1 mutant mitochondria can still oxidise exogenous NADH throughout the pH range and also NADPH at acidic pH. This observation provided evidence for the presence of a second alternative NAD(P)H dehydrogenase, NDE2. Both NDE1 and NDE2 can contribute to the turnover of cytosolic NAD(P)H in *N. crassa* cells.

As deduced from the present work, *N. crassa* mitochondria contain at least three rotenone-insensitive NAD(P)H dehydrogenases. So far, the gene encoding NDE1 is the only gene cloned, sequenced and identified. In a near future it should be possible to identify the other two genes and very interesting to inactivate them and characterise the activity of their mitochondria. This information can be crucial to illuminate the physiological role of the rotenone-insensitive NAD(P)H dehydrogenases. The production of a Neurospora strain lacking the rotenone-insensitive NAD(P)H dehydrogenases will provide models to study the wild type complex I and the complex I deficient in different subunits. In this way it will be possible to establish the importance of each complex I subunit in the activity of the protein.

Chapter I – General Introduction

1. Neurospora, the model

Though presenting some peculiarities, fungi share the basic characteristics of eukaryotic organisms. The narrow range of DNA concentration values among fungi suggests a remarkably uniform mechanism regulating nuclear division as compared with the otherwise great plasticity characterising the organisms from this kingdom. This narrow range of values for DNA composition contrasts with the huge variability in the composition of other materials. The variability for the same fungus with different growth conditions, ages and stages of development is in fact larger than differences between species (Griffin, 1994). Among eukaryotes, fungi are ideal organisms to be used as models for studies of sub-cellular structure, because of their ease of handling, simplicity, short life cycles and the considerable amount of genetic information available on them.

The species *Neurospora crassa* is a multicellular eukaryote that belongs to the kingdom Fungi, division Eumycota, class Ascomycetes, sub-class Euascomycetidae, order Euascomycetales, family Euascomycetacea and genus Neurospora. The mitochondrion of Neurospora was the model used in the present work. The fact that the organism grows strictly as a haploid makes it convenient to study introduced phenotypes, allowing an accurate characterisation of deleted or added genes.

1.1. The life cycle of *N. crassa*

The life cycle of *N. crassa* is typical of the Euascomycetidae (Fig. 1). Ascospores germinate into multinucleate cells that form branched filaments (hyphae). The hyphal system spreads out fast to form a haploid mycelium. Asexual reproduction is accomplished by macroconidia and microconidia produced on specialised aerial hyphae, called conidiophores. The sexual cycle is initiated when nutrients are limiting, particularly when the levels of the nitrogen sources are low.

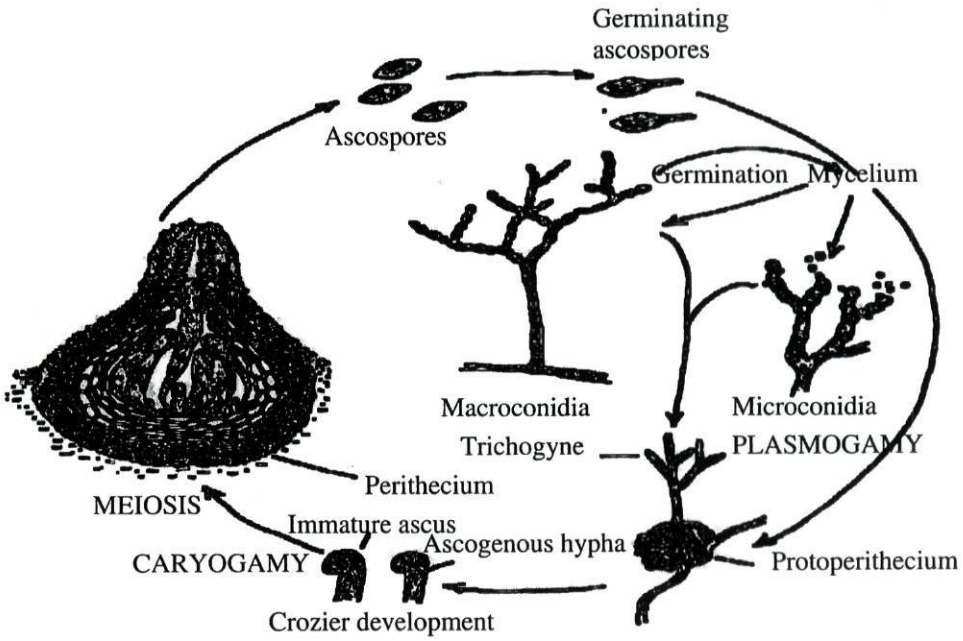

Figure 1. **Neurospora life cycle.** Redrawn from Griffin (1994).

An ascogonium, a swollen coiled hypha with an apical branched trichogyne, differentiates. The differentiated hypha is quickly enclosed by nearby hyphae that grow closely around it, forming a protoperithecium with the trichogyne projecting out. A spermatial element of the opposite mating-type (spermatia can be microconidia, macroconidia or hyphae) fuses with trichogyne. The nucleus of the spermatium enters and migrates along the trichogyne to the ascogonium, creating the "dicaryotic" stage. Ascogenous hyphae grow from the fertilised ascogonium, generating a basal hymeneal layer within the developing perithecium. Most of the tissues of the perithecium arise from the monocaryotic mycelium that formed the ascogonium. The spermatial and ascogonial nuclei migrate into growing ascogenous hyphae, dividing as they go. As development proceeds, nuclei in the tips of the ascogenous hyphae pair together and divide simultaneously, while the tip of the hyphae grows back on it to form a crozier. Two septa form in the crozier. The tip cell is uninucleate, the second cell is binucleate, and the third cell is uninucleate. The first fuses with the third, re-establishing the binucleate condition and a short branch grows from the third cell to repeat crozier formation. The second cell enlarges to become an

ascus; its nuclei fuse, rising a zygote, which immediately proceeds to meiosis. Pre-meiotic synthesis of DNA occurs prior to nuclear fusion, so that when karyogamy takes place, the resulting diploid nucleus immediately undergoes meiosis. Thus the diploid phase of Neurospora life cycle is very short and restricted to a single cell. After meiosis, a single mitosis gives rise to an eight-nucleate ascus and each nucleus is enclosed within a cell wall within the ascus, forming each ascospore. At maturity, the ascus elongates up the perithecial canal, exposing its tips, and the ascospores are ejected. Genetic studies have shown that the nuclei that fused in the ascus were of two genotypes, one from the ascogonial parent and the other from the spermatial one, thus allowing genetic analysis (Selker, 1990; Griffin, 1994).

1.2. The genome of *N. crassa*

The genome of *N. crassa* consists of seven chromosomes ranging in size from 4 to 11 Mbp, as estimated by physical and cytological measurements. Eight percent of the 37 Mbp present in Neurospora genome are repetitive sequences, mainly ribosomal RNA genes. Besides nuclear DNA, Neurospora also contains mitochondrial DNA. It encodes subunits 6, 8 and 9 of ATPase (the latter is a pseudogene, meaning that it does not encode a protein), subunits 1, 2 and 3 from cytochrome *c* oxidase, apocytochrome *b*, seven subunits from complex I and various tRNA, ribosomal proteins, rRNA (Griffin, 1994). Several unidentified open reading frames are also present in the mtDNA of *N. crassa* (Davis, 2000).

1.3. Inactivation of Neurospora genes

A commonly used strategy for protein characterisation is the disruption of their encoding genes and the analysis of the induced mutants. A peculiarity of Neurospora is the phenomenon of repeat-induced point mutations (RIP) acting on nuclear DNA (Selker and Garrett, 1988). DNA duplications in the genome are detected and modified during the sexual cycle between fertilisation and karyogamy. RIP damages

both regions of the duplications introducing multiple GC to AT transition mutations in the DNA. Sequences mutated by RIP are typically methylated at most of the remaining cytosine residues. Any duplicated region bigger than 500 bp is susceptible to RIP'ing. Though timing and effects of this phenomenon are well established, little is known about its mechanism (Watters *et al.*, 1999). The RIP can be used to inactivate genes in *N. crassa.*

Inactivation of genes in Neurospora may also be achieved by homologous recombination. This process is accomplished by introducing a genetic marker, for instance a gene coding for resistance to hygromycin B, in the middle or in the place of a target gene. Neurospora spheroplasts are transformed with the DNA construct, which carries the genetic marker flanked by Neurospora sequences. After transformation, the recombination of the flanking sequences with the homologous sequences in the genome results in an exchange of the host gene with the DNA construct. The DNA sequence of the target gene is thus altered (gene disruption) and the correct protein can not be synthesised (Videira, 1998).

2. Mitochondria: a brief story

The mitochondrion (Fig. 2) of Neurospora is a small organelle (1-2 μm), found in eukaryotic cells, where ribosomes, RNA and DNA are present which makes it a semiautonomous organelle. This organelle is abundant in eukaryotic tissues, easy to purify and a rich source of vital enzymes. The general appearance, structure and organisation of mitochondria are similar for fungi, animals or plants. They are composed of two bilayered membranes, a smooth outer membrane surrounding a highly invaginated inner membrane that contains the electron transport chain. The invaginations of the inner membrane are known as cristae. Inside the inner membrane is a very concentrated protein suspension, the matrix, where the tricarboxylic acid cycle takes place. The space between the two mitochondrial membranes is called the intermembrane space. Intact mitochondria are osmotically active, which means that they take up water and swell when placed in a hypo-osmotic medium. Most inorganic ions and charged organic molecules do not diffuse

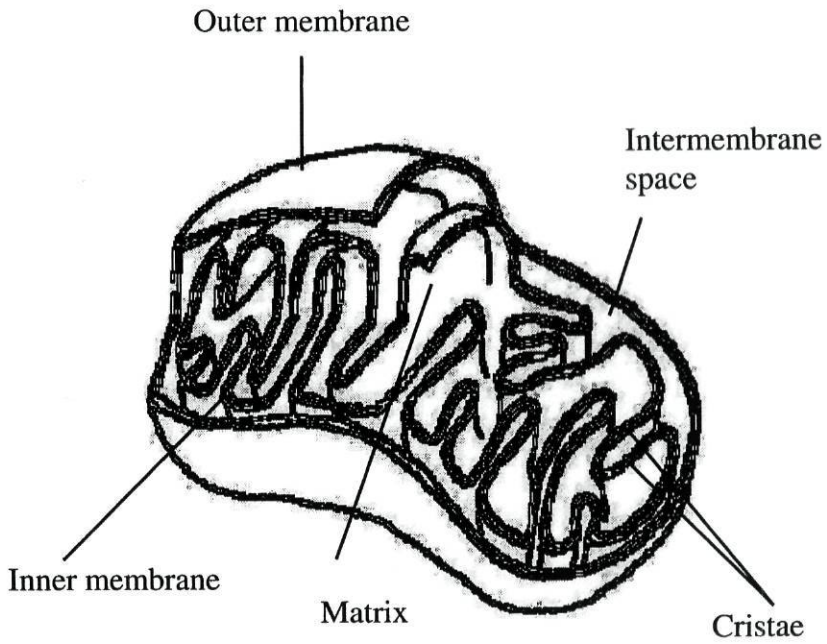

Figure 2. **Three-dimensional drawing of a mitochondrion.**

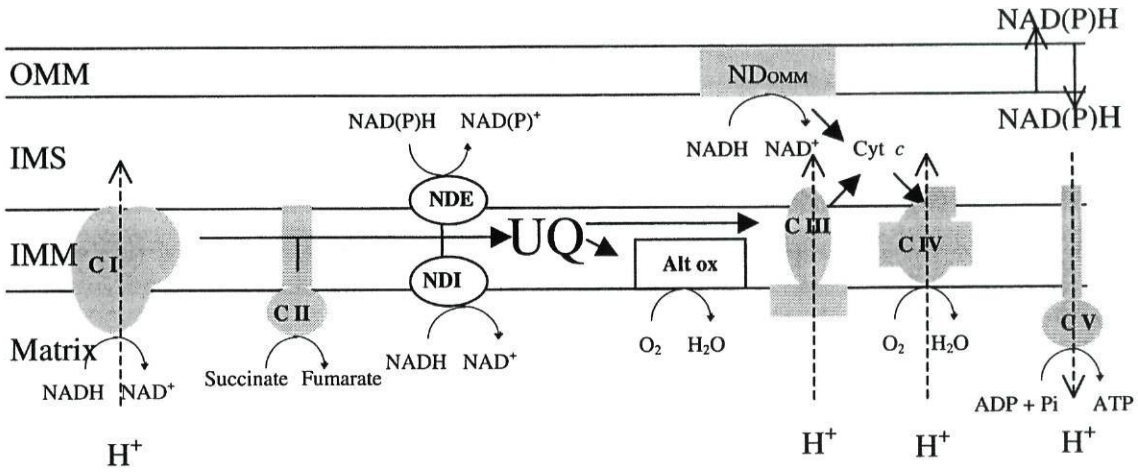

Figure 3. ***N. crassa*** **respiratory chain.** The gray fill represents common features with the mammalian respiratory chain. Alt ox, alternative oxidase; C I, complex I or NADH:ubiquinone oxidoreductase; C II, complex II or succinate dehydrogenase; C III, complex III or cytochrome bc_1; C IV, complex IV or cytochrome *c* oxidase; C V, complex V or ATP synthase; IMM, inner mitochondrial membrane; IMS, intermembrane space; NDE, rotenone-insensitive NAD(P)H dehydrogenase from the outer surface of the inner mitochondrial membrane; NDI, rotenone-insensitive NADH dehydrogenase from the inner surface of the inner mitochondrial membrane; NDOMM, NADH dehydrogenase from the outer mitochondrial membrane; OMM, outer mitochondrial membrane; UQ, ubiquinone.

freely into the matrix due to the inner membrane, which works as an osmotic barrier. In contrast, the outer membrane is permeable to solutes with molecular masses smaller than 10 kDa (Douce, 1985; Taiz and Zeiger, 1991).

In non-photosynthetic eukaryotes, mitochondria are the organelles in charge of producing most of the energy for cellular metabolism by a process called oxidative phosphorylation. Electrons from the oxidation of substrates like NADH, NADPH and $FADH_2$ are passed along the electron transport chain to oxygen (Fig. 3). This transfer is coupled to proton pumping across the inner membrane and when the protons pass back, through the ATP synthase, ATP is synthesised (Hatefi, 1985). Beta-oxidation of fatty acids and regeneration of intermediates for cellular biosynthesis through the tricarboxylic acid cycle also occur in mitochondria.

Another very important task involving mitochondria is programmed cell death, or apoptosis. Though a lot of mystery still surrounds the issue, it is recognised that mitochondria play a central role in the regulation of this process. The organelles can trigger cell death by disrupting electron transport and energy metabolism, releasing or activating apoptosis-mediating proteins, or altering the cellular reduction-oxidation potential (Green and Reed, 1998).

2.1. Description of respiratory chains

There are five inner-membrane complexes in the respiratory chain of mammalian mitochondria (Fig. 3). Complex I, or NADH:ubiquinone oxidoreductase, is the largest complex of the respiratory chain, and is responsible for the transfer of electrons from NADH to ubiquinone. Complex II, or succinate dehydrogenase, is a component of the tricarboxylic acid cycle. The electrons from succinate oxidation are transferred to ubiquinone. Complex II contains FAD, several Fe-S clusters, and a *b*-type cytochrome. Cytochrome bc_1, complex III, transfers electrons from the reduced ubiquinone, ubiquinol, to cytochrome *c* through an iron-sulphur center, two *b*-type cytochromes, and a membrane-bound cytochrome c_1. Complex IV, or cytochrome *c* oxidase, receives electrons from the water-soluble hemoprotein cytochrome *c*, located in the intermembrane space, and transfers them to the heme-, iron- and

cooper- containing active site, where they are used to reduce oxygen to two molecules of water. Complexes I, III and IV couple proton translocation across the inner membrane to the oxidation of their substrates, thus building an electrochemical proton gradient. This is used as a proton-motive force by ATP synthase (complex V) in the synthesis of ATP. The protons are driven back to the matrix simultaneously with ATP synthesis, collapsing the electrochemical gradient. Complex V is a functionally reversible enzyme which also hydrolyses ATP producing ADP (Saraste, 1999). The general mechanistic principle of oxidative and photosynthetic phosphorylation – the chemiosmotic theory – explaining the coupling between respiration and ATP synthesis was proposed by Peter Mitchell (Mitchell and Moyle, 1965). This theory awarded him the Nobel Prize in chemistry, in 1978.

In addition to these inner-membrane complexes, there is an NADH-cytochrome b_5 reductase in the outer membrane of mammalian mitochondria that does not pump protons. This enzyme directs electrons to cytochrome *c*, which will transfer them to complex IV (Bernardi and Azzone, 1981).

Fungal and plant mitochondria present slightly different respiratory chains than those of animals. The former contain additional proteins that oxidise NAD(P)H in a rotenone-resistant manner (Yagi, 1991). Moreover, an alternative way to drive electrons from ubiquinol to water have been described in these respiratory chains, the alternative oxidase (Li *et al.*, 1996; McIntosh, 1994). None of these proteins is involved in pumping protons with the concomitant production of ATP. However, the alternative NAD(P)H dehydrogenases give electrons to ubiquinone, contributing to proton-pumping at complexes III and IV (Yagi, 1991; Vanlerberghe and McIntosh, 1997).

2.2. NADH: The super fuel

NADH (Fig. 4) is the major electron donor to the electron transport chain of mitochondria and many bacteria, playing a crucial role in the synthesis of ATP through oxidative phosphorylation. In eukaryotes, respiratory dissimilation of sugars promotes reduction of NAD^+ to NADH in the cytosol, mainly through glycolysis.

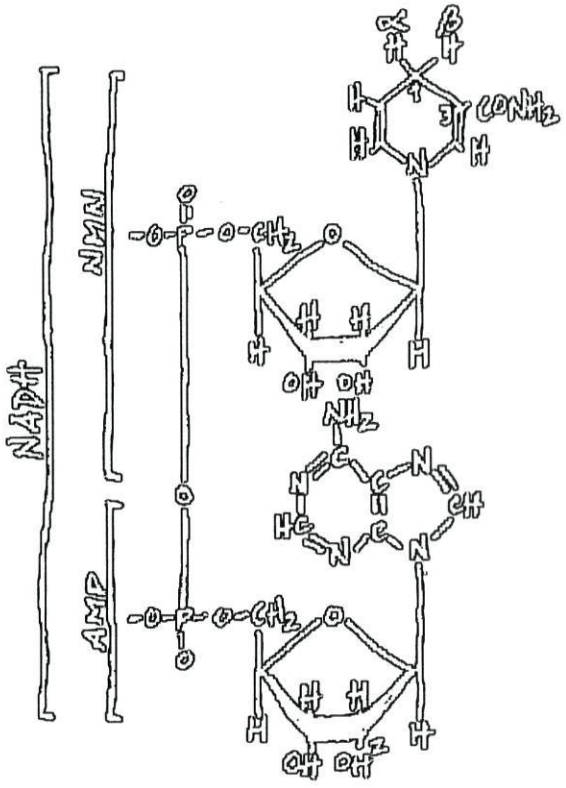

Figure 4. **Structural representation of NAD(P)H.** AMP is coupled to a nicotinamide-containing nucleotide (NMN) through a pyrophosphate bond. The two non-equivalent hydrogens, labelled α and β, in NADH are in position 4 of the nicotinamide ring. NADPH differs from NADH by having a phosphate group instead of a hydroxyl group in position 2 of the ribose in AMP.

NADPH (Fig. 4) is also produced in the cytosol via the pentose phosphate pathway (Overkamp *et al.*, 2000). The generated NAD(P)H can be reoxidised by mitochondria. In plants (Roberts *et al.*, 1995) and fungi (Weiss *et al.*, 1970) this oxidation can be carried out by external NAD(P)H dehydrogenases. In the case of mammalian mitochondria, with the exception of α-glycerol phosphate dehydrogenase from the outer surface of the inner mitochondrial membrane, NAD(P)H needs to be transported, by redox shuttle mechanisms, into the matrix, where it will be oxidised by complex I, (Dawson, 1979), since the inner mitochondrial membrane is impermeable to NAD(P)H (von Jagow *et al.*, 1970). Redox shuttle mechanisms are not unique to mammals. They are also present in

plants (Krömer and Heldt, 1991) and yeast (Overkamp *et al.*, 2000), despite the presence of external NAD(P)H dehydrogenases.

NADH is also produced in the matrix by pyruvate dehydrogenase, and by three enzymes in the Krebs cycle: isocitrate dehydrogenase, alfa-ketoglutarate dehydrogenase and malate dehydrogenase (Stryer, 1995). The oxidation of the matrix substrate can occur via the complex I or the internal rotenone-insensitive NAD(P)H dehydrogenases. Usually, though with different affinity, NADH-oxidising enzymes can also oxidise NADPH, a compound with very similar structure to NADH with the exception of a phosphate group replacing the hydroxyl in position 2 in the ribose of AMP (Fig. 4) (Finel, 1998). At alkaline pH, this oxidation might be prevented by electrostatic repulsion between the phosphate group of NADPH and the phospholipids of the membrane (Møller *et al.*, 1982).

3. Two types of NAD(P)H dehydrogenases

The existence of distinct NADH dehydrogenases was first reported by Bragg and Hou (1967) who partially purified two NADH:menadione reductases from *Escherichia coli* with distinct properties.

3.1. Complex I

The NADH:ubiquinone oxidoreductase, or complex I, is a well-characterised enzyme that catalyses the transfer of electrons from NADH to ubiquinone, through a number of protein-bound prosthetic groups, coupling this transfer to energy transduction (Hatefi, 1985).

Electron microscopy of single complex I molecules revealed this enzyme as an L-shaped structure with two major domains, a hydrophobic arm imbedded in the inner membrane, and a peripheral arm protruding into the matrix. A thin collar, missing in the *N. crassa* complex I, separates the two arms in *E. coli* (Guenebaut *et al.*, 1998) and in the bovine enzyme (Grigorieff, 1998). Independent assembly

pathways have been shown for the peripheral and the membrane arms of Neurospora complex I. In chloramphenicol-treated cells only a smaller form of the complex is present (Friedrich *et al.*, 1989). Pulse-labelling experiments have shown that some complex I subunits are found in intermediate complexes that resemble the membrane arm (Tushen *et al.*, 1990) and in Mn^{-} deficient growth conditions, the peripheral arm was not formed but the membrane arm could be detected (Schmidt *et al.*, 1992).

Complex I has a dual genetic origin: the majority of its protein subunits is nuclear-encoded, synthesised in the cytoplasm and imported into mitochondria, where they are assembled with a few mitochondrially encoded polypeptides (Walker, 1992). The number of subunits that constitute the enzyme varies from 14, in bacteria, which constitute the so-called minimal functional unit, to 43 in the bovine complex I. The Neurospora homologue of the minimal functional unit contains seven mitochondrially encoded subunits, ND1, ND2, ND3, ND4, ND4L, ND5 and ND6, and seven nuclear-encoded subunits. This last group includes the 78, 51, 49, 30.4, 24, 21.3c, and 19.3-kDa subunits. The 51-kDa subunit binds NADH, the FMN and a tetranuclear iron-sulphur cluster. The subunits of 78, 24, 21.3c and 19.3-kDa also bind iron-sulphur clusters (Videira, 1998). In addition to the minimal functional unit, 21 other subunits are estimated to be present in Neurospora complex I, one of which is an acyl-carrier protein (Schulte *et al.*, 1994).

Gene disruption experiments in Neurospora were carried out using the two approaches described above: the replacement of the endogenous gene with defective copies by homologous recombination and the method of RIP. Both methods have been successful and already produced 15 mutants in nuclear encoded subunits: 11 in subunits of the peripheral arm, and 4 in subunits of the membrane arm. The inactivation of these genes gave mutants where the peripheral and/or the membrane arm was detected independently (Table 1) confirming previous observations (Videira, 1998). All mutants are viable in the vegetative state, although with decreased growth rates, meaning that complex I is not essential for the survival of *N. crassa*. In these mutants it is likely that the alternative NADH dehydrogenases replace complex I, performing the turnover of NADH and contributing to the production of ATP although with lower yield. Nevertheless, complex I is required for sexual development in Neurospora (Duarte and Videira, 2000).

Several natural and synthetic substances have been shown to be specific inhibitors of complex I activity. These compounds fall into two groups, those that prevent NADH oxidation and those that prevent reduction of ubiquinone (Esposti, 1998). In the first group, there is the competitive inhibitor ADP-ribose that, due to its

Table 1. **Characteristics of subunits of complex I from Neurospora.** i, intact; m, membrane arm; mt, mitochondrial; n, nuclear; p, peripheral arm; smi, small membrane intermediate; lmi, large membrane intermediate; +, viable mutant. Adapted from Videira *et al.*, 1998.

Neurospora protein (kDa)	DNA origin	Localisation	Mutant / CI assembled
78	n	p	+/m
51	n	p	+/i
49	n	p	+/m
30.4	n	p	+/m
29.9	n	p	+/m
24	n	p	+/i
21.3a	n	p	+/i
21.3b	n	m	+/ p, smi, lmi
21.3c	n	p	+/m
21	n	p	+/i
20.9	n	m	+/p, smi
20.8	n	m	+/p, smi, lmi
19.3	n	cf	+/m
17.8	n	m	
14.8	n	p	
12.3	n	m	+/p, m
10.5	n	p	
9.3	n	m	
ACP	n	p	+/-
ND1	mt		
ND2	mt		
ND3	mt		
ND4	mt		
ND4L	mt		
ND5	mt		
ND6	mt		

analogous structure, can bind to the NADH binding site (Zharova and Vinogradov, 1997) and diphenyleneiodonium (DPI) which binds to the $FMNH_2$, preventing its oxidation (Majander *et al.*, 1994). Rotenone, and capsaicin and piericidin A are in the second group, and each one is representative of a different type of inhibition, non-competitive, competitive and antagonist of the quinol, respectively (Esposti, 1998).

3.2. Rotenone-insensitive NAD(P)H dehydrogenases

Type II NADH dehydrogenases are nuclear-encoded polypeptides without an energy-transducing site (Yagi, 1991). Unlike complex I, they are usually composed of a single polypeptide, though the *Sulpholobus* NDH-2 (Yagi, 1991) and the 43 kDa internal NAD(P)H dehydrogenase from red beetroot mitochondria (Menz and Day, 1996) were reported to be homodimers. These proteins lack FMN and iron-sulphur clusters as co-factors, but contain a non-covalently bound FAD instead. They are resistant to the complex I specific inhibitors rotenone and piericidin A, and no general specific inhibitor has been described though a few compounds can prevent their activity (Yagi *et al.*, 1993).

