

MOLECULAR BIOLOGY CHARACTERISATION OF MITOCHONDRIAL NADH DEHYDROGENASES



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ABBREVIATIONS

ACP	Acyl Carrier Protein
ADP	Adenosine Diphosphate nucleotide
AOX	Alternative Oxidase
ATP	Adenosine Triphosphate nucleotide
ATPase	Adenosine Triphosphatase
bp	base pairs
BN-PAGE	Blue Native-Polyacrylamide Gel Electrophoresis
CN-PAGE	Colorless Native-Polyacrylamide Gel Electrophoresis
cyt	cytochrome
COX	Cytochrome c Oxidase
DDM	n-Dodecylmaltoside
DIG	Digitonin
DNA	Deoxyribonucleic acid
EPR	Electron Paramagnetic Resonance
FAD	Flavin adenine dinucleotide
Fe_xS_x	iron sulfur cluster
FMN	Flavin Mononucleotide
kDa	kilo Dalton
LDAO	Lauryl <i>N,N</i> -dimethylamine <i>N</i> -oxide
LHON	Leber's Hereditary Optic Neuropathy
MALDI-TOF-MS	Matrix-assisted laser desorption ionization Time-Of-Flight Mass Spectrometry
MDa	Mega Dalton
MELAS	Mitochondrial Encephalomyopathy with Lactic Acidosis and Stroke-like episodes
MERRF	Myoclonic Epilepsy with Ragged-Red Fibers
mtDNA	mitochondrial DNA
nDNA	nuclear DNA
Na⁺-NQR	NADH:quinone oxidoreductase capable of pumping Na ⁺
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate) (reduced form)
ND	NADH Dehydrogenase
NDH-1	Type I Bacterial NADH:quinone oxidoreductase or NADH Dehydrogenase
NDH-2	Type II NAD(P)H:quinone oxidoreductase or NADH Dehydrogenase
nuo(x)	<i>N. crassa</i> mutant of the <i>x</i> kDa subunit of the NADH:quinone oxidoreductase
Nuo(x)	subunit <i>x</i> of the bacterial (<i>e.g. Escherichia coli</i>) NADH:quinone oxidoreductase
Nqo(x)	subunit <i>x</i> of the bacterial (<i>e.g. Thermus thermophilus</i>) NADH:quinone oxidoreductase
OXPHOS	Oxidative Phosphorylation System
Pi	Inorganic phosphate
Q	Quinone
QH₂	reduced quinone
rRNA	ribosomal ribonucleic acid
tRNA	transfer ribonucleic acid
ROS	Reactive Oxygen Species
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SHAM	Salicylhydroxamic acid
TX-100	Triton X-100

For consistency, the following rules have been applied concerning the subunit nomenclature: the *N. crassa* nomenclature is used when referring to subunits of the Neurospora enzyme (with the bovine nomenclature in parentheses when different). The bovine nomenclature is used when referring to subunits of bovine complex I, or homologous subunits of human and *E. coli* (with the Neurospora nomenclature in parentheses when different).

SUMMARY

The mitochondrial complex I is a large L-shaped multisubunit structure of approximately 1 MDa, consisting of two perpendicular domains: the hydrophobic membrane arm and the hydrophilic peripheral arm. This complex catalyses the transfer of two electrons from NADH to quinone, coupled to the translocation of four protons across the inner mitochondrial membrane. In most prokaryotic species, the NDH-1 complex (Type 1 Bacterial NADH Dehydrogenase) is composed of 14 dissimilar subunits that represent the minimal functional form of the enzyme, all of which have homologues in the mitochondrial complex I of eukaryotes. Seven of the eukaryotic homologous subunits are encoded by mitochondrial DNA (ND1