3.2.1. Rotenone-insensitive NAD(P)H oxidase activities in different organisms

The respiratory chain of *E. coli*, beyond the energy-transducing NDH-1 (the complex I equivalent in this organism), displays a membrane-bound rotenone-insensitive NADH dehydrogenase (NDH-2) with the catalytic site facing the cytosol. In 1987, Matsushita and others, comparing the deamino-NADH:ubiquinone 1 reductase and the NADH:ubiquinone 1 reductase activities, verified that the former activity showed more sensitivity to piericidin A than the latter. Furthermore, the membranes exhibited two apparent K_ms for NADH, but only one for deamino-NADH. They produced strains, whose inside-out membrane vesicles were deficient in deamino-NADH:ubiquinone 1 reductase activity but could oxidise NADH generating a membrane potential after ubiquinone. In the light of these observations,

they proposed the existence of two species of NADH dehydrogenases. One of these was able to oxidise deamino-NADH and NADH and its turnover lead to the production of a proton gradient at a site between the primary dehydrogenase and ubiquinone. The other enzyme oxidises exclusively NADH and does not generate a proton gradient before ubiquinone. Spiro *et al.* (1989, 1990) suggested that oxygen positively regulates the expression of NDH-2 in *E. coli* but not of NDH1.

In the cyanobacterium *Synechocistys* sp. strain PCC 6803 there are three open reading frames coding for type II NADH dehydrogenases, *ndbA*, *ndbB* and *ndbC*. Their primary structures display sequence identities with other NADH dehydrogenases that do not exceed 30 %. NAD(P)H and FAD binding motifs are conserved in all sequences. The genes have been cloned and deletion mutants produced which lead only to small changes in the respiratory activity. An expression construct of *ndbB* complemented an *E. coli* strain lacking NDH-1 and NDH-2 (Howitt *et al.*, 1999).

The obligate aerobic yeast *Yarrowia lipolytica* has a type II NAD(P)H dehydrogenase on the outer surface of the inner mitochondrial membrane. Deletion mutants of the enzyme were fully viable. Total inhibition of NADH oxidation in mitochondria solubilized with CHAPS was achieved with piericidin A, indicating that complex I activity was the sole NADH oxidation activity left in those strains. The orientation of the alternative NADH dehydrogenase was assessed measuring NADH:5-nonylubiquinone oxidoreductase activity before and after permeabilisation of the inner mitochondrial membrane. In the presence of piericidin A, NADH:5-nonylubiquinone oxidoreductase activity was not affected by permeabilisation, showing that the active site of the enzyme faced the intermembrane space (Kerscher *et al.*, 1999).

The respiratory chain of the facultative aerobic yeast *Saccharomyces cerevisiae* lacks complex I. The oxidation of NAD(P)H from the cytosol and from the matrix is carried out by two external and one internal NAD(P)H dehydrogenases, respectively. de Vries and Grivell (1988) described the purification of a presumptive external NADH dehydrogenase, in mitochondria from *S. cerevisiae*. The protein consisted of a single subunit with molecular mass of 53 kDa that contained a FAD and was insensitive to rotenone and piericidin A. Marres *et al.*, (1991) isolated and disrupted

the nuclear gene coding for one of these NAD(P)H dehydrogenases. A null mutant was constructed and the oxidation of several substrates by mitochondria from wild type and mutant strains was measured. The oxidation of external NADH was not affected in mutant mitochondria, while the oxidation of substrates generating internal NADH was severely decreased (lactate) or missing (pyruvate/malate and ethanol). This study showed that the inactivated enzyme was the internal NADH dehydrogenase. Latter, Small and McAlister-Henn (1998), and Luttik *et al.*, (1998) have identified two other genes coding for mitochondrial NADH dehydrogenases, NDE1 and NDE2, oxidising NADH from the cytosol. Both genes were deleted and the NADH oxidation was followed in mitochondria from wild type and *nde* deletion mutants. Compared to wild type mitochondria, exogenous NADH oxidation was drastically reduced in one of the mutants, albeit the other displayed no difference. However, in mitochondria from the double mutant, oxidation of external NADH was completely absent. In conclusion, the external location of NDE1 and NDE2 was confirmed.

In Neurospora mitochondria, two rotenone-insensitive NAD(P)H dehydrogenases have been reported, one on each side of the inner membrane. Weiss *et al.*, (1970) observed oxygen consumption after addition of rotenone to mitochondria respiring pyruvate/malate. This result indicated the presence of a rotenone-resistant NADH dehydrogenase facing the matrix of Neurospora mitochondria. Weiss *et al.*, (1970) and Møller *et al.*, (1982) showed that Neurospora electron transport chain was able to oxidise external NADH and NADPH in a rotenone-insensitive manner as well.

Rotenone-insensitive NAD(P)H dehydrogenases have also been described in the electron transport chain of plant mitochondria. In plants, several efforts have been done to purify them. There are reports of the purification of a 42-kDa (Luethy *et al.*, 1991), a 26-kDa (Rasmusson *et al.*, 1993) and a 43-kDa (Menz and Day, 1996) NAD(P)H dehydrogenases from red beetroot mitochondria. In red beetroot mitochondria, the purification of a 58-kDa protein was associated to external NAD(P)H oxidation activity (Luethy *et al.*, 1995). The purification of a 32-kDa external NADH dehydrogenase from maize mitochondria has been described (Knudten *et al.*, 1994).

Studies of NADH and NADPH oxidation by intact mitochondria from *Arum maculatum* and potato tubers (Roberts *et al.*, 1995), and by IO-SMP from potato tubers and Jerusalem artichoke (Møller and Palmer, 1982; Rasmusson and Møller, 1991b; Melo *et al.*, 1996) lead to the conclusion that there are four distinct enzymes, two on each side of the inner membrane. Rasmusson *et al.*, (1999) described two different cDNAs, from potato, homologous to genes encoding rotenone-insensitive NADH dehydrogenases in yeast and bacteria. The encoded proteins have approximate molecular masses of 55 and 65 kDa and are located in the inner and in the outer surfaces of the inner mitochondrial membrane, respectively. The latter protein could be homologous to the 58-kDa protein isolated from red beetroot.

3.2.2. Effects of cations on rotenone-insensitive NAD(P)H oxidase activities

In the matrix of mammalian mitochondria, the physiological concentration of free calcium is estimated to be between 0.05 and 5 μM. A few matrix NAD^+-reducing enzymes are stimulated by calcium, namely pyruvate dehydrogenase and the tricarboxylic acid cycle enzymes 2-oxoglutarate dehydrogenase and NAD^+-isocitrate dehydrogenase (McCormack *et al.*, 1990).

Oxidation of matrix NADPH by potato tuber mitochondria is also enhanced by the presence of calcium. The internal NADH oxidation is not really affected by additions of external calcium (Rasmusson and Møller, 1991a, Melo *et al.*, 1996). The presence of a calcium-dependent NADPH dehydrogenase on the matrix side of the inner mitochondrial membrane potato tuber mitochondria, linked to the electron transport chain, opens the possibility of a regulation of the redox potential of NADPH pool, in the matrix, by calcium (Rasmusson and Møller, 1991a).

The stimulation of exogenous NADH oxidation by cations through screening of the negative charges on the surface of the inner membrane was reported in *N. crassa* mitochondria (Møller *et al.*, 1982). Calcium-sensitivity has frequently been associated with these enzymes. Cytosolic NADH oxidation by plant mitochondria is totally dependent on calcium (Coleman and Palmer, 1971; Møller *et al.*, 1981). Edman *et al.*, (1985) made this observation in spinach mitochondria and reported the

same behaviour for external NADPH oxidation. The same authors proposed that cation concentration, in general, and the concentration of calcium, in particular, can regulate exogenous NAD(P)H oxidation. The primary structure of the 65 kDa NAD(P)H dehydrogenase from potato mitochondria (Rasmusson *et al.*, 1999) contains an EF-hand motif, suggesting that this protein might bind calcium. This is in sharp contrast with the primary structure of most alternative NAD(P)H dehydrogenases such as the NDH-2 from *E. coli* (Young *et al.*, 1981), NDI1 (de Vries *et al.*, 1992), NDE1 and NDE2 (McAlister-Henn *et al.*, 1998; Luttik *et al.*, 1998) from *S. cerevisiae*, which all lack calcium-binding domains.

3.2.3. The external NADH dehydrogenase from rat heart mitochondria – a mysterious exception

There are several articles describing the oxidation of exogenous NADH by an external NADH dehydrogenase on the outer surface of the inner membrane of rat heart mitochondria (Nolh and Shönheit, 1996, Oliveira *et al.*, 2000). Nevertheless, this issue is the center of an intense debate, and consensus is far from being achieved.

Studies have been performed regarding the possible physiological role of such an enzyme and its behaviour in different pathological processes. The external NADH dehydrogenase has been associated with pathological conditions related to the production of oxygen free radicals, released during metabolic events under conditions of ischemia/reperfusion. Similar association was found in cardioselective toxicity of adrianmycin, an anticancer drug accepting the electrons from the external NADH dehydrogenase (Nohl, 1998). It was also suggested that this NADH dehydrogenase could be responsible for the oxidation of exogenous NADH when there is an excessive accumulation of that substrate in the cytosol (Nolh and Shönheit, 1996).

Recently, Oliveira *et al.*, (2000) reported a specific inhibitor to the external NADH dehydrogenase, carvedilol, that does not affect the oxygen uptake resulting from complex I activity. In contrast to glutamate/malate or succinate, the oxidation of exogenous NADH oxidation, by rat heart mitochondria, does not generate a

membrane potential. Nonetheless, this activity is inhibited by antimycin A, potassium cyanide, sodium azide and mixothiazol. Surprisingly, exogenous NADH oxidation by rat heart mitochondria is also inhibited by rotenone.

4. Human pathologies associated with NADH oxidation

In humans, complex I is solely responsible for the oxidation of matrix NADH and shuttle mechanisms exist for the oxidation of cytosolic NADH. Mutations in mtDNA genes, for instance complex I genes, can be responsible for drastic phenotypes (Wallace, 1992). An example of a pathology caused by point mutations in mtDNA is the Leber's hereditary optic neuropathy (LHON) (Schapira, 1998). Complex I deficiency and oxidative damage have been identified in the *substantia nigra* (Schapira, 1996) and platelets (Swerdlow *et al.*, 1996; Gu *et al.*, 1998) of patients with Parkinson's disease. Platelets from these patients have been used in genome transplantation experiments with cells lacking mtDNA (ρ^0 cells). This involves fusion of the platelets (mtDNA but no nucleus) from a patient with Parkinson's disease and complex I deficiency with ρ^0 cells (nucleus but no mtDNA). In these experiments, the complex I defect was transferred with the patients mtDNA to the resulting fused cells, indicating that it was due to a defect in the mtDNA (Gu *et al.*, 1998). The defects in mtDNA may be solely responsible for the mitochondrial malfunctioning in Parkinson's disease but it is not known if that malfunctioning is enough to cause the disease, or if other genetic or environmental factors are involved (A. H. V. Schapira (2000) personal communication).

Nuclear gene mutations have also been associated with human pathologies. For instance, a mutation in the nuclear gene coding for the 18-kDa subunit of complex I was identified in a patient with encephalomyopathy (van den Heuvel *et al.*, 1998). Other mutations in nuclear genes from complex I subunits have been described for patients with Leigh's syndrome. These involved two iron-sulphur proteins: the subunits encoded by *NDUFS8* and *NDUFS7* (homologous to the 21.3c-kDa and the 19.3-kDa subunits of the *N. crassa* complex I, respectively), possibly involved in the co-ordination of the iron-sulphur cluster N-2 (Loeffen *et al.*, 1998; Triepels *et al.*,

1999). Three children have been reported with mutations in the gene *NDUFV1* encoding the NADH binding subunit, homologous to the 51-kDa subunit of the *N. crassa* complex I. Two of them developed vomiting, hypotonia, myoclonic epilepsy and psychomotor delay at 5 months and died at 14 and 17 months, respectively. The third child MRI presented with myoclonic epilepsy at 6 months and progressed with psychomotor delay and spasticity. At 12 months psychomotor development stopped and macrocephaly was observed. Still alive at 10 years old, MRI showed macrocytic leukodistrophy. In all cases, the patients were either homozygous or compound heterozygotes for the respective mutations and inheritance was autosomal recessive (Schuelke *et al.*, 1999).

Complex I-defects associated with human pathologies failed to be healed by chemotherapies (Chrazanowska-Lightowlers *et al.*, 1995). The capacity of type II NADH dehydrogenases to carry out the turnover of NADH may be more important to health than their inability to pump protons. Therefore, a possible approach to overcome complex I-defects is to introduce in patient cells a type II NADH dehydrogenase to restore the function of oxidising NADH in their mitochondria (Kitajima-Ihara and Yagi, 1998).

The internal NADH dehydrogenase from *S. cerevisiae* (NDI1) was expressed in *E. coli* (Kitajima-Ihara and Yagi, 1998) and in complex I-deficient chinese hamster cells (Seo *et al.*, 1998), whereby functioned as a member of the respiratory chain in the host cells. NDI1 was able to restore the NADH oxidase activity in the latter case. Human kidney cells were also transfected by the gene encoding NDI1. The transfected enzyme was successfully transcribed and translated to produce a functional enzyme linked to the electron transport chain of the host cell mitochondria (Seo *et al.*, 1999).

5. Final comments

Our understanding of the structure, mechanisms and physiological roles of NAD(P)H:ubiquonone oxidoreductases is far from clear, particularly with respect to the rotenone-insensitive NAD(P)H dehydrogenases. Over the following pages, our

contribution to further characterise the oxidation of NAD(P)H by the respiratory chain of mitochondria from the fungus *N. crassa* is described. This work paid special attention to genetics, biogenesis and physiology of the crucial enzymes responsible for accomplishing this function in Neurospora. Contributing to the development of models where the human mitochondrial disease condition can be simulated and studied, allowing future progress concerning therapeutics, was also the target of our research.

Chapter II – Research Project

1. OBJECTIVES

Although much is already known about the NADH:ubiquinone oxidoreductase (complex I) from Neurospora mitochondria, a full understanding of the structure and function of several subunits of the enzyme has not been accomplished yet. In Neurospora mitochondria, the presence of at least two rotenone-insensitive non-proton-pumping NAD(P)H dehydrogenases has been reported. Nevertheless, the genes encoding these proteins have not been identified and their structure was unknown when this project started.

The overall purpose of the present work was to provide a better understanding of the bioenergetical processes occurring in the mitochondrion, by improving our knowledge of the NAD(P)H dehydrogenases of the *Neurospora crassa* organelle.

This project aimed to detect and characterise the different NAD(P)H dehydrogenase activities in mitochondria from the wild type strain and from strains deficient in complex I subunits. The isolation and sequencing of genes encoding rotenone-insensitive NAD(P)H dehydrogenases was another goal. Envisaging a detailed characterisation of the rotenone-insensitive NAD(P)H dehydrogenases, a special attention was given to the induction and characterisation of mutants and also of double mutants deficient in both the alternative NAD(P)H dehydrogenases and in complex I subunits. The characterisation of the alternative NAD(P)H dehydrogenases and the production of mutants deficient in these enzymes can provide new models for the study of complex I and associated pathologies in Neurospora mitochondria.

2. RESULTS AND DISCUSSION

2.1. Characterisation of matrix NADH oxidation activities

In *N. crassa* mitochondria, two types of matrix activities oxidising NAD(P)H have been described, a rotenone-sensitive NAD(P)H:ubiquinone oxidoreductase, or complex I, and a rotenone-insensitive NADH dehydrogenase (General Introduction). Percoll-purified mitochondria and IO-SMP from wild type and *N. crassa* strains lacking the complex I subunits of 24 kDa (nuo24) and 21 kDa (nuo21) (Almeida *et al.*, 1999; Ferreirinha *et al.*, 1999) were prepared. The oxidation of pyruvate/malate and NADH was followed in mitochondria and IO-SMP, respectively, to establish the consequences of inactivation of the 24-kDa and the 21-kDa subunits of complex I on its rotenone-sensitive NADH oxidation activity (Fig.1).

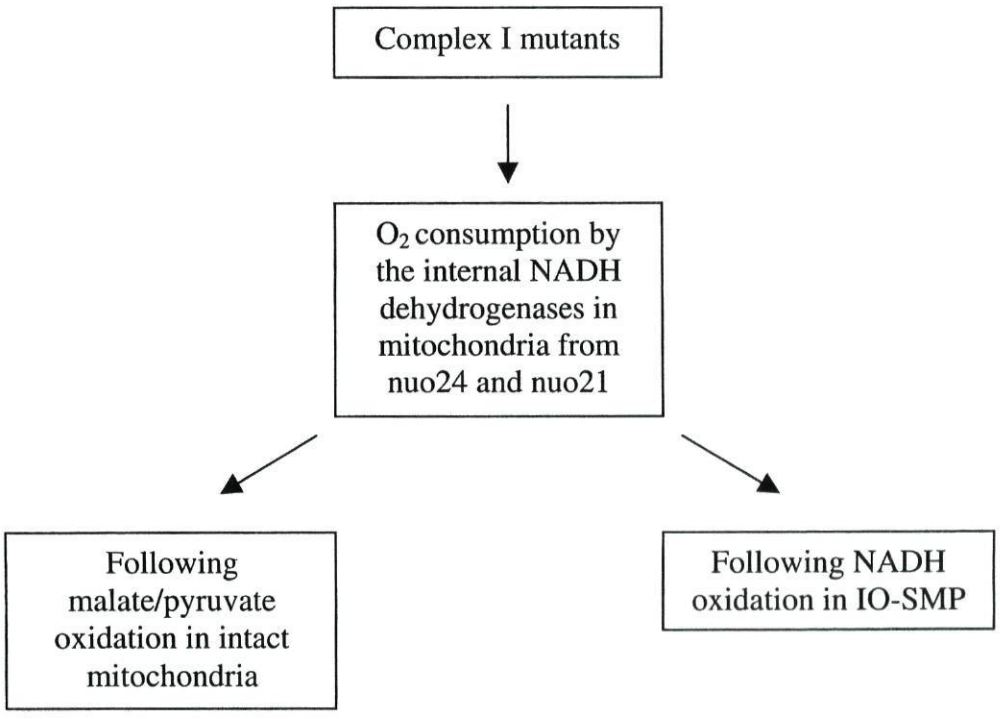

Figure 1. **Strategy used to characterise internal NAD(P)H oxidation activities.**

2.1.1. Characterisation of internal NADH oxidation by nuo24

nuo24 mitochondria assembles an almost intact complex I, lacking only the 24-kDa subunit and presenting severely diminished amounts of the 51-kDa subunit, the subunit containing the NADH binding-site (Walker, 1992). This complex lacks NADH:ferricyanide or NADH:hexaammineruthenium oxidoreductase activity (Almeida *et al.*, 1999). However, it was necessary to verify if the electrons could still be accepted from NADH by the nuo24 complex, used to reduce other iron-sulphur centers of the enzyme and transferred to ubiquinone, proceeding through the rest of the electron transport chain to oxygen.

The oxygen uptake by wild type mitochondria respiring on pyruvate/malate was inhibited by 39% by the complex I inhibitor rotenone (Fig. 2, see also Table 1). In agreement with this, 47% of the oxygen consumption by wild type IO-SMP oxidising directly NADH was inhibited by rotenone. In contrast, when the same experiments were performed using mitochondria and IO-SMP prepared from nuo24, the respiration of the pyruvate/malate and NADH could not be stopped by rotenone (Fig. 2 and Table 2). The oxygen consumption in both strains was completely inhibited by antimycin A. These results indicate that nuo24 complex I cannot transfer electrons from NADH to ubiquinone and that, in the mutant, the oxidation of NADH is performed by an internal alternative NADH dehydrogenase (Weiss *et al.*, 1970) (Table 1). This observation suggests that these enzymes are overexpressed in the mutant, compensating for the complex I failure.

An important finding from the characterisation of this mutant is that absence of the 24-kDa protein compromises the electron-transfer function of complex I and might lead to a reduction in the total energy production by the respiratory chain.

It has been suggested that the reduction of energy production in mitochondria below threshold levels is implicated in human mitochondrial disorders (Wallace, 1992). A decrease in the relative amounts or even undetectable levels of the 24-kDa polypeptide was associated with some of these mitochondrial pathologies (Schapira *et al.*, 1988, Mizuno *et al.*, 1989). Recently, an increased susceptibility to Parkinson's disease was found to be associated with mutations in the mitochondrial sequence of the human homologue to the Neurospora 24-kDa polypeptide,

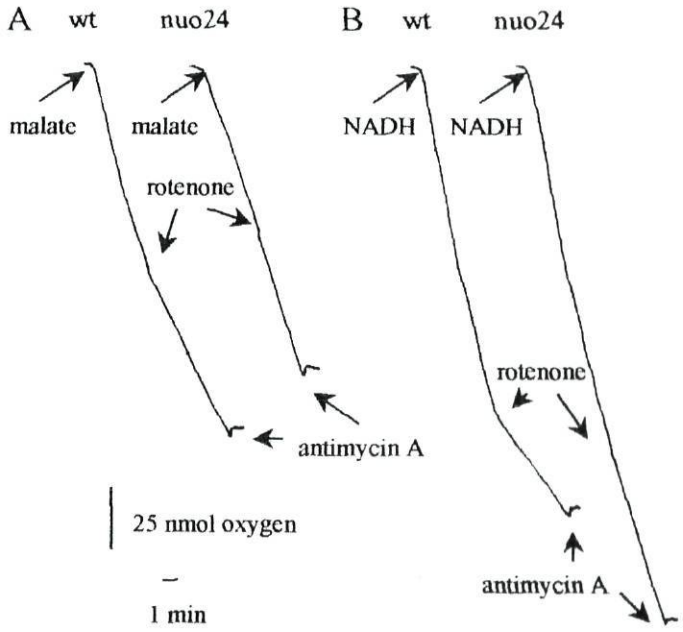

Figure 2. **nuo24 complex I does not carry out NADH oxidation coupled to oxygen consumption.** Polarographic traces of respiration performed by mitochondria (A) or IO-SMP (B), from Neurospora strains wild type (wt) and nuo24, are depicted in the figure. The start of the reactions with either malate or NADH and the addition of respiratory inhibitors are indicated. Purified mitochondria from wild type and nuo24 displayed 95% latent activity for cytochrome *c* oxidase and 88% and 90% for malate dehydrogenase, respectively. The IO-SMP presented 89% and 86% cytochrome *c* oxidase latent activity and 17% and 12% malate dehydrogenase latent activity for wild type and nuo24, respectively. Reproduced from Almeida *et al.* (1999) with permission of the Blackwell Science Ltd.

presumably affecting the levels of the mature protein inside the mitochondria (Hattori *et al.*, 1998). Nevertheless, no mutation affecting the primary structure of the mature protein has been reported. The phenotype of nuo24 can provide an explanation for these findings. When the amounts of the protein are reduced, the contribution of complex I to energy transduction decreases, possibly due to an improper assembly of the 51-kDa protein, that may be responsible for the symptoms of the disease. The nuo24 mutant provides a useful eukaryotic model for further research on the role of the 24-kDa subunit of complex I.

Table 1. **Measurements of oxygen consumption by mitochondria and IO-SMP.** The activities are expressed in nmol O_2/min x mg protein. Values in parenthesis indicate percentage inhibition. The data are an average of at least three independent experiments. Reproduced from Almeida *et al.* (1999) with permission of the Blackwell Science Ltd.

Preparation	Wild type	nuo24
Mitochondria		
+ Malate	49.6	69.2
+ Rotenone	30.4	69.2
	(39)	(0)
+ Antimycin A	0	0
	(100)	(100)
SMP		
+ NADH	51.7	43.4
+ Rotenone	27.5	42.7
	(47)	(1.6)
+ Antimycin A	0	0
	(100)	(100)

2.1.2. Characterisation of internal NADH oxidation by nuo21

This mutant nuo21 assembles an almost intact complex I lacking uniquely the 21-kDa polypeptide (Ferreirinha *et al.*, 1999) but, in contrast to nuo24, its complex I is able to carry out the transfer of electrons from NADH to ubiquinone. The same strategy used to characterise nuo24 was employed in the study of oxygen consumption by mitochondria and IO-SMP from the mutant nuo21 respiring on pyruvate/malate or NADH, respectively.

Intact mitochondria respiring on malate/pyruvate displayed 51% and 39% inhibition by rotenone in wild type and nuo21, respectively (Fig. 3A). IO-SMP from the same strains oxidising NADH were 41% and 42% inhibited by rotenone, respectively (Fig. 3B). These observations suggest that there are no significant

differences between the complex I of the wild type and the nuo21 strains. In addition, the specific activities for the oxidation of both substrates were similar in mitochondria and IO-SMP from wild type and nuo21 (Table 2). An internal rotenone-insensitive NADH dehydrogenase is probably responsible for the remaining oxygen consumption after addition of rotenone (Weiss *et al.*, 1970). The latter activity was fully inhibited by antimycin A, thus confirming that the electrons were transferred to oxygen via the *bc*1 complex (complex III).

The lack of phenotype of the nuo21 complex I, considering its NADH:oxidoreductase activity, was surprising since a disease involving the human homologue of the 21-kDa subunit of complex I, the AQDQ protein, was reported. It was the first identified mutation in a nuclear-coded subunit of complex I (van den Heuvel *et al.*, 1998). The patient presented a multisystemic disorder with a fatal progressive phenotype, owing to a pathological duplication of five base pairs in the gene that altered the C-terminal region and abolished the putative phosphorylation site of the protein (Papa *et al.*, 1996; van den Heuvel *et al.*, 1998). Scacco *et al.* (2000) reported that treatment of serum-starved mouse fibroblasts with cholera toxin, which induces elevated levels of cAMP, promoted serine-phosphorylation of the AQDQ subunit of complex I. A 2- to 3- fold enhancement of the rotenone-sensitive endogenous respiration of fibroblasts, the rotenone-sensitive NADH oxidase and the NADH:ubiquinone activities of complex I was observed upon the serine-phosphorylation of the AQDQ polypeptide. Direct exposure of fibroblasts to dibutyryl cAMP, which also promotes serine-phosphorylation of the 18-kDa subunit, resulted in a similar stimulation of complex I activity. Preliminary results obtained using *N. crassa* mitochondria suggested that the 21-kDa subunit of complex I is phosphorylated in this organism as well, however, no changes in the malate/pyruvate oxidation rates in the absence or presence of different cAMP concentrations were observed (Duarte and Melo, preliminary results).

The fact that the patient carrying the five base pair-mutation in the AQDQ protein was homozygous for the mutation and originated from two heterozygous parents (van den Heuvel *et al.*, 1998) suggests that a "loss of function" phenotype is involved. Thus, the nuo21 mutant of *N. crassa*, where the protein was absent, should simulate the human situation.

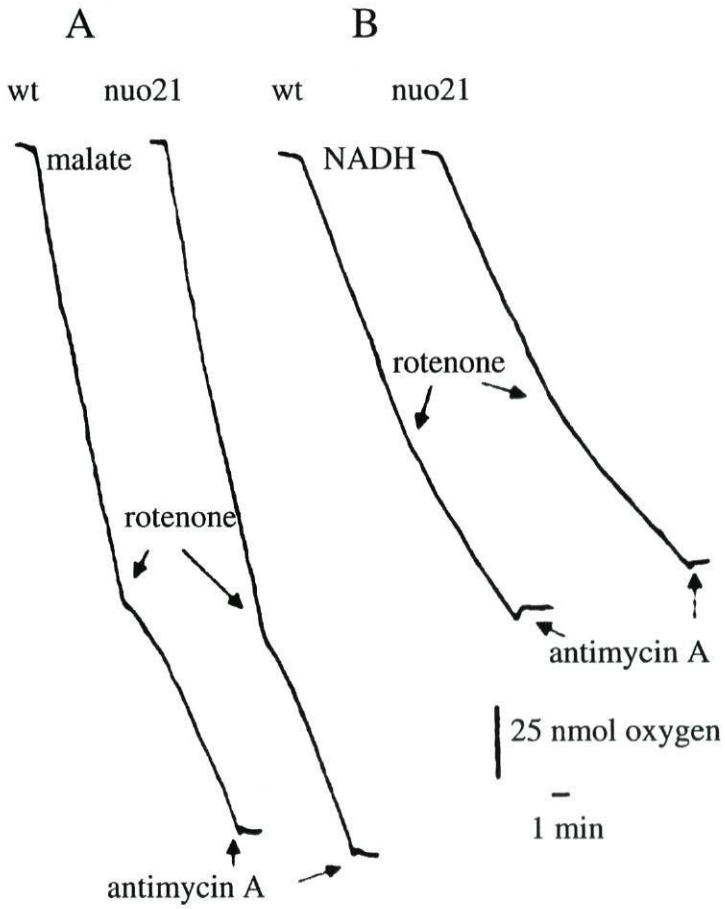

Figure 3. **NADH oxidation by nuo21 mitochondria is inhibited by rotenone.** The polarographic traces were obtained from wild type and nuo21 mitochondria (A) or IO-SMP (B). The assays started by malate or NADH and addition of inhibitors is indicated. Reproduced from Ferreirinha *et al.* (1999) with permission of the Biochemical Society.

One possibility to explain why mutations in the AQDQ protein lead to a severe phenotype in humans, is that it may be involved in the regulation of NADH:ubiquinone activity by complex I, through a cAMP-regulated phosphorylation/dephosphorylation mechanism (S. Papa, oral communication, 2000).

Another explanation is that the mutation affects an unknown function of complex I not directly related to the bioenergetic activity of the enzyme. Maybe complex I of nuo21 does not pump protons so efficiently. Further experiments are

required to clarify this issue of great relevance to human health, and nuo21 appears to be a very useful tool to proceed research in this field.

Table 2. **Measurements of oxygen consumption by mitochondria and IO-SMP.** The activities are expressed in nmol O_2/min x mg protein. The data represent a typical experiment where the mycelia grew for 19 h. The values in parenthesis indicate percentage of inhibition.

Preparation	Wild type	nuo21
Mitochondria		
+ Malate	62.2	59.0
+ Rotenone	30.4	36.2
	(51)	(39)
+ Antimycin A	0	0
	(100)	(100)
SMP		
+ NADH	49.9	40.4
+ Rotenone	29.4	23.2
	(41)	(42)
+ Antimycin A	0	0
	(100)	(100)

2.2. Identification, mapping and inactivation of the gene encoding a putative rotenone-insensitive NAD(P)H dehydrogenase

A partially sequenced cDNA cloned in the pBluescript SK+ vector, NM1C2, displaying homology with the rotenone-insensitive NAD(P)H dehydrogenases from other organisms, was obtained from the Fungal Genetics Stock Center. It was isolated from a mycelial library of *N. crassa* (Nelson *et al.*, 1997). The strategies employed to characterise and inactivate *nde1* gene are outlined in figure 4.

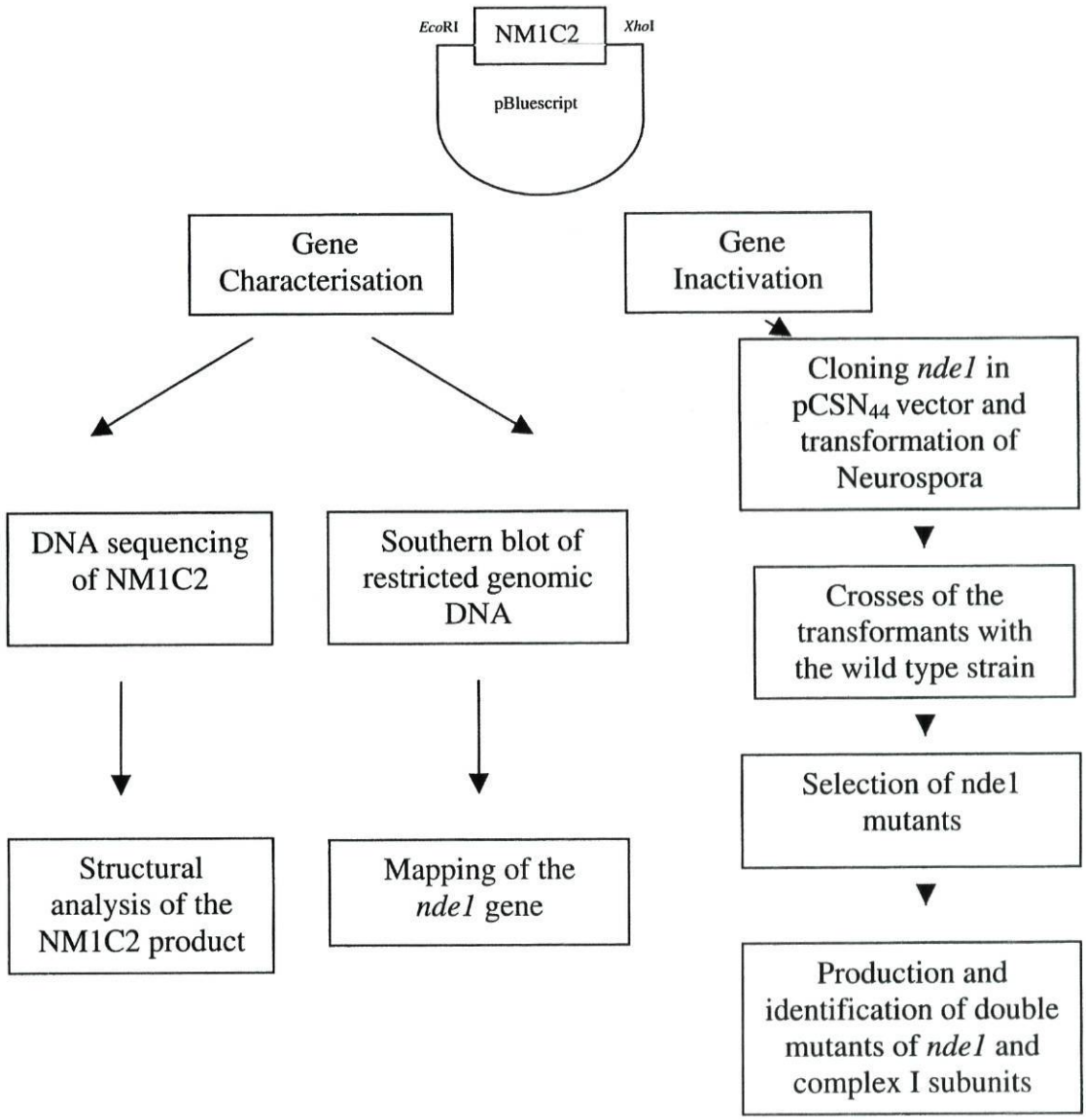

Figure 4. **Strategies used to characterise and disrupt the putative rotenone-insensitive NAD(P)H dehydrogenase**, starting from the clone NM1C2.

2.2.1. Gene characterisation

2.2.1.1. Structural analysis of the NM1C2 product

The two strands of NM1C2 cDNA were completely sequenced using pBluescript primers or specific primers in the vector carrying the intact cDNA. Both kinds of primers were also used to sequence vectors resulting from digestion and re-ligation of the original cDNA. The gene has 2570 bp and contains an open reading frame of 2019 bp. Several in frame stop codons are found upstream of the presumed first ATG codon indicating that, in fact, it represents the initiation codon (Fig. 5). The gene encodes a polypeptide chain of 673 amino acids (Fig. 6) with a molecular mass of 75891 Da. The mature form of the protein (NDE1) displayed an apparent molecular mass of 64 kDa as estimated by SDS-Page followed by Western blot analysis with antibodies against the 64-kDa-polypeptide. For the production of antibodies, a pQE31 vector (Qiagen) containing an insert encoding the 579 C-terminal amino acid residues of the *N. crassa* NAD(P)H dehydrogenase was used to transform *E. coli*. The transformed bacteria were induced to express that region of the Neurospora protein. The protein was purified and used to immunise rabbits.

```
                                                                  GC     1
CGA GTC TTC TTC CTT TAC TGG CTG CAC TAA CAT TAA ACA CTG CAC TGA CAC CGC    56
AAC ACC ACC TAC CTA ACA CCA AGC GCC CGG ACC TTA TCA ACA CTG CCT TGG CTA   110
CCT CTA GGT AGG TAT TGA CGT CAT ACC AAA GCA GCA CAG CAG CAG CAG CAG CAG   164
CAA AAA GTA AAC AAC AGC AGA CAA CCA CAG CAA CAC ACC GCA GCC AAC GCC CAT   218
ACA TAT TGT GAA CAA ACA ATG CTG CGC ACA TAT CGA GTT GCG CGG GCT TCT GGC   272
CTT GCT ACA GCT CCT CGA ACT TTG ACT TTA ACC TCG ACG ACA GCA ACG CGA CAC   326
TTG TTC ACC CTA CCA AAG AGT CTA CAA TTA CGC CGC CCT GAG AAG CTC TCC CTC   380
ATC AGC CAA CGA CAA TTG TCG GGC AGA CCA CTC CCG CGA ACC CAG TCG CGA CTC   434
CTC AAC TTT GGC TAC CGC ACC GCT GCC TGG TTT GGC AGC TCC ATC GCA TTT GTC   488
GGC CTC AGC TTC GTC GCC TTC TTC CTC TAC GAT GCC TCG ACC TAC AGC TCC CAC   542
GCG ACA AAT CAG GGC GAC ATC ACT GTC CCC AAG CTC GCC CTA AAC CCT CGT CGC   596
GGC GGT CCC AAG AAC CTA CCC ATC CTC GAG ATC TTC CTC GAC GAC GAC GAC AGC   650
GAA GAG AAA AAG AAA CAC AAG GAG AAA CCC AGG CTG GTC ATC TTG GGT GGT GGC   704
TGG GGC AGT GTG GCT CTG CTC AAG GAG CTC AAC CCG GAT GAC TAC CAT GTC ACC   758
GTT GTC TCC CCG GCC AAC TAC TTC CTC TTC ACG CCC ATG CTG CCC TCG GCC ACC   812
GTC GGA ACC CTC GAG CTC AAT TCC TTG GTA GAG CCC ATC CGC AAC ATC ATC GAC   866
CGA GTC AAG GGC CAC TAT ATC CGG GCC GCC GCC GAA GAT GTC GAC TTC TCC TCC   920
CGC CTG GTC GAG GTA TCT CAA AAG GAC CCC CGT GGC AAC GAA GTG CGC TTC TAC   974
GTT CCC TAC GAC AAG CTC GTC ATT GCC GTC GGC TCC ACC ACC AAC CCT CAC GGC  1028
GTC AAG GGT CTG GAG AAC TGC CAC TTC CTC AAA GAC ATC AAC GAC GCC CGC CAG  1082
ATC CGC AAC AAG ATC ATC CAA AAC CTC GAG CTC TCC TGC CTG CCC ACC ACC TCG  1136
GAC GAG GAG CGC AAG CGT CTG TTG TCC TTT GTC GTC TGC GGC GGC GGG CCC ACC  1190
GGT GTC GAG TTC GCC GCC GAG CTC TTT GAC CTT CTC AAC GAG GAC CTG ACC CTC  1244
CAC TTC CCG CGT CTC CTG CGC AAC GAG ATC TCC GTC CAC CTC ATC CAG TCC CGC  1298
GAC CAC ATC CTC AAC ACG TAC GAT GAA GCT GTC TCC AAG TAC GCC GAA GAC CGC  1352
TTC TCG CGC GAC CAA GTC GAC GTG CTC GTC AAC TCC CGC GTA GCC GAA GTC CGC  1406
CCC GAG TCC ATC CTC TTC ACA CAG CGG GGC CCC GAC GGC AAG ACC ACC GTG ACC  1460
AAG GAG TGC CCC ATG GGT TTC TGC CTC TGG TCA ACA GGC GTC TCG CAA GCC GAG  1514
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TTC TGC AAG CGC ATC TCG CGC CAA CTC GGT CCC GCC CAA ACG AAC CGA CAC GCC  1568
CTC GAG ACC GAC ACG CAC CTG CGT CTC AAC GGC ACA CCC TTG GGC GAC GTG TAC  1622
GCC ATT GGC GAC TGC TCC ACA ATC CAG AAC AAC GTC GCC GAC CAC ATC ATC ACC  1676
TTC CTC CGC AAC CTC GCC TGG AAG CAC GGC AAG GAC CCG GAA AGC CTC GAG CTG  1730
CAC TTC TCC GAC TGG CGT GAT GTC GCG CAG CAG ATC AAG AAG CGC TTC CCG CAA  1784
GCC ACG GCC CAC TTG AAG CGA CTC GAC AAG CTG TTT GAA GAA TAC GAC AAG GAC  1838
CAG AAC GGC ACC CTC GAC TTT GGC GAG CTG CGC GAG CTG CTG AAG CAG ATC GAC  1892
AGC AAG CTC ACC TCC CTC CCT GCC ACC GCG CAG CGC GCC CAC CAG CAA GGA CAG  1946
TAC CTA GCG CAC AAG TTC AAC AAG CTT GCG CGC GCG GCG CCG GGG CTG TCG GCG  2000
AAT GAG ATT CAC GAG GGC GAT CTG GAT GCG GCT GTG TAC AAG GCG TTT GAG TAC  2054
CGG CAT TTG GGC AGT TTG GCG TAT ATT GGC AAC AGC GCC GTG TTT GAT TGG GGT  2108
GAC GGC TGG AAC TTT TCC GGA GGG CTG TGG GCC GTG TAT GCA TGG AGG AGC ATT  2162
TAC TTT ACG CAG TGC GTG AGT TTG CGG ACG AGG GTT CTC ATG GCC ATT GAC TGG  2216
GGC AAG AGA GCG TTG TTT GGA CGC GAT TTG ATG AGA TAC TAA AGC CGA CTA CTA  2270
CTG ACT CTT TTG CTG TTT TCG GCA CAA TAA GAA AAG CAA GGA CGG TTG AGC TAA  2324
GCG GGA AAG CGA GCG AGC GGA CGA GCA ACT TTC GGC GGT TGT TAT TAT TTA TCG  2378
GGG GCT TTA CCT ACA TAC AAA CCT TCA TCT CAC CTC ACT TAT CTT ACC TCT TCA  2432
TGA AGT CAT CAT CCT TTT TTT GAT TAT TCA TTG CGG TTC GGT TTT CTA GAC GTG  2486
GGA ATT GGT TGA GAG GCA GTC GTT GTT ACC AAA AGC AAG TTC TAA ACT ACA TAG  2540
ATT TTC AGA TAA TTA AGG TCC TTG CCA CTT                                  2570
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Figure 5. **Open reading frame of the *nde1* cDNA from *N. crassa*.** The stop codons upstream the initiation codon are depicted in bold. Initiation and stop codons are shadowed.

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M L R T Y R V A R A S G L A T A P R T L T L T S T T A T R H L F T L P K S L Q L  40
R R P E K L S L I S Q R Q L S G R P L P R T Q S R L L N F G Y R T A A W F G S S  80
I A F V G L S F V A F F L Y D A S T Y S S H A T N Q G D I T V P K L A L N P R R 120
G G P K N L P I L E I F L D D D D S E E K K K H K E K P R L V I L G G G W G S V 160
A L L K E L N P D D Y H V T V V S P A N Y F L F T P M L P S A T V G T L E L N S 200
L V E P I R N I I D R V K G H Y I R A A A E D V D F S S R L V E V S Q K D P R G 240
N E V R F Y V P Y D K L V I A V G S T T N P H G V K G L E N C H F L K D I N D A 280
R Q I R N K I I Q N L E L S C L P T T S D E E R K R L L S F V V C G G G P T G V 320
E F A A E L F D L L N E D L T L H F P R L L R N E I S V H L I Q S R D H I L N T 360
Y D E A V S K Y A E D R F S R D Q V D V L V N S R V A E V R P E S I L F T Q R G 400
P D G K T T V T K E C P M G F C L W S T G V S Q A E F C K R I S R Q L G P A Q T 440
N R H A L E T D T H L R L N G T P L G D V Y A I G D C S T I Q N N V A D H I I T 480
F L R N L A W K H G K D P E S L E L H F S D W R D V A Q Q I K K R F P Q A T A H 520
L K R L D K L F E E Y D K D Q N G T L D F G E L R E L L K Q I D S K L T S L P A 560
T A Q R A H Q Q G Q Y L A H K F N K L A R A A P G L S A N E I H E G D L D A A V 600
Y K A F E Y R H L G S L A Y I G N S A V F D W G D G W N F S G G L W A V Y A W R 640
S I Y F T Q C V S L R T R V L M A I D W G K R A L F G R D L M R Y *             673
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Figure 6. **Primary structure of the NDE1 protein from *N. crassa*.** The amino acid residues representing the putative mitochondrial targeting pre-sequence are shadowed. The first amino acid residue (nº 95) of the fragment used to obtain the antibodies is underlined. The amino acid residues integrating the most likely transmembrane domain are depicted in bold.

The N-terminus of the deduced polypeptide sequence displays 74 amino acid residues with the characteristics of a mitochondrial targeting pre-sequence (Fig. 6, shadowed). One to five transmembrane domains are predicted in this protein, by different computer analysis (TMpred, ISREC, Switzerland; Sosui, Tokyo). Two of these coincide with the ADP-binding domains therefore should be excluded. The other possibilities are from residues 73-94, 173-196 and 620-639. The domain located closer to the N-terminus (from residues 73 to 94) shows higher probability of existing than the others, as can be observed in the hydrophobic profile of the NDE1

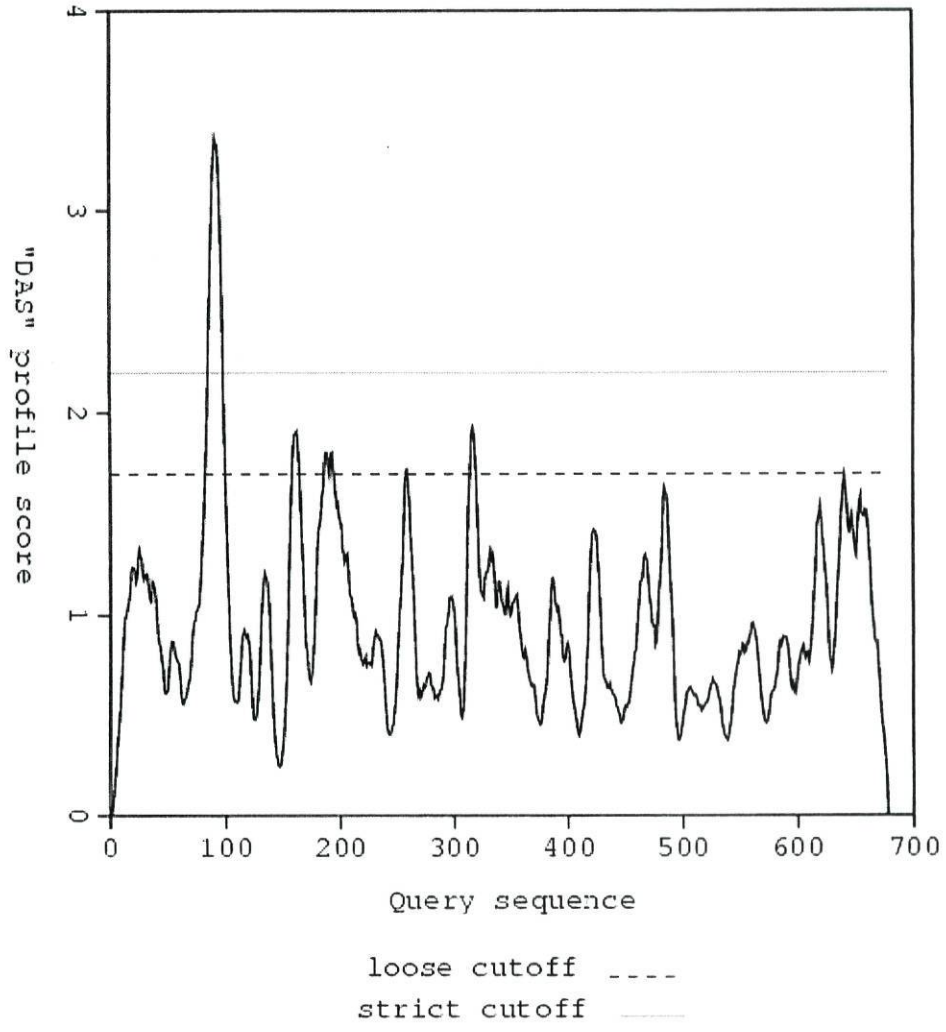

Figure 7. **Transmembrane domains prediction for NDE1.** "DAS" prediction of transmembrane segments.

protein (Fig. 7). Both the putative mitochondrial location and the membrane spanning domains of NDE1 require experimental evidence.

The primary structure of NDE1 is homologous to other prokaryotic and eukaryotic NAD(P)H dehydrogenases. Figure 8 depicts the alignment of the primary structure of NDE1 from *N. crassa* with those of NDB1 and NDB2 from potato, NDH2 from Yarrowia, NDI1, NDE1 and NDE2 from *S. cerevisiae* and NDH2 from *E. coli*. These proteins display around 35% identity and two very well conserved motifs for binding the ADP region of NAD(P)H or FAD (Walker, 1992). Among other conserved amino acid residues in the surroundings, three invariant glycine residues separated by one or two amino acids are observed within regions potentially forming the beta-alpha-beta structures involved in the binding of FAD and NAD molecules (Wierenga *et al.*, 1986). Compared to the other sequences, the *N. crassa* polypeptide is the largest and has an internal extension of 80-90 amino acid residues that presents a perfectly conserved consensus sequence for the binding of calcium (Kretsinger, 1992). This motif is also present in the sequence of the NDB1 protein from potato but absent in the other NAD(P)H dehydrogenases. The presence of this domain suggests that NDE1 and NDB1 proteins might be regulated by calcium (see below). Calcium-dependent NAD(P)H oxidising activities have been reported in plant mitochondria (Møller *et al.*, 1993).

Phylogenetic analysis of the eight alternative NAD(P)H dehydrogenases (Fig. 9) revealed the *E. coli* homologue protein apart from the eukaryotic proteins. It also separated the rotenone-insensitive NAD(P)H dehydrogenases from unicellular organisms like *Y. lipolytica* and *S. cerevisiae* in a distinct branch of the proteins from the multicellular organisms potato and Neurospora. The external NAD(P)H dehydrogenases from *S. cerevisiae* (SCNDE1, SCNDE2) constitute a sub-group of

```
1 NCNDE1 ----MLRTYRVARAS GLATAPRTLTLTSTT ATRHLFTLPKSLQLR     41
2 STNDB1 -----MRGFTYLSKV LHSHSSYSKLLVLCS VSTGGLLVYAESNVE     40
3 STNDB2 ----MPWFKNLIKIS KTITNQSSSYKSITP LASPLLTQFLQFTK-     40
4 YLNDH2 -MLRLRPAVRAVSVA RSVALTRSLHVSVAK FNKIEGTAPAGLPKE     44
5 SCNDE1 MIRQSLMKTVWANSS RFSLQSKSGLVKYAK NRSFHAARNLLEDKK     45
6 SCNDE2 ----MLPRLGFARTA RSIHRFKMTQISKPF FHSTEVGKPGPQQK-     40
7 SCNDI1 ----MLSKNLYSN-- KRLLTSTNTLVRFAS TRSTGVENSGAGPT-     38
8 ECNDH2 -------------- -------------- --------------      0
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1 NCNDE1 RPEKLSLISQRQLSG RPLPRTQSRLLNFGY RTAAWFGSSIAFVGL      86
2 STNDB1 --------------- --------------- -S-------------      41
3 STNDB2 --------------- --------------- ---------------      40
4 YLNDH2 VKQTAGHQGHHQEIP KPDENHPRRKKFHFW RSLWRLTYLSAIASL      89
5 SCNDE1 VILQK-------VAP TTGVVAKQSFFKRTG KFTLKALLYSALAGT      83
6 SCNDE2 --------------- LS--KSYTAVFKKWF VRGLKLTFYTTLAGT      68
7 SCNDI1 --------------- --------------- ---------------      38
8 ECNDH2 --------------- --------------- ---------------       0

1 NCNDE1 SFVAFFLYDASTYSS HATNQGDITVPKLAL NPRRGGPKNLPILEI     131
2 STNDB1 ---GKQVVEQNQP-- --------------- ---------------      51
3 STNDB2 ---QYSTNDHVVG-- --------------- ---------------      50
4 YLNDH2 GYIGYRIYVIRNPSD Q-------------- ---------------     105
5 SCNDE1 AYVSYSLYREANPST QVPQ----------- ---------------     102
6 SCNDE2 LYVSYELYKESNPPK QVPQ----------- ---------------      87
7 SCNDI1 ---SFKTMKVIDP-- --------------- ---------------      48
8 ECNDH2 --------------- --------------- ---------------       0

1 NCNDE1 FLDDDDSEEKKKHKE KPRLVILGGGWGSVA LLKELNPDD-----Y     171
2 STNDB1 -----------ES-K KKRVVVLGTGWGGTS FLKDVDISS-----Y      79
3 STNDB2 -------LEATKSDQ KPRIVVLGSGWAGCR LMKDIDTNI-----Y      83
4 YLNDH2 ---------LPADPS KKTLVVLGSGWGSVS FLKKLDTSN-----Y     136
5 SCNDE1 ------SDTFPNGSK RKTLVILGSGWGSVS LLKNLDTTL-----Y     136
6 SCNDE2 ------STAFANGLK KKELVILGTGWGAIS LLKKLDTSL-----Y     121
7 SCNDI1 ----------QHS-D KPNVLILGSGWGAIS FLKHIDTKK-----Y      77
8 ECNDH2 -----------MTTP LKKIVIVGGGAGGLE MATQLGHKLGRKKKA      34

1 NCNDE1 HVTVVSPANYFLFTP MLPSATVGTLELNSL VEPIRNIIDRVKG--     214
2 STNDB1 DVQVVSPRNYFAFTP LLPSVTCGTVEARSI VEPVRNIIKKRSGE-     123
3 STNDB2 DVVCVSPRNHMVFTP LLASTCVGTLEFRSV AEPIGRIQPAVSTQP     128
4 YLNDH2 NVIVVSPRNYFLFTP LLPSCPTGTIEHRSI MEPIRGIIRHKQAE-     180
5 SCNDE1 NVVVVSPRNYFLFTP LLPSTPVGTIELKSI VEPVRTIARRSHGE-     180
6 SCNDE2 NVTVVSPRSFFLFTP LLPSTPVGTIEMKSI VEPVRSIARRTPGE-     165
7 SCNDI1 NVSIISPRSYFLFTP LLPSAPVGTVDEKSI IEPIVNFALKKKGN-     121
8 ECNDH2 KITLVDRNHSHLWKP LLHEVATGSLDEG-V DALSYLAHARNHG--      76

1 NCNDE1 --HYIRAAAEDVDFS SRLVEVSQKDPRG-- ----------NEVRF     245
2 STNDB1 -IQFWEAECLKIDPV NRTVSCRSGINDN-- --------LAGHNDF     157
3 STNDB2 ASYFFLANCNAIDFD NHMIECETVTEG--- ------VETLEAWKF     164
4 YLNDH2 -CQYLEADATKIDHE KRIVTIRSAVSENS- ---------KEEVIK     214
5 SCNDE1 -VHYYEAEAYDVDPE NKTIKVKSSAKN--- ----------NDYDL     211
6 SCNDE2 -VHYIEAEALDVDPK AKKVMVQSVSED--- ----------EYFVS     196
7 SCNDI1 -VTYYEAEATSINPD RNTVTIKSLSAVSQL YQPENHLGLHQAEPA     165
8 ECNDH2 -FQFQLGSVIDIDRE AKTITIAELRDEKG- --------ELLVPER     111

1 NCNDE1 YVPYDKLVIAVGSTT NPHGVKGLEN-CHFL KDINDARQIRNKIIQ     289
2 STNDB1 SLQYDYLVVAVGAQV NTFNTPGVMEHCHFL KEVEDAQRIRRTVID     202
3 STNDB2 NVSYDKLVIASGAHA LTFGIKGVNEHATFL REVHHAQEIRRKLLL     209
4 YLNDH2 EIPFDYLVVGVGAMS STFGIPGVQENACFL KEIPDAQQIRRTLMD     259
5 SCNDE1 DLKYDYLVVGVGAQP NTFGTPGVYEYSSFL KEISDAQEIRLKIMS     256
6 SCNDE2 SLSYDYLVVSVGAKT TTFNIPGVYGNANFL KEIEDAQNIRMKLMK     241
7 SCNDI1 EIKYDYLISAVGAEP NTFGIPGVTDYGHFL KEIPNSLEIRRTFAA     210
8 ECNDH2 KIAYDTLVMALGSTS NDFNTPGVKENCIFL DNPHQARRFHQEMLN     156
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1 NCNDE1 NLELSCLPTTSDEER KRLLSFVVCGGGPTG VEFAAELFDLLNEDL   334
2 STNDB1 CFEKSVIPGLSEEER RTNLHFVIVGGGPTG VEFAAELHDYVYEDL   247
3 STNDB2 NLMLSDVPGVSEEEK RRLLHCVVVGGGPTG VEFSGELSDFILKDV   254
4 YLNDH2 CIEKAQFE-KDPEVR KRLLHTVVVGGGPTG VEFAAELQDFFEDDL   303
5 SCNDE1 SIEKAASLSPKDPER ARLLSFVVVGGGPTG VEFAAELRDYVDQDL   301
6 SCNDE2 TIEQASSFPVNDPER KRLLTFVVVGGGPTG VEFAAELQDYINQDL   286
7 SCNDI1 NLEKANLLPKGDPER RRLLSIVVVGGGPTG VEAAGELQDYVHQDL   255
8 ECNDH2 LFLKYSAN----LGA NGKVNIAIVGGGATG VELSAELHNAVKQLH   197

1 NCNDE1 TLHFPRLLRNEISVH LIQSRDHILNTYDEA VSKYAEDRFSRDQVD   379
2 STNDB1 VKIYP-SVKDFVKIT VIQSGDHILNTFDER ISSFAEQKFQRDGIE   291
3 STNDB2 HQRYA-HVKDYIHVT LIEAN-EILSSFDDR LRVYATNQLTKSGVR   298
4 YLNDH2 RKWIP-DIRDDFKVT LVEALPNVLPSFSKK LIDYTEKTFSDEKIS   347
5 SCNDE1 RKWMP-ELSKEIKVT LVEALPNILNMFDKY LVDYAQDLFKEEKID   345
6 SCNDE2 RKWMP-DLSKEMKVI LIEALPNILNMFDKT LIKYAEDLFARDEID   330
7 SCNDI1 RKFLP-ALAEEVQIH LVEALPIVLNMFEKK LSSYAQSHLENTSIK   299
8 ECNDH2 SYGYKGLTNEALNVT LVEAGERILPALPPR ISAAAHNELTKLGVR   242

1 NCNDE1 VLVNSRVAEVRPESI LFTQRGPDGKTTVTK ECPMGFCLWSTGVSQ   424
2 STNDB1 VSTGCRVTSVSDHFI NMKVKSTG----KHV EVPYGMVVWSTGVGT   332
3 STNDB2 LVRGLVQHVQPDNII LSDGTNVP------- --YG-LLVWSTGVGP   332
4 YLNDH2 ILTKTMVKSVDENVI RAEQTKGDG-TKETL EMPYGTLVWATGNTV   391
5 SCNDE1 LRLKTMVKKVDATTI TAKTGDGD-----IE NIPYGVLVWATGNAP   385
6 SCNDE2 LQVNTAVKVVEPTYI RTLQNGQT-----NT DIEYGMLVWATGNEP   370
7 SCNDI1 VHLRTAVAKVEEKQL LAKTKHEDG-KITEE TIPYGTLIWATGNKA   343
8 ECNDH2 VLTQTMVTSADEGGL HTKDGEYI------- -EAD-LMVWAAGIKA   278

1 NCNDE1 AEFCKRISRQLGPAQ TNRHALETDTHLRLN GTPLGDVYAIGDCST   469
2 STNDB1 RPFVKDFMEQVGQEK R--RILATDEWLRVK GC--SNVYALGDCAS   373
3 STNDB2 SPFVNSLDIPKAKGR IG----ID-EWLRVP SVQ--DVYSIGDCSG   371
4 YLNDH2 RPVVRELMSKIPAQK GSRRGLLVNEYLVVE GT--EGIWALGDC--   432
5 SCNDE1 REVSKNLMTKLEEQD S-RRGLLIDNKLQLL GAK-GSIFAIGDCT-   427
6 SCNDE2 IDFSKTLMSRIPEQT N-RRGLLINDKLELL GSE-NSIYAIGDCT-   412
7 SCNDI1 RPVITDLFKKIPEQN SSKRGLAVNDFLQVK GS--NNIFAIGDN--   384
8 ECNDH2 PDFLKDIGGLETNRI N---QLVVEPTLQTT RDP--DIYAIGDCAS   318

1 NCNDE1 IQNN-VADHIITFLR NLAWKHGKDPESLEL HFSDWRDVAQQIKKR   513
2 STNDB1 VDQHKVMEDISTIFE AADKDDSGTLSVEEF RDVLEDIIIRYPQVD   418
3 STNDB2 ---------------- ---------------- ----------------   370
4 YLNDH2 ---------------- ---------------- ----------------   432
5 SCNDE1 ---------------- ---------------- ----------------   427
6 SCNDE2 ---------------- ---------------- ----------------   412
7 SCNDI1 ---------------- ---------------- ----------------   384
8 ECNDH2 CP-------------- ---------------- ----------------   320

                    nxxnn xxnDxDxDGxIDxxE nxxnnxxn
1 NCNDE1 FPQATAHLKRLDKLF EEYDKDQNGTLDFGE LRELLKQIDSKLTSL   558
2 STNDB1 LYLKNKHLLEAKDLF RDSEGNEREEVDIEG FKLALSHVDSQMKSL   463
3 STNDB2 ---------------- ---------------- -----FLESTGRQVL   380
4 YLNDH2 ---------------- ---------------- ----------SATKY   437
5 SCNDE1 ---------------- ---------------- ----------FHPGL   432
6 SCNDE2 ---------------- ---------------- ----------AHTGF   417
7 SCNDI1 ---------------- ---------------- ----------AFAGL   489
8 ECNDH2 ---------------- ---------------- --------RPEGGFV   327
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1 NCNDE1 PATAQRAHQQGQYLA HKFNKLARAAPGLSA NEIHEG---------     594
2 STNDB1 PATAQVAAQQGTYLA RCLNRWDQCKSNPEG PRRFKS---------     499
3 STNDB2 PALAQVAERQGKYLA SLLNKVGKQGGGHAN CAQNIN---------     416
4 YLNDH2 APTAQVASQEGSYLA NLLNGIAKTEDLNNE ITNLEKQSEHTFDEQ     482
5 SCNDE1 FPTAQVAHQEGEYLA QYFKKAYKIDQLNWK MTHAKDDSEVARL--     475
6 SCNDE2 FPTAQVAHQEGEYLA KILDKKLQIEQLEWD MLNSTDETEVSRL--     460
7 SCNDI1 PPTAQVAHQEAEYLA KNFDKMAQIPNFQKN LSSRKD---------     425
8 ECNDH2 PPRAQAAHQMATCAM NNILAQMNGKPLKNY QYKDHG---------     363

1 NCNDE1 --------------- DLDAAVYKAFEYRHL GSLAYIGNSAVFDWG     624
2 STNDB1 --------------- -SGRHEFLPFEYRHL GQFAPLGGDQAAAEL     528
3 STNDB2 --------------- -----LGDPFVYKHL GSMATIGRYKALVDL     441
4 YLNDH2 ERKNIFAQLESKSRK LRRSRAMLPFEYSHQ GSLAYIGSDRAVADL     527
5 SCNDE1 -----------KNQI VKTQSQIEDFKYNHK GALAYIGSDKAIADL     509
6 SCNDE2 -----------QKEV NLRKSKLDKFNYKHM GALAYIGSETAIADL     494
7 SCNDI1 -----------KIDL LFEENNFKPFKYNDL GALAYLGSERAIATI     459
8 ECNDH2 --------------- ----SLVSLSNFSTV GSLMGNLTRGSMMIE     389

1 NCNDE1 DG-------WNFSGG LWAVYAWRSIYFTQC VSLRTRVLMAIDWGK     662
2 STNDB1 PG------DWVSMG- HSTQWLWYSVYASKQ VSWRTRYLVVGDWVR     566
3 STNDB2 RES--KEAKGVSLAG FTSFFVWRSAYLTRV VSWRNKIYVLINWLT     484
4 YLNDH2 SFNFWGIMNWSSGG- TMTYYFWRSAYVSMC FSMRNKILVCIDWMK     571
5 SCNDE1 AV---GEAKYRLAG- SFTFLFWKSAYLAMC LSFRNRVLVAMDWAK     550
6 SCNDE2 HM---GDSSYQLKG- MFAFLFWKSAYLAMC LSIRNRILIAMDWTK     535
7 SCNDI1 RS---GKRTFYTGGG LMTFYLWRILYLSMI LSARSRLKVFFDWIK     501
8 ECNDH2 GR----------IAR FVYISLYRMHQIALH GYFKTGLMMLVGSIN     424

1 NCNDE1 RALFGRDLMRY-    673
2 STNDB1 RYIFGRDSSRI-    577
3 STNDB2 TLVFGRDISRI-    495
4 YLNDH2 VRVFGRDISRE-    582
5 SCNDE1 VYFLGRDSSRI-    561
6 SCNDE2 VYFLGRDSSV--    545
7 SCNDI1 LAFFKRDFFKGL    513
8 ECNDH2 RVIRPRLKLH--    434
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Figure 8. **Clustal alignment of the primary structure of the NDE1 protein from *N. crassa* with rotenone-insensitive NAD(P)H dehydrogenases from diferent organisms.** The shadowed sequences depict the conserved ADP binding-motifs (Wierenga *et al.*, 1986). A consensus sequence for calcium binding is shown above the sequence of NCNDE1 and STNDB1. NCNDE1, *N. crassa* (Melo *et al.*, 1999), STNDB1 and STNDB2, *S. tuberosum* (Rasmusson *et al.*, 1999), YLNDH2, *Y. lipolytica* (Kersher *et al.*, 1999), SCNDE1, SCNDE2 and SCNDI1, *S. cerevisiae* (de Vries *et al.*, 1992; Small *et al.*, 1999 and Luttik *et al.*, 1999) and ECNDH2, *E. coli* (Matsushita *et al.*, 1987).

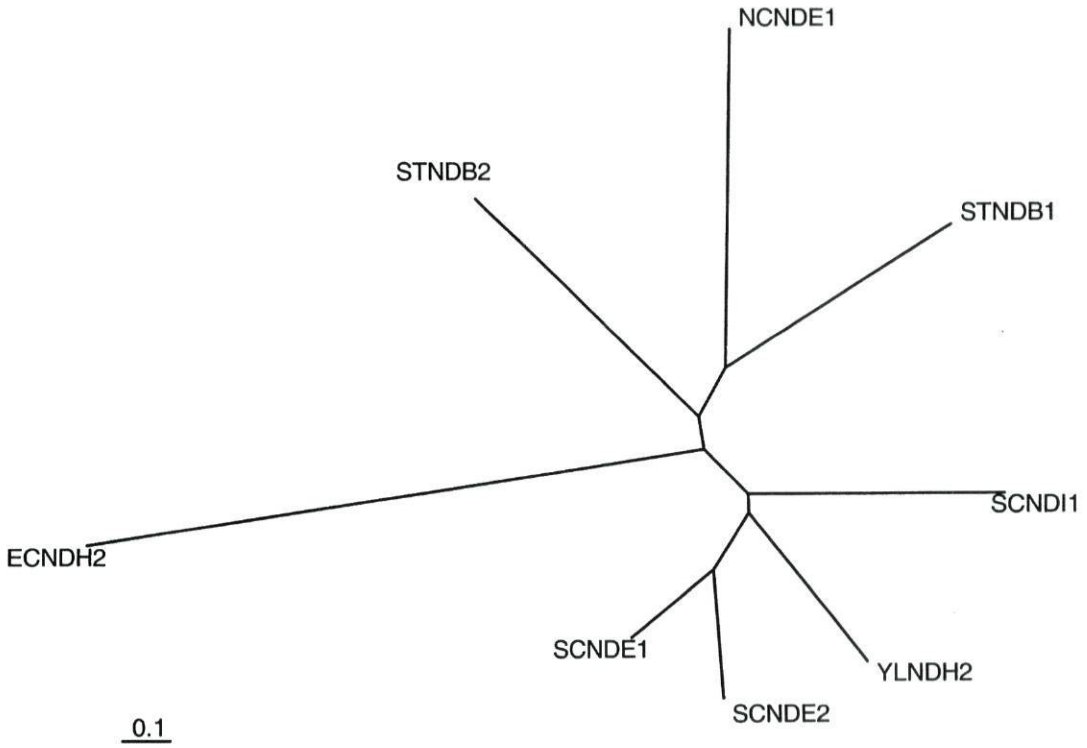


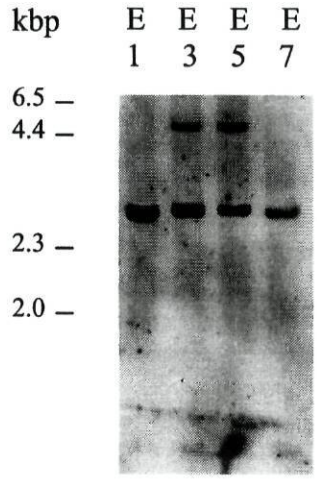
Figure 9. **Phylogenetic relationship between the alternative NAD(P)H dehydrogenases.** (Neighbour-joining method, Phylip, Felsenstein, 1995). *N. crassa* (NCNDE1), (Melo *et al.,* 1999), *S. tuberosum* (STNDB1and STNDB2), (Rasmusson *et al.,* 1999), *Y. lipolytica* (YLNDH2), (Kersher *et al.,* 1999), *S. cerevisiae* (SCNDE1), (de Vries *et al.,* 1992), (SCNDE2, SCNDI1), (Small *et al.*, 1999; Luttik *et al.*, 1999), and *E. coli* (ECNDH2), (Matsushita *et al.,* 1987). The sequences used were N-terminally truncated, starting at the 1st amino acid of ECNDH2.

unicellular organisms that also contains the Yarrowia enzyme (YLNDH2). These proteins were grouped apart from the internal NAD(P)H dehydrogenase (SCNDI1) suggesting a divergent evolution for different topologies on the inner membrane. NCNDE1 and STNDB1 were also set in a distinct group from STNDB2, presumably due to the fact that both the former enzymes contain a consensus sequence for binding calcium; it is also possible that they share the same topology on the inner membrane. These data point to a high diversity among the type II NAD(P)H dehydrogenases that compose the electron transport chain of different organisms which is not surprising, considering their differences with respect to topology and calcium sensitivity (General Introduction).

2.2.1.2. Chromosomal location of the gene encoding NDE1

The segregation of polymorphic restriction fragments among the 38 strains of the Multicent-2 cross kit is typical of their position in the genome (Metzenberg *et al.*, 1984). To determine the chromosomal location of the gene encoding NDE1, genomic

A.



B.

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

Figure 10. **Chromosomal mapping of the gene encoding NDE1.** A. Southern blot of genomic DNA, from four strains of the Multicent-2 cross kit restricted with *Sal*I, presenting a polymorphism. The detection was performed with the Gene Images detection kit (Amersham Pharmacia Biotech). B. Segregation pattern of the *Sal*I restriction length polymorphism among the 38 strains of the Multicent-2 cross kit. Restriction fragments (RF) of the Mauriceville type (M) and Oak Ridge type (O) are indicated. Adapted from Melo *et al.* (1999) with permission of Elsevier Science.

DNA of these strains was prepared, digested with different restriction enzymes and separated by agarose gel electrophoresis. The DNA was transferred onto a nylon membrane, which was hybridised with a probe in order to find polymorphisms. The probe used was a 2 kbp *Eco*RI/*Hind*III fragment of the cDNA labelled with the Gene Images random prime kit (Amersham Pharmacia Biotech).

A polymorphism with the restriction enzyme *Sal*I among the progeny of the cross is shown in figure 10A. Genomic DNA from strains E1 and E3 display a different restriction pattern than strains E5 and E7, which present an extra band around 5 kbp that is lacking in the former group. The pattern of segregation of *nde1* among the progeny of the cross is shown (Fig. 10B).

A comparison between the segregation pattern of *nde1* and that of genes with known chromosomal location (Metzenberg and Grotelueschen, 1995) located the gene encoding NDE1 at the right side of the centromere of the linkage group IV, near the *Fsr*-4. These results also indicated that NDE1 is encoded by a single-copy gene in *N. crassa*.

2.2.2. Inactivation of the *nde1* gene

In order to investigate the specific role of NDE1, we disrupted its encoding gene by the generation of repeat-induced point mutations (RIP) (Selker, 1990).

The cDNA clone NM1C2 carrying the *nde1* gene in the vector pBluescript SK+ (Fig. 11A) was excised with the restriction enzyme *Pvu*II, cloned in the *Eco*RV restriction site of pCSN44 (Fig. 11B) and the new construct (Fig. 11C) was used to transform Neurospora. When the vector carrying the *nde1* gene enters the host cell, the molecule of recombinant DNA is cleaved and integrates into the host DNA. To proceed with the RIP protocol the cleavage of the DNA must not occur in the region of the insert but somewhere in the vector. In this way it is ensured that the exogenous copy of the gene is intact. The transformants were selected by resistance to hygromycin B. Genomic DNA from the wild type and the transformant strains was isolated and analysed by Southern blot to trace a transformant containing a unique and intact copy of the foreign DNA. The digestion fragments were separated by

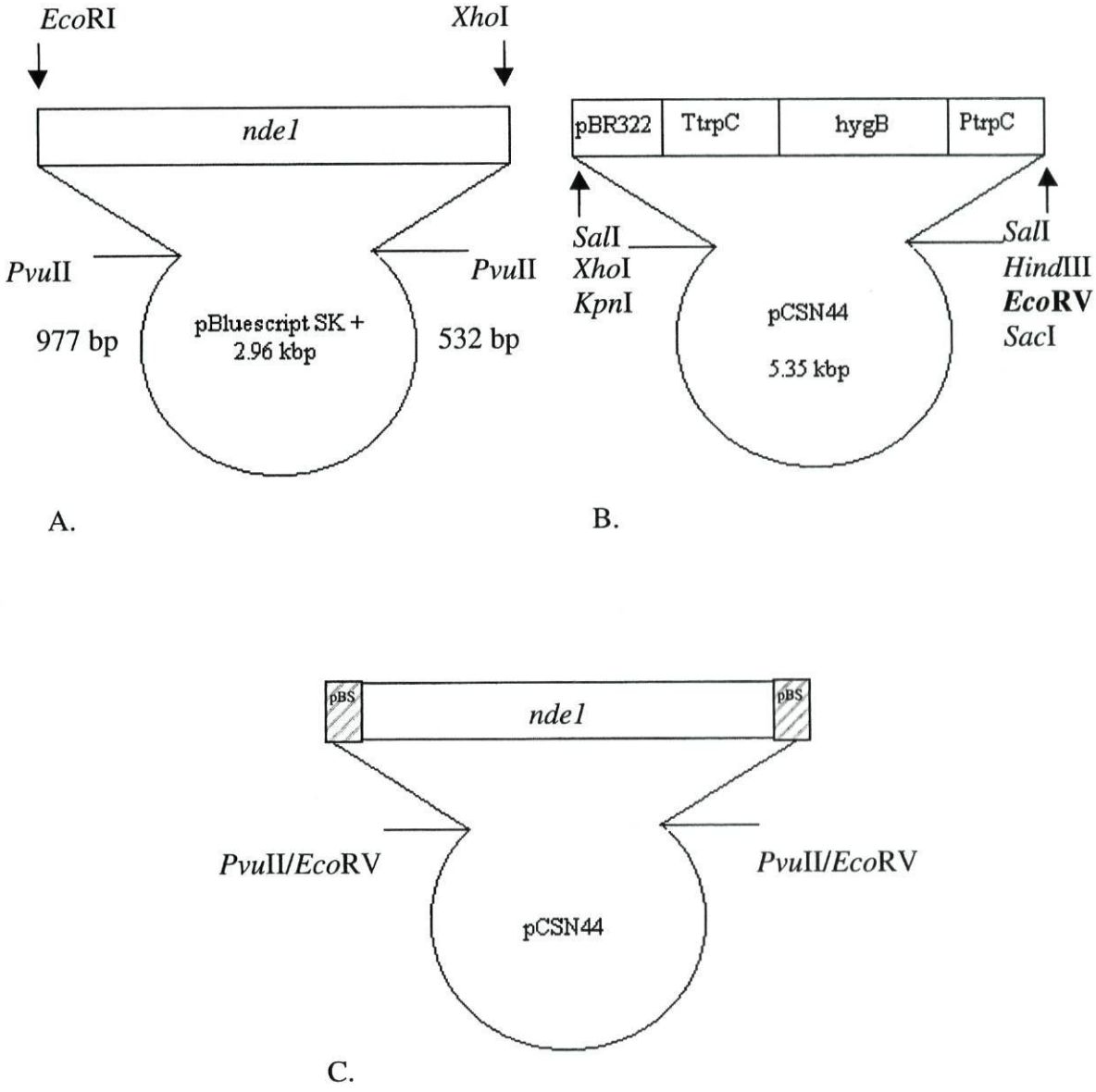


Figure 11. **Construction of a pCSN44 vector carrying the *nde1* gene.** A, pBluescript SK+ (pBS) carrying *nde1* flanked by the sites *Eco*RI/*Xho*I, B, pCSN44 carrying the gene of resistance to hygromycin B (hygB) flanked by the *Aspergillus nidulans* promoter and terminator sequences (PtrpC and TtrpC, respectively), C, pCSN44 carrying *nde1* at the restriction site for *Eco*RV, which disappeared after cloning.

agarose gel electrophoresis and transferred onto nylon membranes. These were hybridised with the *nde1* probe and detected with the Gene Images detection kit (Amersham Pharmacia Biotech). Figure 12 shows the restriction pattern of genomic DNA from the wild type and the transformant strains with *Bam*HI (A) *Sph*I (B) and *Sal*I (C). Restricting the DNA with *Bam*HI an extra band of ~3 kbp is observed in the transformant. This band corresponds to the size of *nde1* thus indicating that an intact copy of the gene was integrated into the host genome. The restrictions with *Sph*I and *Sal*I demonstrated that the transformant integrated a single copy of *nde1* since, in both cases, only an extra band is found. The strain carrying the exogenous copy of *nde1* cDNA was crossed with the wild type strain to achieve the inactivation of both copies of the duplication.

Mitochondria from each individual spore of the progeny of the cross were isolated. Mitochondrial proteins were separated by SDS-PAGE and analysed by Western blotting with antibodies against NDE1 (Melo *et al.*, 1999) in order to identify mutant strains. Figure 13 depicts a mutant where the polypeptide encoded by *nde1* is missing (compare lanes 1 and 2).

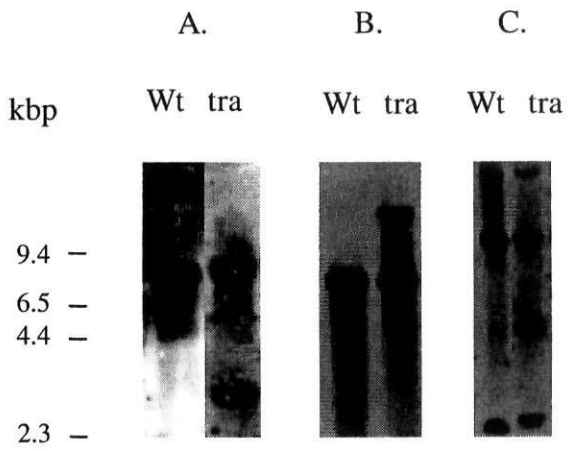


Figure 12. **Southern blot of genomic DNA from the wild type (Wt) and the strain transformed with *nde1* (tra).** The DNA was restricted with *Bam*HI (A), *Sph*I (B) and *Sal*I (C). The Southern blot was hybridised with a 2 kbp *Eco*RI/*Hind*III cDNA probe.

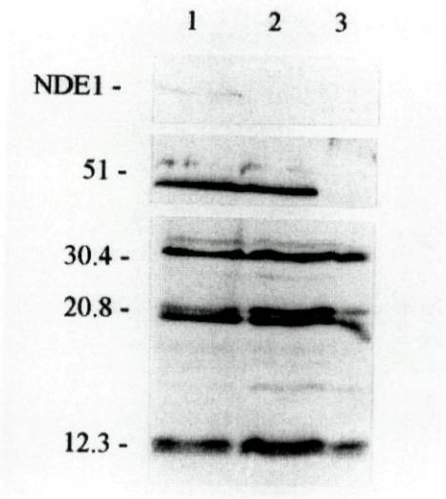


Figure 13. **nde1 mutant and double mutant nde1/nuo51.** Total mitochondrial proteins (100 μg) from the wild type (1), the nde1 mutant (2) and the double mutant nde1/nuo51 (3) were analysed by Western blotting with an antiserum against the NDE1 protein and a mixture of antisera against the 51, 30.4, 20.8 and 12.3 kDa polypeptide subunits of complex I. Reproduced from Melo *et al.* (2001) with permission of The Journal of Biological Chemistry.

2.2.3. Analysis of crosses involving the nde1 mutant

The nde1 mutant was crossed with several mutants in subunits of complex I and double mutants were obtained in crosses between the strain lacking NDE1 and the complex I mutants nuo51 (Fecke *et al.*, 1994), nuo24 (Almeida *et al.*, 1999), nuo21.3a (Alves and Videira, 1994), nuo21.3c (Duarte and Videira, 2000), nuo20.8 (da Silva *et al.*, 1996) and nuo12.3 (Duarte *et al.*, 1995), which display different phenotypes in terms of complex I assembly and function. Figure 13 (lane 3) depicts the analysis of the double mutant nde1/nuo51 which carries a non-functional complex I. In the respiratory chain of *N. crassa* a sole matrix rotenone-resistant NAD(P)H dehydrogenase was described (Weiss *et al.*, 1970). If NDE1 was this enzyme, a double mutant of *nde1* and *nuo51* should not be viable, since in such mutant there would not be any enzyme oxidising matrix NAD(P)H. However, it cannot be excluded that there might be other internal rotenone-insensitive NAD(P)H dehydrogenases, as verified in plant mitochondria (Møller and Palmer, 1982;

Rasmusson and Møller, 1991b; Melo *et al.*, 1996). Another and simpler hypothesis to interpret these results is NDE1 being an enzyme that oxidises cytosolic NAD(P)H.

Homozygous crosses were also carried out with the nde1 mutant yielding spores, thus indicating that in, contrast with complex I (Duarte *et al.*, 1998), the *nde1* gene is not crucial for sexual reproduction. Neither complex I nor NDE1 are essential for Neurospora vegetative growth under standard conditions. The expression of NDE1 and complex I proteins throughout the life cycle of Neurospora was carried out performing 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 proteins are constitutively expressed throughout the Neurospora life cycle (not shown). This is corroborated by the findings of the different cDNA clones in three different cDNA libraries, representing both vegetative and sexual stages of development.

2.3. Characterisation of NAD(P)H oxidation in Neurospora mitochondria

The mitochondrial location of the NDE1 protein was experimentally verified (Fig. 13, lane A). The protein could also be observed in mitochondrial membrane preparations, obtained by sonication followed by centrifugation of the organelles (not shown). An accurate localisation of the protein and its functional characterisation are described in the present section. A brief summary of the approaches used clarifying these issues is presented in figure 14.

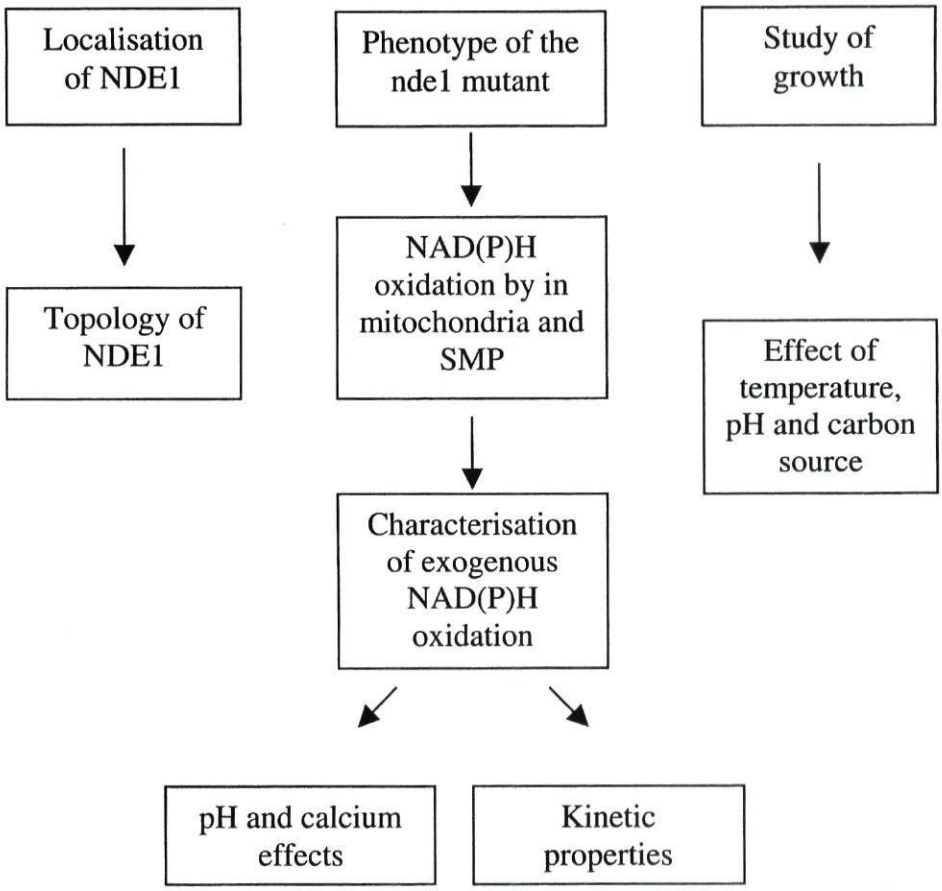


Figure 14. **Diagram of the strategies used to carry out the characterisation of NDE1.**

2.3.1. Localisation of the NDE1 protein

2.3.1.1. Localisation of NDE1 in the inner mitochondrial membrane

To achieve an accurate localisation of NDE1 within the mitochondrion, purified mitochondria were incubated with increasing concentrations of digitonin in order to obtain a sequential opening of the mitochondrial compartments. After a 30-sec incubation with digitonin on ice, the samples were diluted 10 times and the incubation proceeded for 30 min. The mitochondrial fractions were resolved into pellet and supernatant by centrifugation. Malate dehydrogenase and adenylate kinase activities were measured in the pellet fractions. SDS-PAGE was used to separate the mitochondrial proteins in the pellet and the supernatant, for each digitonin concentration. The proteins were then transferred onto nitrocellulose membranes. Immunodecoration with antibodies against NDE1 and the 20.8-kDa (Videira *et al.*, 1990a) and 30.4 kDa (Videira *et al.*, 1990b) subunits of complex I was carried out. The opening of the external and internal mitochondrial membranes was controlled by the release of adenylate kinase and malate dehydrogenase, from the intermembrane space and the matrix, respectively (Fig. 15A). In these experiments, the pattern of the 64-kDa protein paralleled that of the subunits of the respiratory chain complex I, an inner membrane complex, showing that NDE1 is located in the inner mitochondrial membrane (Fig. 15B,C).

The topology of NDE1 was further characterised by protease treatment of intact mitochondria, sonicated mitochondria and mitochondria submitted to hypotonic swelling, which destroys the integrity of the outer membrane, without any damage to the inner membrane. After separating the proteins by SDS-PAGE, Western blot analysis was carried out. The mitochondrial processing peptidase from the matrix (Hawlitschek *et al.*, 1988) and the cytochrome *c* heme lyase from the intermembrane space (Lill *et al.*, 1992) were used as controls in the assays (Fig. 16). It can be observed that NDE1 is resistant to externally added proteinase K in intact and swollen mitochondria. In sonicated mitochondria the protein is digested by the protease, indicating that it is not naturally resistant to proteinase K. These results first

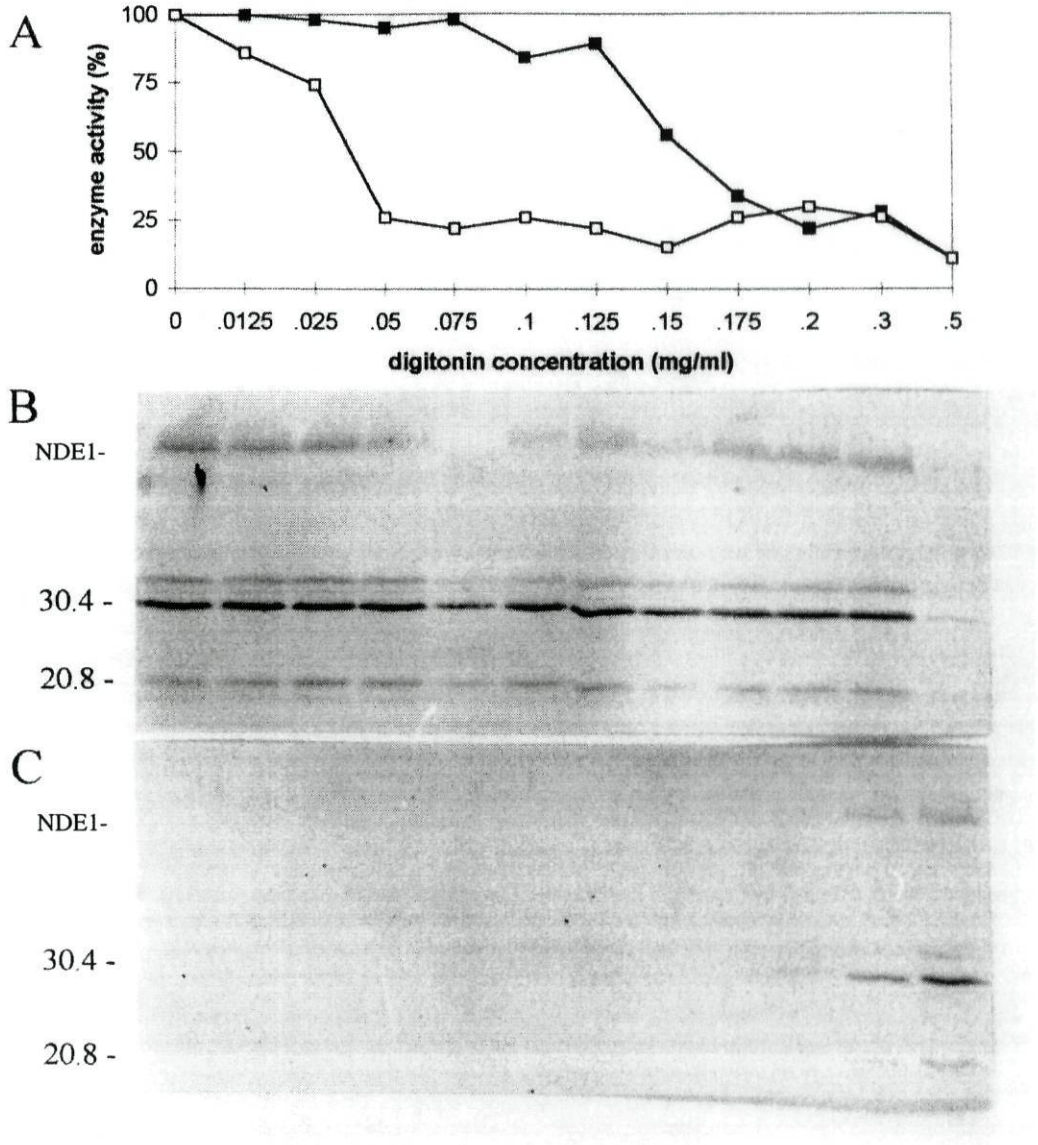


Figure 15. **NDE1 is released from the inner mitochondrial membrane simultaneously with the 30.4-kDa and 20.8-kDa subunits of complex I.** Aliquots of a mitochondrial preparation were treated with increasing concentrations of digitonin as indicated, and resolved by centrifugation into pellet and supernatant. The activities of malate dehydrogenase (closed squares) and adenylate kinase (open squares) were determined in the pellet fractions (A). Both pellet (B) and supernatant (C) were resolved by SDS-PAGE and analysed by Western blotting with a mixture of antisera against NDE1, and the 30.4-kDa and 20.8-kDa subunits of complex I. Reproduced from Melo *et al.* (1999) with permission of Elsevier Science.

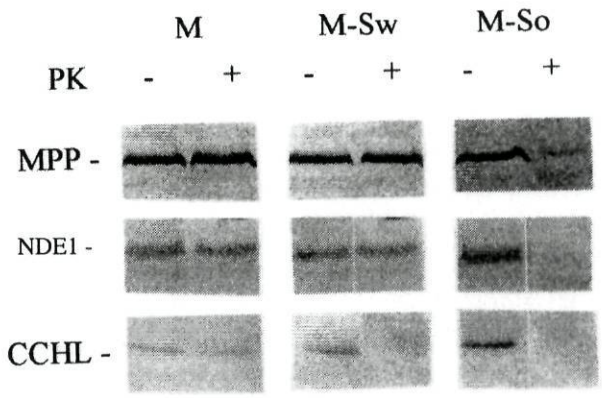


Figure 16. **Treatment of mitochondrial fractions with proteinase K.** Intact mitochondria (M), swollen mitochondria (M-Sw) and sonicated mitochondria (M-So) were incubated in the absence (-) or presence (+) of proteinase K (PK). Samples were resolved by SDS-PAGE and analysed by Western blot with antisera against the mitochondrial processing peptidase (MPP), the 64-kDa protein (NDE1) and the cytochrome *c* heme lyase (CCHL). Reproduced from Melo *et al.* (1999) with permission of Elsevier Science.

suggested that NDE1, or most of it, is located in the mitochondrial matrix. However, the possibility that some regions of the protein could be resistant to proteases in NDE1 native form, what might mislead our interpretation of the results, should not be excluded. In fact, other experiments will confirm a very folded conformation to the protein that can explain its resistance to protease treatment in native conditions (see below).

Characterising the kind of interactions of NDE1 with the inner mitochondrial membrane, extraction of mitochondria with Na_2CO_3 was performed (Fig. 17). After high speed centrifugation, the 30.4-kDa subunit of the peripheral arm of complex I can be extracted while the 20.8-kDa subunit of the membrane arm of the enzyme remains in the membrane pellet. In the case of NDE1, roughly half of the protein remained in the pellet, suggesting that it is an integral membrane protein. This is consistent with the computer analysis, which predicted at least a transmembrane helix in the N-terminal region of the protein (amino acid residues 73-94).

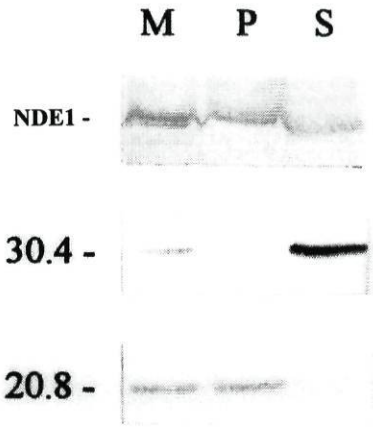


Figure 17. **NDE1 is divided between the pellet and the supernatant after alkaline extraction.** Mitochondria were incubated under alkaline conditions and resolved by centrifugation into pellet and supernatant fractions. Mitochondria before alkaline treatment (M), pellet (P) and supernatant (S) fractions were resolved by SDS-PAGE and analysed by Western blot with a mixture of antibodies against NDE1 and the subunits of 30.4 and 20.8 kDa from complex I. Reproduced from Melo *et al.* (1999) with permission of Elsevier Science.

2.3.1.2. NDE1 faces the intermembrane space

Other approaches were employed to obtain a detailed characterisation of the topology of NDE1. In order to characterise the accessibility of the protein to proteinase K, mitochondria were treated with the protease after being incubated with increasing concentrations of digitonin. The experiment was carried out essentially as described in 2.3.1.1. but in the presence of 50 μg/ml proteinase K. The reactions were stopped after 30 min adding 1 mM PMSF. The mitochondrial protein were resolved by SDS-PAGE and analysed by Western blot. The opening of the mitochondrial membranes was controlled with antisera against the mitochondrial processing peptidase (Hawlitschek *et al.*, 1992), the ADP/ATP carrier (Kubrich *et al.*, 1998), cytochrome *c* heme lyase (Lill *et al.*, 1992) and TOM20 (Söllner *et al.*, 1989), as markers for the matrix, inner membrane, intermembrane space and outer membrane, respectively.

TOM20, with domains facing the cytosol, is readily digested by proteinase K in intact mitochondria. The disappearance of the intermembrane space enzyme

cytochrome *c* heme lyase, at a digitonin concentration of 0.2%, confirmed that total solubilization of the outer mitochondrial membrane was achieved. When the final concentration of digitonin was 2%, the inner mitochondrial membrane was dissolved, as attested by the digestion of the mitochondrial processing peptidase. The behaviour of NDE1 parallels that of cytochrome *c* heme lyase indicating that the protein has domains exposed to the intermembrane space. Nevertheless, two NDE1 fragments remain after proteinase K exposure, when the outer membrane is opened, at 0.2% digitonin (Fig. 18, the left panel). The largest fragment (~57 kDa) remains membrane bound upon a 60-min incubation with Na_2CO_3, confirming previous indications that NDE1 is a membranous protein. The smallest fragment is extracted to the

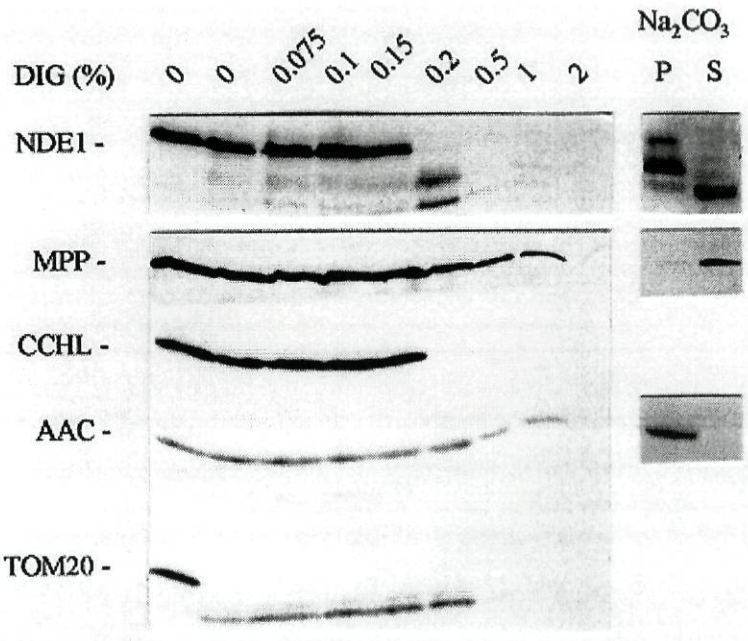


Figure 18. **NDE1 is accessible to proteinase K in digitonin-generated mitoplasts.** In the left panels, 100 μg of mitochondria were treated with different digitonin (DIG) concentrations and afterwards with proteinase K (except the first sample) and analysed by Western blotting with antisera against NDE1, the mitochondrial processing peptidase (MPP), the cytochrome *c* heme lyase (CCHL), the ADP/ATP carrier (AAC) and the outer membrane protein TOM20. In the right panels, mitochondria (100 μg) treated with 0.3% digitonin and afterwards with proteinase K were incubated with Na_2CO_3 and separated by centrifugation into pellet (P) and supernatant (S) before the Western blotting analysis. Reproduced from Melo *et al.* (2001) with permission of The Journal of Biological Chemistry.

supernatant, probably cleaved by protease K after the transmembrane domain (Fig. 18, the right panel). In this experiment, a mitochondrial sample incubated with 0.3% digitonin and proteinase K underwent alkaline extraction and was resolved into pellet and supernatant to discriminate intrinsic membrane proteins. The mitochondrial processing peptidase and the ADP/ATP carrier were used as markers for soluble and membranous proteins, respectively.

The precursor of NDE1 was synthesised *in vitro* using radioactively labelled methyonine, and imported into Neurospora mitochondria with and without a membrane potential. After import, the mitochondria were incubated with proteinase K for 30 min, on ice. The proteins were resolved in SDS-PAGE, transferred onto a nitrocellulose membrane and exposed to a Kodak film. Import and processing to the mature form of NDE1 was only achieved in the presence of a membrane potential, as deduced by the resistance of the mature form of the protein to added proteinase K in intact mitochondria (not shown). This is the expected result for an inner membrane protein like NDE1 (Neupert, 1997). The precursors of NDE1 and F1β were also successfully imported and processed into yeast mitochondria. As observed in

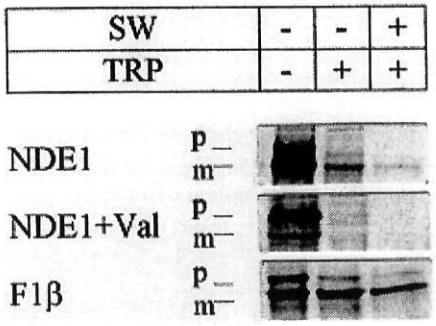


Figure 19. **NDE1 is imported into yeast mitochondria and sorted into the intermembrane space.** The radioactively labelled NDE1 and F1β precursors were imported into yeast mitochondria, in the presence of a membrane potential, generated by succinate or NADH, respectively. The precursor of NDE1 was also imported without a membrane potential, which was prevented by valinomycin (VAL). After import, mitochondria were incubated with trypsin (TRP) under swelling (SW) and non-swelling conditions. The samples were electrophoresed, blotted onto a membrane and exposed to a sensitive film. p, precursor; m, mature. Reproduced from Melo *et al.* (2001) with permission of The Journal of Biological Chemistry.

Neurospora mitochondria, the NDE1-processed precursor was resistant to trypsin, in intact yeast mitochondria, only when the import took place in the presence of a membrane potential (Fig. 19). In addition and in contrast to F1β, mature NDE1 was made accessible to the protease in the mitoplasts generated by swelling of mitochondria. These observations corroborated the results obtained in the digitonin/proteinase K experiments, which indicated that NDE1 has domains facing the intermembrane space.

The computer analysis of NDE1 primary structure predicted that amino acids residues 73-94 represent the most likely transmembrane domain of NDE1. Taking advantage of the fact that the protein contains seven cysteines located C-terminal to this segment, it was decided to label them with AMDA, a sulfhydryl-reagent that can cross the outer but not the inner mitochondrial membrane. An increase in the size of a protein is expected when the reagent reacts with cysteine residues (Spannagel *et al.*, 1998, Roudeau *et al.*, 1999).

Intact mitochondria or mitochondria solubilized either with Triton X-100 or SDS were incubated with AMDA and analysed by Western blot with NDE1 antiserum (Fig. 20). Apparently, the cysteines of NDE1 do not bind AMDA in intact organelles, what was unexpected considering the location of the protein on the outer surface of the inner mitochondrial membrane (Fig. 20, lane 2). However, similar results were obtained in the experiment with the non-denaturing detergent Triton X-

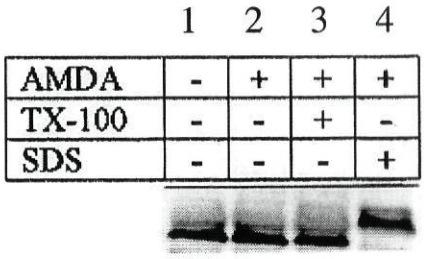


Figure 20. **Labelling of mitochondrial proteins with AMDA.** Samples containing 100 μg of intact mitochondria (lanes 1 and 2), mitochondria solubilized with 0.2% Triton X-100 (TX-100) (lane 3) and mitochondria solubilized with 1% sodium dodecyl sulphate (SDS) (lane 4), were incubated in the presence of AMDA at 30° C for 30 min. The material was analysed by Western blot using an antibody against NDE1.

100 (Fig. 20, lane 3). This rather suggests that NDE1 might be facing outwards in a tightly folded conformation, with the cysteines buried inside the protein structure and not able to react with AMDA. In fact, the SDS-denatured protein readily binds AMDA, as deduced from the shift in its mobility in the gel (Fig.20, lane 4). This very folded structure might explain the misleading results obtained in the experiment where swollen Neurospora mitochondria were incubated with proteinase K and NDE1 was not digested (Fig. 16).

2.3.2. Phenotype of the nde1 mutant

In order to characterise the activity of NDE1, IO-SMP and mitochondria from wild type and the nde1 mutant were prepared and tested for different activities under various conditions. The obtained IO-SMP were always more than 85% inside-out, as deduced from cytochrome *c* oxidase latent activity of the preparations (Møller *et al.*, 1987). The integrity of the mitochondrial membranes was very good as inferred from the latent activities of cytochrome *c* oxidase (EC 1.3.9.1) and malate dehydrogenase (EC 1.1.1.37) for the outer or inner mitochondrial membrane, respectively (Table 3).

Table 3. **The outer and inner mitochondrial membranes from wild type and nde1 mutant mitochondria are more than 90% intact.** The data refer to the mitochondria used in the experiments described in table 4. Similar results were obtained in other mitochondrial preparations.

Enzyme parameter		wt	nde1
MDH	Latency (%)	97	96
	Specific Activity (μmol/min.mg)	4.4	4.2
CCO	Latency (%)	93	92
	Specific Activity (μmol/min.mg)	1.3	2.0

Table 4. **External NAD(P)H oxidation activity is missing in nde1 mutant mitochondria at pH 7.2.** The data are from a typical mitochondrial preparation. Similar results were observed in 3 independent preparations.

	Additions	Oxygen consumption (nmol/min x mg)	
		wild type	nde1
1)	NADH	152	144
	+ Antimycin A	8	5
2)	NADPH	35	6
	+ Antimycin A	0	10

The oxidation rates of NADH and NADPH were followed polarographically using an oxygen electrode. Rotenone-insensitive oxidation rates for both substrates at pH 7.2 were similar in IO-SMP from both strains. The oxidation of NADH by wild type and nde1 mitochondria was inhibited 66% and 73% by rotenone, respectively. Concerning NADPH oxidation, the inhibition was 55% and 54% in wild type and nde1 mitochondria, respectively. These results indicate that the oxidation of NAD(P)H by the internal alternative NAD(P)H dehydrogenase was not affected by the disruption of *nde1*. In addition, when intact mitochondria were assayed with NADH at pH 7.2, no significant difference was found in the oxygen uptake rates between the two strains (Table 4, experiment 1). However, NDE1-defective mitochondria showed a very reduced oxidation activity relative to the wild-type organelles when NADPH was used as substrate at the same pH (Table 4, experiment 2). All activities were inhibited by antimycin A, confirming that the electrons, from NAD(P)H oxidation by the internal and external alternative NAD(P)H dehydrogenases, are transferred to oxygen via the cytochrome pathway of the electron transport chain. These results associate the external topology of NDE1 to a specific function in the oxidation of cytosolic NADPH, further supporting the previous indications that the protein has domains facing the intermembrane space.

Since mitochondria from the nde1 mutant are able to carry out exogenous NAD(P)H oxidation as well, though under different conditions, the mutant itself is the evidence for the presence of a second external rotenone-insensitive NAD(P)H dehydrogenase, NDE2, in the electron transport chain of *N. crassa* mitochondria.

2.3.3. Characterisation of exogenous NAD(P)H oxidation by Neurospora mitochondria - pH and calcium effects

To achieve a detailed characterisation of the exogenous NAD(P)H oxidation by mitochondria from wild type and nde1 mutant, the oxidation rates of these substrates were followed in the pH range of 4.7 to 9.2. The assays were performed in the presence or absence of calcium, the latter condition being achieved by the addition of EGTA. Data from three independent preparations of wild type and nde1 mutant mitochondria were considered. The percentage of latent activities of wild type and nde1 mutant mitochondria were 86% and 89% for cytochrome *c* oxidase, and 92% and 93% for malate dehydrogenase, respectively.

Table 5. **Comparison of NADH oxidation by wild type and nde1 mutant mitochondria.** The oxygen uptake is expressed in nmol/minxmg.

pH	NADH WT+Ca	NADH WT+EGTA	NADH nde1+Ca	NADH nde1+EGTA
4.7	23	21	26	27
5.0	47	51	77	76
5.3	84	77	88	77
5.6	101	102	113	115
5.9	104	101	141	135
6.2	137	123	131	128
6.5	134	119	156	132
6.8	146	128	158	154
7.1	163	133	175	190
7.4	158	161	161	196
7.7	164	107	189	163
8.0	141	85	164	88
8.3	144	51	175	118
8.6	136	66	144	89
8.9	146	58	128	70
9.2	103	47	111	46

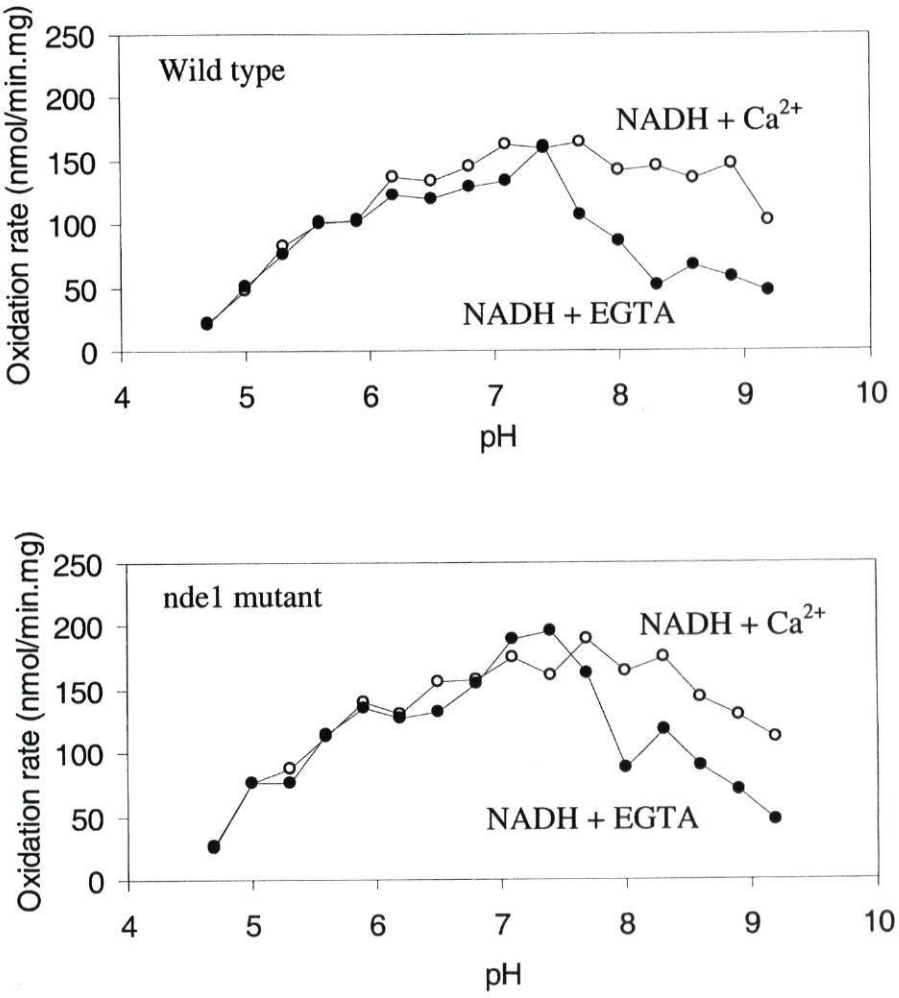


Figure 21. **Exogenous NADH oxidation by mitochondria from wild type and nde1 mutant is affected by calcium under alkaline conditions.** The results are an average of at least three independent preparations of mitochondria. Reproduced from Melo *et al.* (2001) with permission of The Journal of Biological Chemistry.

When respect to NADH oxidation and when calcium is present in the reaction medium, wild type and nde1 mutant mitochondria showed similar patterns throughout the pH range (Table 5, Fig. 21). Figure 22 depicts wild type minus nde1 oxidation rates when mitochondria are using NADH. The graph stresses the idea of an unaffected function of exogenous NADH oxidation in the nde1 mutant. By contrast, under the same conditions and as expected, the profile of oxygen uptake due to NADPH oxidation by nde1 mitochondria is different from the wild type, from pH

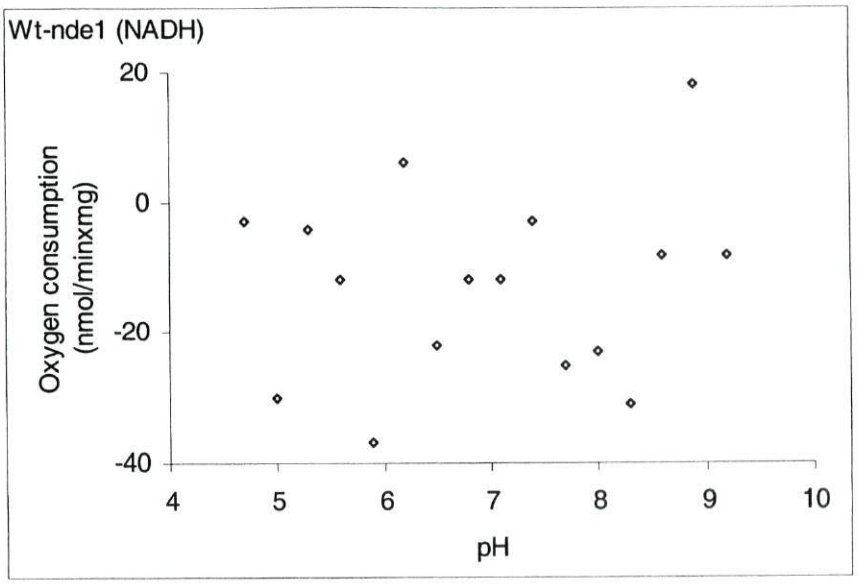


Figure 22. **Mitochondrial NADH oxidation of wild type minus nde1 mutant**, in the presence of calcium. The data represent an average of three independent mitochondrial preparations.

Table 6. **Comparison of NAPDH oxidation by wild type and nde1 mutant mitochondria.** The oxygen uptake is expressed in nmol/minxmg.

pH	NADPH WT+Ca	NADPH WT+EGTA	NADPH nde1+Ca	NADPH nde1+EGTA
4.7	26	13	12	20
5.0	21	21	29	25
5.3	33	25	27	33
5.6	35	23	33	28
5.9	43	23	29	25
6.2	39	19	32	20
6.5	35	15	13	12
6.8	26	15	12	6
7.1	32	15	4	1
7.4	18	12	1	0
7.7	15	4	0	0
8.0	10	1	0	0
8.3	4	0	0	0
8.6	0	0	0	0
8.9	0	0	0	0
9.2	0	0	0	0

6.5 to 8.3. In the presence of calcium, wild type mitochondria can oxidise NADPH from pH 7.4 to pH 8.3, while nde1 mutant mitochondria had no activity above pH 7.1 (Table 6).

Concerning the effect of calcium, NADH oxidation by mitochondria from wild type and nde1 mutant strains was unaffected by the cation at acidic pH, whereas decreased activity in the absence of calcium was observed under alkaline conditions (Table 5; Fig 21).

A distinct behaviour was found in NADPH oxidation. Under calcium depletion conditions, a drastic reduction in NADPH oxidation rate was observed in wild type mitochondria throughout the pH range (Fig. 23). At pH 8, this activity was totally dependent on calcium. In contrast, the oxidation of NADPH by mitochondria from the nde1 mutant was not affected by calcium throughout the pH range (Fig. 23).

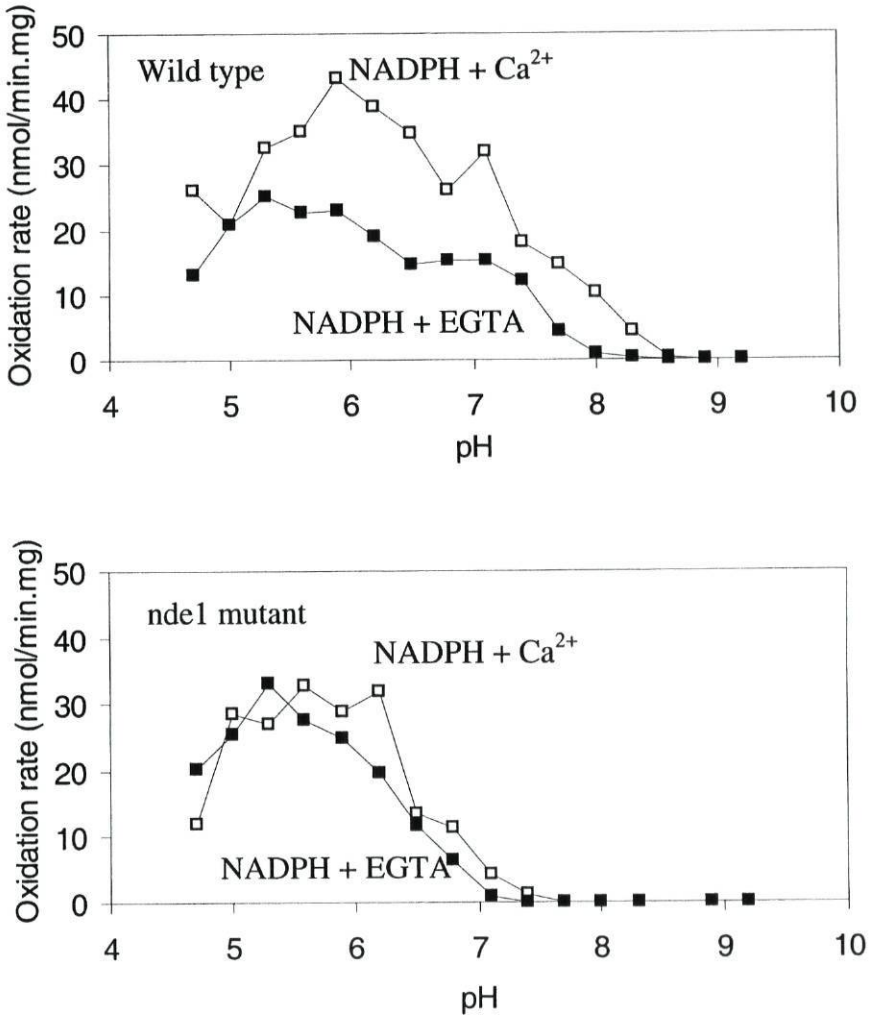


Figure 23. **NADPH oxidation by NDE1 is calcium dependent.** The results are an average of at least three independent preparations of mitochondria. Reproduced from Melo *et al.* (2001) with permission of The Journal of Biological Chemistry.

Figure 24 shows the oxidation of NADPH by wild type minus nde1 mutant mitochondria in the presence of calcium, illustrating the stimulation of NADPH oxidation by the cation in wild type in contrast to nde1 mutant mitochondria.

The data were examined by the statistical analysis of variance-covariance (ANCOVA) using as covariate the value of pH (Zar, 1996). The statistical parameters confirmed that there was no significant difference in the pattern of NADH oxidation between mitochondria from the two strains throughout the pH range, neither in the presence ($p=0.29$) nor in the absence of calcium ($p=0.089$) (Fig. 21). 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. 21). The differences of the oxygen uptake rates in the presence of calcium or EGTA were not statistically significant neither in wild type ($p=0.62$) nor in nde1 mutant ($p=0.89$) in the pH range 4.7 to 7.4. However these were significant both in wild type ($p<0.001$) and nde1 mutant ($p<0.01$) when the pH ranged from 7.7 to 9.2. Considering the oxidation of NADPH, the statistical analysis revealed that calcium had a highly significant effect on the activity of wild type mitochondria all over the pH range ($p<0.001$). In contrast, the oxidation of NADPH by mitochondria

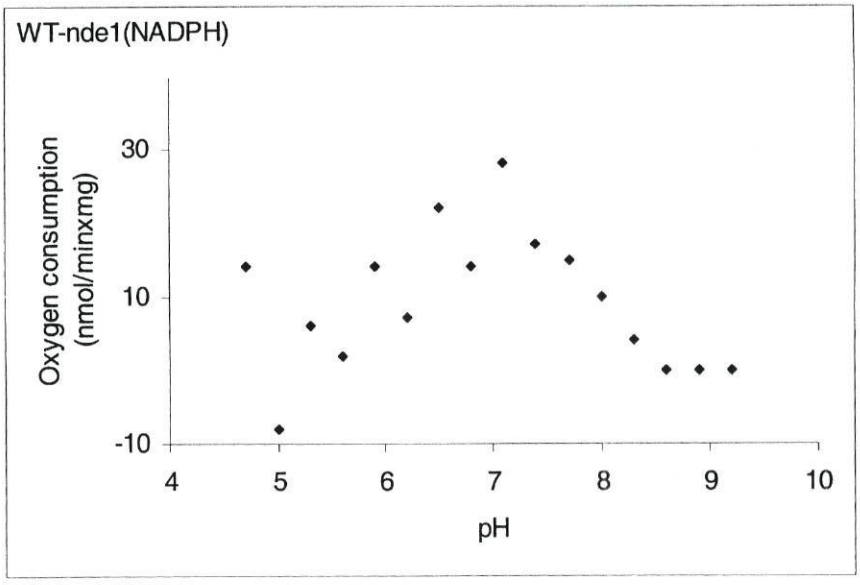


Figure 24. **Mitochondrial NADPH oxidation of wild type minus nde1 mutant in the presence of calcium.** The data represent an average of three independent mitochondrial preparations.

from the nde1 mutant was not significantly affected by calcium (Fig. 23), as confirmed by the statistical analysis (p=0.63). These results clearly indicate that NDE1 is an external calcium-dependent NADPH dehydrogenase.

This is the first time that the gene for a mitochondrial NADPH dehydrogenase has been identified and that evidence for at least two external NAD(P)H dehydrogenases in *N. crassa* mitochondria was obtained. The inability of the NDE2 to oxidise NADPH at pH higher than 6.8 may explain the need for a separate enzyme, NDE1, to regenerate the cytosolic $NAD(P)^+$ required in several biosynthetic pathways.

Experiments were carried out in order to find a specific inhibitor to distinguish between the external NAD(P)H dehydrogenases. DPI (Majander *et al.*, 1994; Roberts *et al.*, 1995) and carvedilol (Oliveira *et al.*, 2000) were used to inhibit the oxygen uptake due to NADH and NADPH oxidation by wild type mitochondria. Both compounds inhibited all the antimycin A sensitive oxygen uptake, thus failing to be useful to distinguish among the external rotenone-insensitive NAD(P)H dehydrogenases.

2.3.4. Kinetic properties of the external rotenone-insensitive NAD(P)H dehydrogenases

The kinetic properties of NAD(P)H oxidation by NDE1 and NDE2 were determined as Lineweaver and Burk plots (Lineweaver and Burk, 1934) (maximal velocities [V_{max}] and Michaelis constants [K_m]) measuring the mitochondrial oxidation rates from wild type and nde1 mutant at pH 7.4, in the presence of calcium, at different substrate concentrations. The protein content was kept constant, the concentration of NADH ranged from 0.1 μM – 1 mM and the concentration of NADPH from 5 μM – 1 mM, when determining the respective parameters. At least seven different concentrations of substrate were used to draw each plot.

NDE2 (nde1 mitochondria) displayed a K_{mNADH} of 12.0 ± 1.7 μM and a $V_{maxNADH}$ of 184 ± 17.8 nmol/min.mg, while NDE1 + NDE2 (wild type mitochondria) presented the values of 10.7 ± 0.1 μM and 268 ± 24.7 nmol/min.mg

for the K_{mNADH} and the $V_{maxNADH}$, respectively. The catalytic constants (K_{cat}) for NDE2 and NDE1 + NDE2 were 1.5 x 10^{-2} and 2.5 x 10^{-2}, respectively. Each result is an average of two independent mitochondrial preparations and the standard deviation for the K_m was ≤1. Despite the remarkable similarity among the patterns of NADH oxidation by mitochondria from both strains, these data are not sufficient to predict the existence of a single enzyme oxidising NADH. Therefore the ability of NDE1 to oxidise NADH remains open.

Our results were generally of the same order of those determined for the external rotenone-insensitive NADH oxidation by plant mitochondria (Table 7). Jerusalem artichoke mitochondria presented the highest K_{mNADH} and also higher $V_{maxNADH}$ than the *N. crassa* parameter. Arum mitochondria displayed the highest $V_{maxNADH}$ value, about 5 times higher than that determined in wild type mitochondria from Neurospora.

Despite the value obtained with Jerusalem artichoke mitochondria, it is remarkable the similarity of the K_{mNADH} determined for the same activity in mitochondria from different organisms from different taxa. The variability of $V_{maxNADH}$ values can probably be explained by differences in the physiological requirements of the different organisms and tissues from which mitochondria were obtained.

Table 7. **Kinetic parameters of mitochondrial external NADH oxidation of Neurospora**, in the presence of calcium. The experiments were carried out at pH 7.4 in Neurospora, at pH 7.2 in Arum and Jerusalem artichoke (Møller and Palmer, 1981) and at pH 7.0 in spinach (Edman *et al.*, 1985).

Mitochondria from	*K*m (μM)	*V*max (nmol/minxmg)
Neurospora NDE1+NDE2 (wt)	11	268
NDE2 (nde1 mutant)	12	184
Spinach	14	250
Jerusalem artichoke	54	555
Arum maculatum	19	1490

Table 8. **Kinetic parameters of mitochondrial NADPH oxidation**, in a medium containing calcium. The experiments were carried out at pH 7.4 in Neurospora, at pH 7.2 in potato (Rasmusson and Møller, 1991a) and at pH 7.0 in spinach organelles (Edman *et al.*, 1985).

Mitochondria from	*K*m (μM)	*V*max (nmol/minxmg)
Neurospora mitochondria NDE1 (wt)	38	24
Spinach mitochondria	14	183
Potato IO-SMP	41	1040

Assays to determine the kinetic properties of NADPH oxidation by NDE1 were carried out with mitochondria from wild type at pH 7.4. The enzyme was characterised with a 38.0 ± 15 μM K_{mNADPH}, a $V_{maxNADPH}$ = 24.2 ± 11 nmol/min.mg and a K_{cat} of 6,4 x 10^{-4}. The determination of the homologous oxidation in spinach mitochondria showed a smaller K_{mNADPH} value, in contrast with the $V_{maxNADPH}$, which was about 6 times higher than in Neurospora mitochondria (Table 8). In potato mitochondria, the kinetic parameters of the internal rotenone-insensitive NADPH oxidation differed from the external NADPH dehydrogenase from Neurospora with respect to the $V_{maxNADPH}$, which was 50 times higher than NDE1 (Table 8).

The similarity of the results obtained for the exogenous NAD(P)H oxidation parameters may suggest a meaningful conservation in respect to the affinity of these enzymes to the substrate during evolution. And the distinct values obtained for the oxidation of matrix NAD(P)H by potato mitochondria can be due to a very different enzyme working in a different environment.

2.3.5. Physiological role of NDE1

The physiological role of rotenone-insensitive NAD(P)H dehydrogenases is far from understood. In order to comprehend this issue, growth of the Neurospora

mutant lacking NDE1 was compared to growth of the wild type strain on different carbon sources, at different pH and at different temperatures.

Growth in Vogel's minimal medium at 4° C was not observed in any strain. Growth of wild type and nde1 mutant in minimal medium of Vogel's at pH 5, 6, 7, 8, and 9 was followed at 26° C and 37° C, and no significant difference was observed between the two strains (data not shown). Glycerol, mannitol and acetate were assayed as alternative carbon sources to sucrose. In the case of glycerol, growth was assayed at 30 and 40° C without significant differences in the performance of wild type and nde1 mutant strains. A 60% decrease in the growth rate was observed at 40° C in both strains (data not shown).

Growth of wild type and nde1 mutant strains, at 30 and 40° C, using acetate as source of carbon was carried out. At 30° C it was not possible to distinguish any differences between the behaviour of the two strains. However, when the experiment was carried out at 40° C, nde1 mutant grew faster than wild type between 50 to 100 h (Fig. 25). When Neurospora is grown with acetate as the source of carbon and energy, the metabolic pathways differ from those occurring in hyphae grown in sucrose. In the former conditions the activities of acetyl Co-A synthase, malate synthase and phosphoenolpyruvate carboxykinase are considerably enhanced, what is essential for the utilisation of acetate and gluconeogenesis, while the rate of glycolysis is reduced (Schwitzguébel and Palmer, 1981). In addition, Neurospora mitochondria grown in acetate, in contrast to mitochondria grown in sucrose, oxidise

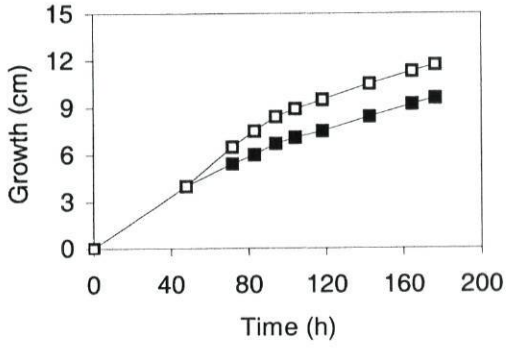


Figure 25. **Growth of Neurospora wild type and nde1 mutant strains in Vogel's minimal medium plus 39 mM ammonium acetate, at 40° C.** Closed squares, wild type; open squares, nde1.

citrate and *cis*-aconitate at high rates, displaying strong inhibition by malonate, the succinate dehydrogenase inhibitor, but only slightly inhibition by rotenone (Schwitzguébel *et* al., 1981). Exogenous NADPH oxidation is twice as fast in acetate-grown mitochondria, whereas the rate of exogenous NADH remains equal. Nonetheless, the oxidation of NADH is still faster than NADPH oxidation (Schwitzguébel and Palmer, 1981). It is possible that, when NDE1 is absent, uniquely NADH is oxidised therefore the energetic production becomes faster, what could be vital for enhancement of the growth rate, in a tissue where complex I activity is diminished, until a steady-state or a different growth stage is achieved. It is tempting to speculate that the presence of two external NAD(P)H dehydrogenases could regulate the energy production, according to the cellular demands.

Another situation where a distinct performance of growth was found was when Neurospora wild type and nde1 mutant strains were grown with mannitol instead of sucrose (Fig. 26). In this case, both strains grew in parallel, presenting slower rates than with sucrose, in the first 30 h, upon which the growth of the wild type strain became considerably faster reaching the rates achieved on sucrose. Mycelia from the nde1 mutant strain also displayed a rate enhancement, though smaller than wild type.

According to Hult and Gatenbeck (1978), one turn of the mannitol cycle gives the following net result:

$$NADH + NADP^+ + ATP \rightarrow NAD^+ + NADPH + ADP + Pi.$$

From this equation it is possible to understand how important the presence of NDE1 may be, in order to regenerate $NADP^+$, thus allowing the cycle to proceed. However, mannitol dehydrogenase (EC 1.1.1.67) generates fructose from mannitol that can be phosphorylated by hexokinase and enter glycolysis, thus contributing to NADH generation. This mechanism allows the use of mannitol as an alternative source of carbon even for the nde1 mutant, though at a slower rate.

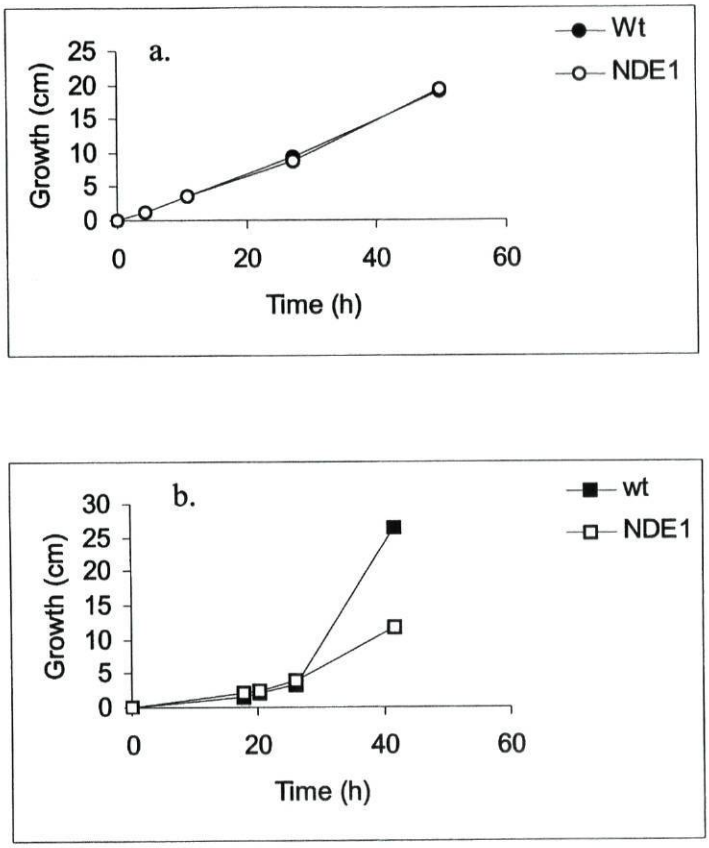


Figure 26. **Growth of Neurospora wild type and ndel mutant strains in Vogel's minimal medium plus sucrose, a, or plus 44 mM mannitol, b, at 37° C.** The data refer to a single experiment which was repeated twice with reproducible results.

2.3.6. Final considerations

The combination of data from several approaches used in this research indicated that the NDE1 polypeptide is an intrinsic protein of the inner mitochondrial membrane with its catalytic site facing the intermembrane space. In *N. crassa*, the protein is likely anchored to the membrane by an N-terminal domain (amino acid residues 73-94), the region displaying the highest probability of being transmembranous according to the computer prediction. A second predicted transmembrane domain (residues 173-196) is unlikely since it would place the two dinucleotide binding-sites (presumably for NAD(P)H and FAD) on opposite sides of

the inner membrane. The existence of a third predicted transmembrane domain at the C-terminal (residues 620-639) is still an open question.

The protein contains two GXGXXG motifs within beta sheet- alpha helix- beta sheet structures for binding NAD(P)H and FAD (Melo *et al.*, 1999). Probably, in NDE1, the first motif binds the substrate NADPH. The third G in NADPH-binding proteins is generally replaced by S, A or P and a conserved negative charge at the end of the second beta sheet is missing (Howitt *et al.*, 1999). The latter might be replaced by an N, avoiding the unfavorable interaction between the negatively charged residue with the negatively charged 2'-phosphate of NADPH (Møller *et al.*, 1981). These features are present in the first and absent in the second dinucleotide-binding motif of NDE1 (Melo *et al.*, 1999). Moreover, the apparent lack of labeling of non-denatured NDE1 with AMDA may suggest that the second motif, which has cysteine residues nearby, is more buried inside the protein structure and thus less likely to bind the NADPH substrate and more likely to bind the FAD.

The NDE1 protein is homologous to the type II NAD(P)H dehydrogenases from *S. tuberosum* (Rasmusson *et al.*, 1999), *Y. lipolytica* (Kerscher *et al.*, 1999), *S. cerevisiae* (de Vries and Marres, 1987, Luttik *et al.*, 1998, Small and McAlistair-Henn) and *E.coli* (Matsushita *et al.*, 1987). In contrast to most of them, it contains a sequence insertion predicted to bind calcium (Melo *et al.*, 1999). This E-F hand motif is also present in the external NDB protein from *S. tuberosum* mitochondria (Rasmusson *et al.*, 1999) and in a putative NADH:ubiquinone reductase from *Arabidopsis thaliana* (AC006234.3). NADH oxidation by intact mitochondria from both the nde1 mutant and the wild type strain was not significantly affected by calcium at acidic pH whereas at alkaline conditions calcium depletion caused a decrease in the NADH oxidation rates. In contrast, NADPH oxidation was stimulated by calcium throughout the pH range where the NDE1 was active. It is possible that calcium plays a particular role avoiding the electrostatic repulsion between the catalytic site, localised in a net negatively charged environment (outer surface of the inner mitochondrial membrane), and the phosphate groups of NADPH, as suggested by Edman *et al.* (1985). In this way, the cation increases the apparent affinity of NDE1 for NADPH by screening of the negative charges. It is also likely that the binding of calcium induces conformational changes in the protein that increase the

enzyme/substrate affinity. This is probably the case for NDE1 whose structure contains a calcium-binding domain. Wild-type mitochondria displayed strong calcium sensitivity and the presence of the cation was absolutely required for activity at physiological pH. Since the oxidation of NADPH by nde1 mutant mitochondria was not affected by calcium, it was demonstrated experimentally that the NADPH oxidation activity, missing in this strain, is calcium-dependent.

The presence of non-proton-pumping alternative NAD(P)H dehydrogenases varies between different organisms. Their specific role is unclear but they might be involved in situations of NAD(P)H stress. In addition to metabolic functions (Luttik *et al.*, 1998, Small and McAlistair-Henn, 1998, Kercsher *et al.*, 1999), a regulatory role in response to the redox state of the plastoquinone pool has been suggested in cyanobacteria (Howitt *et al.*, 1999). In Neurospora, these enzymes might play an important role in metabolic adaptation of the organism to different carbon sources, when sucrose, the most effective substrate, is not available.

3. CONCLUDING REMARKS

This project focused on the characterisation of NAD(P)H dehydrogenases of the electron transport chain in *N. crassa* mitochondria.

A contribution to the characterisation of the role of the 24-kDa and 21-kDa polypeptides in the structure and function of the complex I was made. The nuo24 complex I can not perform its NADH:ubiquinone oxidoreductase activity, as attested by the lack of inhibition of the oxygen consumption by rotenone. An interesting finding in nuo24 was that the rates of NADH or pyruvate/malate oxidation by IO-SMP or intact mitochondria, respectively, were similar to wild type, suggesting that a rotenone-insensitive NAD(P)H dehydrogenase was overexpressed. In contrast to nuo24 and like the wild type strain, the oxidation of NADH or pyruvate/malate oxidation by IO-SMP or intact mitochondria, respectively, from the nuo21 mutant

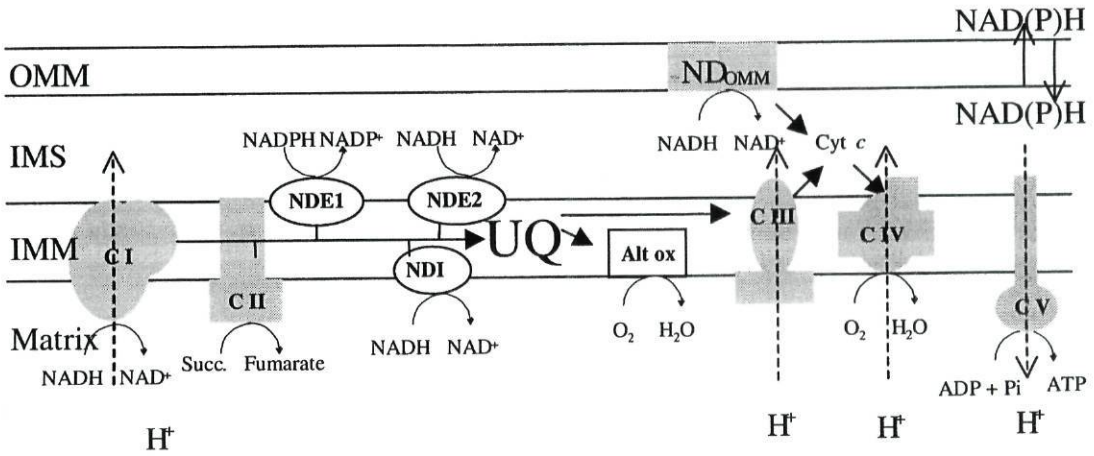


Figure 27. **Schematic drawing of *N. crassa* respiratory chain.** The gray fill represents common features with the mammalian respiratory chain. Alt ox, alternative oxidase; C I, complex I or NADH:ubiquinone oxidoreductase; C II, complex II or succinate dehydrogenase; C III, complex III or cytochrome bc_1; C IV, complex IV or cytochrome *c* oxidase; C V, complex V or ATP synthase; IMM, inner mitochondrial membrane; IMS, intermembrane space; NDE, rotenone-insensitive NAD(P)H dehydrogenase from the outer surface of the inner mitochondrial membrane; NDI, rotenone-insensitive NADH dehydrogenase from the inner surface of the inner mitochondrial membrane; NDOMM, NADH dehydrogenase from the outer mitochondrial membrane; OMM, outer mitochondrial membrane; UQ, ubiquinone.

was sensitive to rotenone, thus showing that the nuo21 complex I is functional concerning its NADH oxidoreductase activity.

Special emphasis was given to exogenous NAD(P)H oxidation with the identification, characterisation and disruption of *nde1*. The encoded enzyme, containing an EF-hand in the primary structure, was shown to be calcium-dependent. The nde1 mutant was crucial to the identification and characterisation of NDE1 and to evidence the presence of a second external rotenone-insensitive NADH dehydrogenase, NDE2. This was the first time that the gene for a mitochondrial NADPH dehydrogenase has been identified and that evidence for at least two external NAD(P)H dehydrogenases in *N. crassa* mitochondria has been obtained. In figure 27, a scheme of the electron transport chain from *N. crassa* mitochondria, in the light of the presented achievements, is attempted.

In the future, cloning and disruption of the gene encoding NDE2 as well as the genes coding for the internal rotenone-insensitive NAD(P)H dehydrogenases will be helpful to clarify their function and physiological roles in the mitochondrial and cellular metabolism. This will constitute a step forward in the characterisation of the Neurospora electron transport chain, concerning the NAD(P)H oxidising enzymes, and should make it possible to produce strains where complex I is the sole enzyme responsible for NAD(P)H:ubiquinone oxidoreductase activity. These strains can provide suitable models to study the consequences of mutations in complex I polypeptides in human mitochondrial pathologies, through the characterisation of the phenotypes of mutants deficient in each particular complex I subunit, thus simulating the situation in human mitochondria.

Purification and crystalisation of these enzymes will also enlarge our knowledge of their structure, thus contributing to a better understanding of the mechanisms through which they operate. The knowledge of the latter properties can be important in the search for therapeutics for human mitochondrial diseases.

Chapter III – Experimental Procedures

1. CHEMICALS

The chemicals used in this study were purchased from the indicated companies:

[^{35}S] methionine (1000 Ci/mmol) from Amersham-Buchler, Braunschweig. Restriction enzymes, Gene Images, ECL system, Thermosequenase, and dNTPs from Amersham Pharmacia Biotech. Protein reagents for Bradford protein determination from BioRad. Adenylate kinase, alkaline phosphatase (calf intestine), AMP and creatine kinase from Boeringher Mannheim. Agar, Tryptone and Yeast extract from Difco. Long Ranger gel solution from FMC BioProducts. *Taq* DNA polymerase, agarose – ultrapure, and Klenow fragment from *E. coli* DNA polymerase I from Gibco BRL. X-ray films (Biomax MR) from Kodak. Ampicilin, hygromycin B, antimycin A, APS, ATP, BSA, CCCP, creatine phosphate, DNA from salmon sperm, MES, MOPS, PEG 8000, NADH, NADPH, rotenone, soybean trypsin inhibitor, trypsin and TES from Sigma. All other reagents were obtained from MERCK.

2. MEDIA USED

Alkaline phosphatase buffer
100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$.

Destain solution for Coomassie stained gels
25% ethanol, 8% acetic acid in H_2O.

Electrophoresis buffer
16.5 mM Tris, 133 mM glycine, 0.03% SDS.

Hybridisation solution for Southern blotting
5 x SSC, 0.1% SDS, 5% dextran sulfate, 1:20 blocking agent (Gene Images™, Amersham Pharmacia Biotech) and 100 µg/ml of denatured salmon sperm DNA.

Import buffer
3% (w/v) BSA, 80 mM KCl, 0.5 M sorbitol, 1 mM $MnCl_2$, 2.5 mM EDTA, 2 mM phosphate buffer pH 7.2, 10 mM Mg $(COO)_2$, 50 mM Hepes, 0.6 mM ATP, 0.6 mM NADH (0.75 M succinate), 1% ethanol, 0.3 mM creatine phosphate, 30 µg creatine kinase.

Isolation medium for mitochondria
0.44 M sucrose, 2mM EDTA, 30 mM Tris-HCl pH 7.3.

LB medium
1% Bacto-tryptone, 0.5% yeast extract, 0.5% NaCl; LB-plates plus 1.5% agar.

Lysis solution for Neurospora mycelia
50 mM Tris-HCl pH 8.0, 50 mM EDTA, 3% SDS and 1% 2-mercaptoethanol.

MCS
10 mM MOPS pH 6.3, 50 mM $CaCl_2$ and 1 M sorbitol.

Non-swelling buffer
0.6 M sorbitol, 20 mM Hepes pH 7.2.

Percoll gradient
Bottom solution: Sucrose 0.3 M/ 10 mM TES-KOH pH 7.2, 28% percoll
Top solutions: Mannitol 0.3 M/ 10 mM TES-KOH, pH 7.2, 28% percoll.

PMC
40% PEG 4000, 10 mM MOPS pH 6.3, 50 mM $CaCl_2$.

Polyacrylamide-gel solutions

Table 1. **Composition of 10% and 17.5% polyacrylamide gels.**

Solution	Bottom gel (ml)	Running Gel (ml) 10%	17.5%	Stacking gel (ml)
30% acrylamide 0.2% bisacrylamide	6.700	4.200	7.500	0.830
1.875 M Tris-HCl pH 8.8	2.000	2.250	2.250	-
0.6 M Tris-HCl pH 6.8	-	-	-	0.500
H_2O	1.100	5.900	2.600	3.600
10% (w/v) SDS	0.100	0.125	0.125	0.050
TEMED	0.025	0.025	0.005	0.005
10% APS	0.050	0.062	0.062	0.025

Sample buffer for protein electrophoresis
50 mM Tris-HCl pH 6.8, 2% SDS, 0.1% bromophenol blue and 10% glycerol; when necessary, 2-mercaptoethanol is added at a concentration of 5%.

SEM-buffer
0.25 M sucrose, 2 mM EDTA, 10 mM MOPS-KOH pH 7.2

SEMK-buffer
0.25 M sucrose, 2 mM EDTA, 10 mM MOPS-KOH pH 7.2, 80 mM KCl

SOB medium
2% Bacto-tryptone, 0.5% yeast extract, 0.5% NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$.

SOC medium
SOB medium plus 0.4% glucose.

Solution A for plasmid DNA extraction
20% glucose, 20 mM EDTA, 25 mM Tris-HCl pH 8.0.

Solution B for plasmid DNA extraction
200 mM NaOH, 0.8% SDS

Sonication buffer for IO-SMP
0.4 M sucrose, 5 mM MOPS-KOH pH 7.2-7.4, 20 mM $MgCl_2$.

Southern blotting denaturing solution
1.5 M NaCl, 0.5 M NaOH.

Southern blotting neutralisation solution
1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 1 mM EDTA.

Start buffer for His-trap column (Pharmacia)
20 mM phosphate buffer pH 7.2, 0.5 M NaCl, 10 mM imidazole.

TAE buffer
40 mM Tris-acetate pH 7.5, 1 mM EDTA.

TBS buffer
10 mM Tris-HCl, pH 8.0, 150 mM NaCl.

TFB buffer
10 mM MES/KOH pH 6.2, 100 mM $RbCl_2$, 45 mM $MnCl_2$, 10 mM $CaCl_2$, 3 mM hexaminecobalt chloride.

Top-agar
1 M sorbitol, 1 x Vogel's minimal medium, 1.5% agar, 2% sorbose, 0.05% frutose, 0.05% glucose, 0.02% myoinositol.

Trace elements stock
240 mM citric acid, 170 mM zinc sulphate, 26 mM ammonium iron (II) sulphate, 10 mM cooper sulphate, 3 mM manganese sulphate, 8 mM boric acid, 0.05% sodium molibdate, 0.001% cobalt chloride, 1% chlorophorm.

Transfer buffer for Western blot
20 mM Tris, 150 mM glycine, 20% methanol and 0.01% SDS.

Vogel's minimal medium
0.42 M sodium citrate, 2 M dihydrogen phosphate, 1,25 M ammonium nitrate, 64 mM magnesium sulphate, 34 mM calcium chloride, 0.2% chlorophorm, 5 x 10^{-4}% biotine, 0.5% trace elements stock.

5 x loading buffer for DNA agarose gels
15% Ficoll 400, 50 mM EDTA, 1 x TAE, 0.5% (w/v) SDS, 0.05% (w/v) bromophenol blue, 0.05% xylenecyanol.

10 x TBE
1 M Tris-borate pH 7.5, 20 mM EDTA.

20 x SSC
0.3 M tri-sodium citrate, 3 M NaCl.

3. STRAINS OF *E. coli* AND *N. crassa*

The *E. coli* strain XL1-blue (Stratagene) was used as a host for the cDNA of the *nde1* cloned at the polylinker of pBluescript (Stratagene), and for the pCRII-Topo (Invitrogen) with the open reading frame of *nde1*. DH5α and M15 (Qiagen Inc.) were used to host the

recombinant plasmid pCSN44 and the pQE-31 carrying the C-terminal region of *nde1*, respectively.

For creating bacterial stocks, 400 µl of 80% glycerol were added to 1 ml of the grown culture, and store at –20 ºC.

The *N. crassa* strains used were: the wild type strains 74-OR23-1A and 74-OR8-1a (Perkins, 1986), the mutants of complex I nuo12.3 (Duarte *et al.*, 1995), nuo20.8 (da Silva *et al.*, 1996), nuo21 (Ferreirinha *et al.*, 1999), nuo21.3a (Alves and Videira, 1994), nuo21.3c (Duarte and Videira, *in press*), nuo24 (Almeida *et al.*, 1999), nuo51 (Fecke *et al.*, 1994) and nde1 mutant from this work (Melo *et al.*, 2001).

4. GROWTH OF NEUROSPORA STRAINS

General manipulation of *N. crassa,* including crosses, was carried out according to Davis and de Serres (1970) and Perkins (1986). Race tubes were performed according to White and Woodward (1995). When required, the usual carbon source - sucrose- was replaced by mannitol, glycerol or acetate. The tubes were incubated at different temperatures (4, 26, 37 and 42 ºC) and the growth was registered along the time.

5. ISOLATION OF PLASMID DNA (Sambrook *et al.*, 1982)

5.1. Small-scale preparation of plasmid DNA for analytical purposes

LB-medium (1.5 ml) containing the adequate antibiotic was inoculated with a single bacterial colony, carrying the required plasmid and incubated overnight at 37 ºC with vigorous agitation. The cells were harvested by a 5-min centrifugation, at 16000 x *g*. The resulting pellet was resuspended in 80 µl solution of A, and left at room temperature for 5 min. Solution B (160 µl) was than added, carefully mixed and left on ice. After 5 min, 120 µl of 3 M potassium acetate was added, gently mixed, and incubated on ice for 5 min. The samples were centrifuged, at 16000 x *g*. Extraction with phenol:chloroform: isoamylalcohol (25:24:1, v/v) was carried out. One hundred and fifty microliters of the phenol mixture were added to the supernatant of each sample, mixed by shaking for 5 min and centrifuged for 5 min. The aqueous phases were precipitated with 800 µl of ethanol, at room temperature, for 5 min. After a 5-min centrifugation, the pellets were washed with 70% ethanol and re-centrifuged. The DNA was resuspended in 50 µl of H_2O plus 1 µl of RNase (Sigma) 2 mg/ml and incubated at 37 ºC for 10 min.

5.2. Large-scale preparation of plasmid DNA for preparative purposes

LB-medium (40 ml) with the adequate antibiotic was inoculated with 50 µl of a glycerol stock culture and incubated at 37 ºC, overnight, with vigorous agitation. Cells were harvested by a 10-min centrifugation at 2600 x *g*, resuspended in solution A (4 ml), and left at room temperature for 5 min. Eight ml of solution B was than added, mixed by carefully inverting the tubes and left on ice for 5 min. Four ml of 3 M potassium acetate was added and the samples were centrifuged at 4200 x *g*. After filtration, 0.6 volumes of isopropanol was added to the supernatant, and incubated at room temperature for 15 min. The samples were centrifuged at room temperature for 15 min, at 4200 x *g*, and the pellets washed with 70% ethanol, carefully dried and resuspended in 2 ml of H_2O at 65 ºC. RNA was precipitated with incubation of 2 ml of 5 mM LiCl on ice, for 5 min. A 4200 x *g* centrifugation, at 4 ºC, was performed to the samples. The supernatant was transferred to a new tube and

precipitated with 2 volumes of ethanol. After centrifugation, the pellets were washed and dried. The pellets were resuspended in 200 µl of H_2O at 65 °C and transferred into 1.5 ml tubes. Incubation with 5 µl of RNase 2 mg/ml at 37 °C, for 30 min was performed, followed by a 2-min centrifugation at 16000 x *g*. The supernatants were placed on ice with 300 µl 13% PEG 8000 and 1.6 µl 5M NaCl, for 20 min. A 16300 x *g* centrifugation at 4 °C was done. The pellets were washed with 70% ethanol and dried. After suspending, the samples were extracted first with phenol: chloroform:isoamylalcohol (25:24:1, v/v) and then with chlorophorm:isoamylalcohol (24:1). Upon centrifugation, the supernatant was precipitated by the addition of 0.1 volumes of 3 M sodium acetate plus ethanol and placed at –70 °C, for 30 min. The samples were centrifuged, washed with 70% ethanol and dried, before being resuspended in 200 µl of H_2O.

6. ENZYMES USED IN CLONING

6.1. Digestion of DNA with restriction enzymes (Sambrook *et al.*, 1982)

For analytical purposes, usually ~1 µg plasmid DNA was restricted with 1 unit of a specific endonuclease at 37 °C (unless otherwise indicated), for 3 h, in a final volume of 10 µl. For preparative purposes (DNA insert purification or vector isolation) 10 µg were digested in a volume of 50 µl using the desired restriction enzyme at a concentration of 2 units/µg DNA. The restriction enzymes used were purchased from Pharmacia and the 10 x *One Phor All* Buffer PLUS (100 mM Tris-acetate, pH 7.5, 100 mM magnesium acetate and 500 mM potassium acetate) was used. All digestions were stopped with 1/5 volume of 5 x loading buffer.

6.2. Dephosphorylation of DNA (Chaconas and van de Sande, 1980)

The 5' phosphates were removed from DNA by treatment with calf intestinal alkaline phosphatase (CIP) from Pharmacia. Plasmid DNA was completely digested by restriction enzymes producing a linearised vector. The reaction mixture was separated by agarose gel electrophoresis, the band corresponding to the cut vector was excised and eluted, and the DNA was incubated with 44 µl H_2O, 5 µl 10 x CIP buffer (0.5 M Tris-HCl, pH 9.0, 10 mM $MgCl_2$, 1 mM $ZnCl_2$ and 10 mM spermidine) and 0.5 µl CIP. To dephosphorylate the protruding 5' ends, the reaction started at 37 °C, for 30 min, after which a second aliquot of CIP (0.5 µl) was added, and the incubation continued for another 30 min. To dephosphorylate DNA with blunt ends or with recessed 5' termini, the reaction was incubated for 15 min at 37 °C and then 15 min at 56 °C, another 0.5 µl of CIP were added and the incubations repeated. After extracting the mixture with phenol:chlorophorm:isoamylalcohol (25:24:1, v/v), the DNA in the aqueous phase was precipitated with ethanol and resuspended in a suitable volume of H_2O.

6.3. Ligation of DNA

When theDNA carried cohesive ends, their ligation into CIP-treated vectors was performed in a reaction mixture containing ~50 ng DNA in an approximate proportion of 1(vector): 5 (insert) in a final volume of 20 µl of 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM DTT, 1 mM spermidine, 1 mM ATP and 0.1 mg/ml BSA, plus 5 units of T4 DNA-ligase and incubated overnight at 4 °C. In the case of blunt-ended DNA the same conditions were used except that the ratio vector:insert was 1:10.

6.4. PCR of DNA

The PCR took place in a reaction mixture containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.2 mM nucleotice mix (dATP, dCTP, dGTP, dTTP), 2.5 ng DNA template, 1.25 pmol/µl each primer, 1.25 units of *Taq* DNA polymerase (GibcoBRL) in a final volume of 50 µl. The reaction mixture was incubated for 5 min, at 95 °C, to completely denature the template. After denaturation, 30 cycles of 95 °C, 1 min, 60 °C, 2 min, 72 °C, 1 min were performed. A final extension step of 10 min at 72 °C was carried out. The annealing period and temperature varied with the size of the PCR product and the specificity of the primers to the template, respectively.

6.5. Transformation of *E. coli* with plasmid DNA (Hanahan, 1983)

SOB medium (100 ml) was inoculated with 1 ml of a stationary phase culture of *E. coli* DH5α, XL1-blue or M15. The culture was grown at 37 °C with vigorous agitation for 2-3 h until the $O.D._{550\ nm}$ reached 0.6. Cells were left on ice for 15 min and centrifuged at 4 °C, 10 min at 1500 x g. The supernatant was discarded and cells carefully resuspended in 30 ml TFB and placed on ice for 15 min. After centrifugation, the bacteria were resuspended in 2 ml TFB and placed on ice. Addition of 70 µl DMSO was carried out and the suspension was mixed and left on ice for further 5 min. Then 70 µl DTT (2.25 M) was added, incubated for 10 min and finally another 70 µl DMSO was added. After 5 min, cells were divided into aliquots (200 µl) and 10 µl of the ligation mixture was added and incubated for 30 min, on ice. Samples were moved into a water bath (42 °C) for 90 sec and immediately re-placed on ice for 2 min). SOC medium (800 µl) was added and the cells were shaken at 37 °C for 1 h. Usually, 100 µl of each transformation sample were plated onto LB-agar plates containing the appropriate antibiotic (50 µg/ml ampicilin). The agar plates were incubated at 37 °C overnight. Single cell colonies were picked and plasmid DNA was extracted.

6.6. Labelling a DNA probe (Gene images, Amersham Pharmacia Biotech)

Denatured DNA (50 ng), Klenow (1 U), nucleotide mix (10 µl), primer (5 µl) and H_2O up to 50 µl were gently mixed in a 1.5 microcentrifuge tube and incubated at 37 °C, for 1 h.

7. GEL ELECTROPHORESIS OF DNA

7.1. Agarose gel electrophoresis

Agarose gel electrophoresis was routinely used to separate, identify and purify DNA fragments. Agarose 0.8% and 2% (w/v) in TAE buffer was dissolved in the microwave oven and cooled to below 50 °C. Ethidium bromide was added to a final concentration of 0.5µg/ml. The gel was poured and allowed to set. Gels were run in a horizontal gel trough and the electrophoresis buffer was 1 x TAE. Samples to be run on the gel were mixed with 1/5 volume of 5 x loading buffer. Usually, lambda phage digested with *Hin*dIII was loaded (~0.5 µg) as a molecular weight marker. The gels were run at 120 mV, at room temperature, and progress monitored with UV light.

7.2. DNA sequencing gels

Urea-containing polyacrylamide gels were used to resolve DNA sequencing reactions. Urea (21 g) was dissolved in 6 ml 10 x TBE and 6 ml Long Ranger gel solution from FMC BioProducts and H_2O (in a final volume of 50 ml). The solution was filtered and degassed, 250 µl of 10% APS and 25 µl TEMED was added, mixed gently and poured between two glass plates (58 cm x 21 cm x 0.4 mm). Before using, the glass plates were stringently cleaned with ethanol and polished afterwards, one of them was polished with 1 ml of Repel-Silane from Pharmacia. The gels polymerised overnight before use. The gels were pre-warmed by running for 20 min (45 W; electrophoresis buffer 0.6 x TBE) and the wells were washed with the buffer prior to loading 3-4 µl of the sequencing reaction. Samples were pre-incubated at 95 °C for 3 min and rapidly cooled on ice. The running time depended on which region of the sequence reaction to be read. After electrophoresis, the glass plates were carefully separated, the gel was transferred onto Whatman 3MM paper and dried under vacuum at 80 °C for 2 h. The gels were autoradiographed (Kodak, BioMax, MR Film), allowing visualisation of the sequence.

7.3. Isolation of DNA bands from agarose gels

DNA inserts or linearised vectors to be purified were separated from other contaminating DNA by agarose gel electrophoresis. The desired fragment was identified under the UV lamp (366 nm) and a slice of agarose containing the band was cut. The DNA was then extracted using a Concert Nucleic Acid Purification System (GibcoBRL).

8. ISOLATION OF GENOMIC DNA FROM *N. crassa* (Lee *et al.*, 1988; Videira, *et al.*, 1993)

Conidia were inoculated in 40 ml of Vogel's minimal medium and set to grow for 24 h, with agitation. The grown mycelium was harvested in a Buchner funnel and washed with H_2O. The dried mycelium was transferred to a pre-cooled mortar, in the presence of liquid nitrogen and macerated with a pestle. The macerate was resuspended in 750 µl lysis solution, transferred to a 2 ml cup and incubated for 1 h at 65 °C. It was extracted with phenol:chlorophorm:isoamylalcohol (25:24:1, v/v) as follows: 700 µl of phenol:chlorophorm:isoamylalcohol were added and vortexed with the former mixture. The sample was centrifuged at room temperature, at 16300 x *g* for 10 min. The aqueous phase (600 µl) is transferred to another cup and vortexed with 700 µl of chlorophorm:isoamyloalcohol (24:1, v/v). The centrifugation step was repeated for 5 min. The aqueous phase was transferred to another cup and the DNA was precipitated with 20 µl sodiumacetate, pH 8.0 and 1 ml isopropanol, the cups were gently inverted several times until the DNA was observed. The DNA was recovered in the pellet after a 30 s centrifugation in the same conditions as before. The pellet was carefully resuspended in 300 µl H_2O with 1 µl RNAse (10 mg/ml) and incubated at 37 °C for 15 min. The extractions with phenol:chlorophorm:isoamylalcohol and chlorophorm:isoamyloalcohol were repeated and the resulting supernatant precipitated with 10 µl sodium acetate plus 1 ml ethanol. The cups were gently inverted until the precipitation of DNA was observed. Centrifugation was performed for 30 s, and the pellet was washed with 70% ethanol. The pellet was dried under vacuum for 15 min and resuspended in 100 µl of H_2O. DNA was stored at –20 °C.

9. ANALYSIS OF GENOMIC DNA BY SOUTHERN BLOTTING (Southern, 1979)

Genomic DNA isolated from the wild type and the transformants of *N. crassa* was digested with various restriction enzymes and the DNA fragments were separated on a 0.8% (w/v) agarose gel. After electrophoresis, the gel was rinsed with distilled water and soaked with denaturing solution with gentle agitation, for 30 min at room temperature and then twice for 15 min in neutralisation solution. The denatured DNA was transferred to a Hybond-N+ membrane (Amersham Pharmacia Biotech) with a Vacuum Blotter (Appligene) for 45-60 min, at 40-60 mbar. Over the transfer, the gel was covered with 20 x SSC. The slots of the gel were marked in the nylon membrane and the DNA was fixed under UV light for 2 min. The membrane was rinsed with 5 x SSC and incubated at 65 °C with hybridisation solution. After 30 min, the denatured DNA probe was added and the incubation proceeded overnight. The used probe was a 5' 2000 bp *Eco*RI/*Hind*III fragment of the *nde1* cDNA, labelled with the Gene Images random prime-labelling module (Amersham Pharmacia Biotech). The membranes were washed for 15 min at 65 °C, first in 1 x SSC plus 0.1% SDS and then with 0.5 x SSC plus 0.1% SDS. Detection was carried out using Gene Images CDP-*star*™ (Amersham Pharmacia Biotech).

9.1 Mapping of the *nde1* gene from *N. crassa*

For gene mapping, the segregation of a *Sal*Irestriction fragment length polymorphism was analysed in the 38 strains (FGSC nos. 4450-4487) of the Multicent-2 cross kit (Metzenberg *et al.*, 1984; Videira *et al.*, 1990a). The probe used was a 2 kb *Eco*RI/*Hind*III fragment of the cDNA labelled with Gene Images (Amersham Pharmacia Biotech).

10. TRANSFORMATION OF NEUROSPORA SPHEROPLASTS (Vollmer and Yanofsky, 1986)

10.1. Preparation of spheroplasts

The strain of Neurospora from which we wished to isolate the spheroplasts was set to grow for 7 days, on solid medium, after which a suspension of conidia was prepared. Vogel's (0.5 x) minimal medium (1 l) with the appropriate supplements was inoculated with 5 x 10^6 conidia per ml and incubated at 26 °C with mild agitation. When more than 80% conidia were germinated, the suspension was centrifuged at 4 °C, 4200 x *g* for 10 min. The pellet was resuspended in 10 ml ice-cold H_2O and re-centrifuged at 4 °C, 5000 x *g* for 10 min. Conidia were resuspended in 10 ml cold 1 M sorbitol and centrifuged twice as before. Sorbitol-washed conidia were resuspended in 1 M sorbitol at a final concentration of 10^9 cells/ml and added Novozyme (InterSpex Products) to a final concentration of 15 mg/ml. The conidia were incubated for 1-2 h with gentle agitation, at 30 °C. The formation of spheroplasts was checked adding 10 µl of 10% SDS to a drop of the incubation mixture, looking for the formation of ghosts. When more that 90% spheroplasts were achieved, the mixture was centrifuged at 4 °C, for 10 min at 120 x *g*. Spheroplasts were washed twice in 1 M sorbitol and once in MCS (Section 2., this chapter), and resuspended in 6 ml MCS. The concentration was adjusted to 5 x 10^8 cells/ml with MCS (13 µl DMSO, 0.325 mg heparin and 275 µl of PMC (Section 2., this chapter) were added to each ml of spheroplasts) and spheroplasts aliquoted and stored at –70 °C.

10.2. Transformation of spheroplasts

The DNA was added to the spheroplasts (previously thawed on ice) in a volume about 60% spheroplasts'. The transformation solution was gently mixed and left on ice for 30 min. PMC was added in a volume about 10 times that of spheroplasts plus DNA, gently mixed and incubated at room temperature for 20 min. The transformation reaction was mixed with 10 ml of top-agar, poured into plates of Vogel's minimal medium containing the adequate antibiotic and incubated at 30 to 40 ºC until germination was observed.

11. DISRUPTION OF THE *nde1* GENE BY REPEAT-INDUCED POINT MUTATIONS

The cDNA of the gene to be disrupted was cloned in the pCSN44 plasmid and the recombinant vector was used to transform Neurospora spheroplasts. Individual transformants were selected on hygromycin B (Sigma) plates (200 μg/ml). These were purified by several asexual transfers in slants with Vogel's minimal medium plus 150 μg/ml of hygromycin B, and used to prepare genomic DNA. A single copy transformant was selected, by Southern blotting of genomic DNA restricted with several endonucleases, and used to cross with the wild-type strain 74-OR23-1A, to obtain mutants by repeat-induced point mutations (Selker and Garrett, 1988; Alves and Videira, 1994). Identification of nde1 mutants was performed using Western blots of total mitochondrial protein immunodecorated with an antiserum against NDE1 (Melo *et al.*, 1999).

12. ISOLATION OF MITOCHONDRIA FROM *N. crassa*

12.1. Preparation of mitochondria (Werner, 1977)

Hyphae (1 g wet weight) were ground with quartz sand (1.5 g) in isolation medium plus 1 mM PMSF, added from a freshly prepared stock solution in ethanol, with a mortar and pestle on ice (small scale preparation) or using a grind-mill in a cold room (large scale preparation). PMSF was omitted in experiments where protease treatment was involved. Cell-free homogenate was obtained by centrifuging the resulting slurry twice for 5 min at 2000 x *g* and discarding pellets. A crude mitochondrial fraction was obtained by centrifuging the resulting supernatant for 20 min at 17400 x *g*. The pellet was washed in isolation medium and re-centrifuged under the same conditions. All the steps were carried out at 4 ºC.

12.2. Purification of mitochondria (Struglics *et al.*, 1993)

When required, a purification step was added to the isolation procedure. A discontinuous Percoll gradient (section 2., this chapter) was prepared. Crude mitochondria were loaded on top of the gradient. A brown band was formed in the middle of the Percoll gradient, formed by centrifugation at 48000 x *g* for 1 h. Mitochondria were taken from the gradient and washed in isolation medium as many times as necessary to get remove of Percoll.

12.3. Preparation of IO-SMP (Rasmusson and Møller, 1991)

In order to obtain IO-SMP mitochondria were sonicated 5 x 5 s in sonication buffer and centrifuged for 16 min at 48000 x *g*. The supernatant was then centrifuged at 105000 x *g*, for 65 min, and the final pellet was resuspended in a suitable volume of isolation medium.

13. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (Laemmli, 1970)

Vertical gel electrophoresis was performed according to Laemmli (1970). The gels were prepared between two glass plates (130 mm x 160 mm x 0.75 mm) according to the table in section 2. Samples were prepared in sample buffer for electrophoresis. The gel was run for 3 h at 25 mA in electrophoresis buffer. Gels were then stained with 0.8% Coomassie Brilliant Blue in destain solution for 20 min, washed with water and destained by shaking in the destaining solution or used for Western blot (see below).

14. WESTERN BLOTING (Towbin *et al.*, 1979; Videira and Werner, 1989)

14.1. Semi-dry

After electrophoresis, the gel was placed onto a nitrocellulose membrane, in turn placed on 5 pieces of Whatman paper 3MM imbedded in transfer buffer. Over the gel lay another set of 5 pieces of Whatman paper 3MM imbedded in blot buffer. 250 V were applied to the semi-dry apparatus, for 75 min.

14.2. Wet-blot

After electrophoresis, the gel was placed on 3 pieces of Whatman paper 3MM imbedded in blot buffer. A nitrocellulose membrane was placed on the gel and covered by 3 imbedded pieces of Whatman paper 3MM. After sealing, the blot chamber was lowered into a tank containing blot buffer where the transfer took place overnight, at 60 mA.

15. IMMUNODECORATION OF PROTEINS

After electric transfer of the proteins, the nitrocellulose membrane was blocked at room temperature with agitation, with 5% light milk powder in TBS. After 1 h, it was transferred into the mixture of antibodies and the incubation proceeded for 3 h. The membrane was then washed 5 min in TBS, 5 min in TBS plus 0.05% TX-100 and again 5 min in TBS. The membrane was then incubated with a second antibody, which was an anti-rabbit alkaline phosphatase-conjugated (Sigma) or an anti-rabbit peroxidase-conjugated (Amersham Pharmacia Biotech). After 1 h, the membrane was washed as before. Detection took place in alkaline phosphatase buffer with 1:1:1000 75 mg/ml nitroblue tetrazolium, 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, or with the ECL detection system (Amersham Pharmacia Biotech), when using the anti-rabbit alkaline phosphatase-conjugated or the anti-rabbit peroxidase-conjugated, respectively.

16. EXPRESSION AND PURIFICATION OF A NDE1-FUSION PROTEIN AND PREPARATION OF ANTIBODIES

16.1. Expression of NDE1

In order to express NDE1, the 3' 1794 base pairs of its open reading frame were amplified by PCR using the primer 5'-CCTCTAGGATCCCTCGACCTACAGC-3' (the underlined bases were modified from the *nde1* cDNA to introduce a *Bam*HI restriction site) and a vector-specific primer. The PCR product was restricted with *Bam*HI and *Kpn*I, cloned in pQE-31 previously digested with the same enzymes and transformed into M15. The stock of M15 carrying the recombinant plasmid was inoculated with 50 μg/ml ampicilin and 25 μg/ml kanamycin overnight. The overnight culture was diluted 1:200 and grown for a further 3 h, at 37 °C, with agitation, period after which 1 mM IPTG was added. After 3 h, the cells were harvested by centrifugation (10 min at 2600 x *g*). The pellet was resuspended in 1/10 of the inicial volume in a buffer containing 50 mM Tris-HCl pH 8.0, 0.2 mM EDTA, 100 μg/ml lisozyme, 1/10 of the volume 1% TX-100. Upon a 30-min incubation at 37 °C, it was sonicated 2 x 10 s. The suspension was centrifuged for 15 min, at 12000 x *g*, and the pellet and supernatant fraction were analysed by SDS-PAGE. All centrifugations were run at 4 °C.

16.2. Purification of NDE1

The pellet (of the last centrifugation, section 16.1.) with the recombinant protein (the 579 C-terminal amino acid residues of NDE1 fused to a His-tag), was resuspended in 15 ml of start buffer plus 8 M urea. A sonication of 3 x 10 s was done and the mixture was placed on ice. After 1 h, a centrifugation (10 min, 34500 x *g*) was carried out followed by a 0.45 μm filtration of the supernatant, and the protein was purified by chromatography in a His-trap column (Pharmacia). The obtained protein was further purified by SDS-PAGE, transferred onto a nitrocellulose membrane (see below) and used to immunise rabbits.

16.3. Rabbit immunisation (Videira and Werner, 1989)

The band corresponding to the expressed protein (300-500 μg) was cut from the nitrocellulose membrane, dissolved in a suitable volume of DMSO and mixed with the same volume of complete Freund's adjuvant. After homogenisation through a needle, the mixture was subcutaneously injected into the rabbit. New injections were performed every 15 days (complete adjuvant of Freund was then replaced by incomplete adjuvant of Freund) and 10 days after the third immunisation a sample of blood was taken to test the reactivity of the serum. Several injections were necessary to get good reactivity.

To purify the antiboddies, blood was left at room temperature for 2 h and placed at 4 °C for further 2 h. The supernatant was then decanted, centrifuged to remove cells, and stored at –20 °C.

17. *IN VITRO* TRANSLATION OF PRECURSORS IN A RABBIT RETICULOCYTE SYSTEM

The reticulocyte lysates containing the *in vitro* synthesised precursors were prepared with the single-step transcription-translation system – TNT from Promega using the ORF of *nde1* gene, cloned at pCR II TOPO, as template for sp6 RNA polymerase. ^{35}S-Methionine

was the radioactively labeled amino acid used. The lysates were divided into 20 µl aliquots and stored at –80 °C.

18. IMPORT OF NDE1 AND F1β PRECURSORS INTO NEUROSPORA AND YEAST MITOCHONDRIA

Neurospora (500 µg protein) and yeast (60 µg protein) mitochondria were incubated at 25 °C in import buffer (final volume 150 µl and 300 µl, respectively). 0.3 µM valinomycin was used when necessary to abolish the membrane potential. In that case the NADH (Succinate), ethanol, creatine phosphate and creatine kinase were not present in the medium. After 10 min, 3-5 µl of the lysates were added to the import mixture, and the incubation proceeded for 20 min. The import mixtures were then divided in 3 cups (A, B and C), centrifuged for 10 min at 12900 x *g* at 4 °C, resuspended in non-swelling buffer and placed on ice.

18.1. Neurospora mitochondria

A. 50 µl import reaction
B. 50 µl import reaction + 80 µg/ml proteinase K, 30 min on ice. Stopped with 1 mM PMSF.
C. 50 µl import reaction + 10 mM Na_2CO_3, 30 min on ice. The sample was resolved into pellet and supernatant by centrifugation at 105000 x *g* at 4 °C.

Samples A and B were centrifuged once more and resuspended in sample buffer for electrophoresis. The pellet C was resuspended in sample buffer for electrophoresis, while the supernatant was precipitated with 10% trichloroacetic acid, incubated on ice for 30 min, centrifuged for 20 min at 12900 x *g* at 4 °C and washed with acetone. After drying, the pellet was resuspended in sample buffer for electrophoresis.

18.2. Yeast mitochondria

A. 90 µl import reaction + 810 µl buffer 1
B. 90 µl import reaction + 810 µl buffer 1 + 50 µg/ml proteinase K
C. 90 µl import reaction + 810 µl (20 mM Hepes pH 7.2) + 50 µg/ml proteinase K

Samples were incubated on ice for 30 min, 1 mM PMSF was added and the incubation proceeded for further 5 min. Following incubation, mitochondria were centrifuged and resuspended in buffer 1 plus 80 mM KCl. The centrifugation step was carried out once more and the samples were resuspended in sample buffer for electrophoresis. All samples were cooked at 95 °C and resolved by SDS-PAGE.

Note: Sometimes, instead of proteinase K, trypsin was used at a concentration of 30 µg/ml, 15 min on ice, and the reaction was stopped by a 15-min incubation with 0.8 mg/ml soybean trypsin inhibitor.

19. PROTEIN DETERMINATION (Bradford, 1976)

Mitochondrial suspension (10 µl) was solubilized with Triton X-100 (6 µl) in H_2O (84 µl). Appropriately diluted standards of BSA and mitochondrial samples were mixed with 1 ml of 1:4 diluted dye reagent:H_2O (BioRad Protein Assay). Absorbency at 595 nm was

measured at room temperature, after 5 min. The protein concentration was calculated from the standard curve obtained.

20. ENZYME ASSAYS

The quality of the preparations was assessed measuring the latent activities of malate dehydrogenase and cytochrome oxidase.

20.1. Cytochrome *c* oxidase (EC 1.9.3.1) (Rasmusson and Møller, 1990)

Cytochrome *c* oxidase was measured spectrophotometrically (Shimazu 300) at 550 nm in a medium containing 0.3 M sucrose, 50 mM Tris-acetate (pH 7.2), 45 μM cytochrome *c*, and 100 mM KCl. Triton X-100 (0.025% (w/v) was added to solubilize the membranes, which allowed the measurement of total activity. The assays started with the addition of 5 μl of mitochondrial protein. Latency (%) was calculated as:

$$100 \times [(\text{Rate} + \text{Triton}) - (\text{Rate} - \text{Triton})]/\text{Rate} + \text{Triton}.$$

20.2. Malate dehydrogenase (EC 1.1.1.37) (Møller *et al.*, 1987)

Malate dehydrogenase activity was measured spectrophotometrically (Shimazu 300) at 340 nm, in a medium containing 0.3 M sucrose, 20 mM MOPS (pH 7.0), 0.2 μg/ml antimycin A, 2 mM oxaloacetate and 0.2 mM NADH. Triton X-100 (0.025% (w/v) was added to solubilise the membranes allowing the measurement of total activity. The assays started with the addition of 5 μl protein. Latency was calculated as for 18.1.

20.3. Adenylate kinase (EC 2.7.4.3) (Schmidt *et al.*, 1984)

Adenylate kinase activity was assayed following the oxidation of NADH at 366 nm. A mixture containing 54 μl of solution A, 9 μl of 100 mM ATP, 9 μl of 3mg/ml oligomycin, 9 μl of 1 mM rotenone, 18 μl of the mix pyruvate kinase/lactate dehydrogenase, 50-100 μl of sample and 1.8 ml of solution B was prepared. The mixture was divided in two cuvettes (850 μl each) and the reaction was started with the addition of 5 μl 21 mM AMP to one of them.

Solution A: 700 μl of 28 mM NADH + 350 μl of 51 mM phophoenolpyruvate.
Solution B: 130 mM KCl, 6 mM $MgCl_2$, 100 mM Tris-HCl, pH 7.5.
Mix pyruvate kinase (EC 2.7.1.99)/lactate dehydrogenase (EC 1.1.2.4): 50 μl of 2mg/ml pyruvate kinase plus 7 μl of 5 mg/ml lactate dehydrogenase plus 1 ml 50% (v/v) glycerol in water.
Sample: 17.5 μg mitochondrial protein solubilized with 1% Triton X-100.
Oligomycin and rotenone were prepared in ethanol and acetone, respectively.

21. OXYGEN ELECTRODE MEASUREMENTS

Respiration was measured polarographically at 25 °C with an Hansatech oxygen electrode in a total volume of 1 ml. For IO-SMP (0.3-0.5 mg protein) a medium containing 0.3 M sucrose, 10 mM potassium phosphate, pH 7.2, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM KCl, 4 μM CCCP and 0.02% (w/v) BSA was used. Assays with 0.3-0.5 mg of mitochondrial protein were performed in a medium as described for IO-SMP or for the pH experiment in a

medium containing 20 mM MES, 20 mM Tris, 20 mM MOPS, 0.3 M sucrose, 0.1 mM $CaCl_2$, 4 µM CCCP and 0.02% (w/v) BSA, adjusted to pH 4.7 – 9.2 with KOH. For calcium depletion 1 mM EGTA was added. The assays started with the addition of either 1 mM NADH or 1 mM NADPH. When used, rotenone and antimycin A were added to final concentrations of 20 µM and 0.2 µg/ml, respectively.

22. DIGITONIN FRACTIONATION OF MITOCHONDRIA

Prior to use, digitonin was recrystalised in hot ethanol and dissolved in a buffer containing 0.25 M sucrose, 2 mM EDTA and 10 mM MOPS-KOH, pH 7.2 (SEM-buffer) with 80 mM KCl (SEMK). For fractionation of mitochondria, 10 cups of 1.5 ml were placed on ice with 7 µl of digitonin solution of increasing digitonin concentrations and, when desired, with protease (50 µg/ml proteinase K or 30 µg/ml trypsin). The assay was started by the addition of 13 µl of 10 mg/ml mitochondria. After 30 s, 80 µl SEMK was added and the incubation proceeded to complete protease treatment. The reactions were stopped after 30 min by the addition of 1 mM PMSF, or after 15 min by the addition of 800 µg/ml soybean trypsin inhibitor, when using proteinase K or trypsin, respectively. Mitochondria were isolated by centrifugation and resuspended in sample buffer for electrophoresis.

23. ALCALINE EXTRACTION OF MITOCHONDRIAL PROTEINS (Videira *et al.*, 1993)

Mitochondria (300 µg) were incubated with 0.1 M Na_2CO_3 on ice. After 1 h, the sample was resolved into pellet and supernatant by a 1h centrifugation at 105000 x *g*. The proteins from the resulting fractions were separated by SDS-PAGE and analysed by Western blot with the desired antibodies.

24. PROGRAMS USED IN DATA PROCESSING

Programs available at the Infobiogen Internet site (www.infobiogen.fr) were used for molecular biology analysis of data. The program Blast, at NCBI, (www.ncbi.nlm.nih.gov/BLAST) was used in DNA and protein homology searches. The MIPS Internet site (www.mips.mpg.de) was employed in searching for mitochondrial targeting sequences, and the hydrophobic pattern of proteins as well as other protein structural information was obtained at Pedro's Biomolecular Research Tools site of the Internet (www.public.iastate.edu/~pedro/research_tools.html).

Chapter IV - References

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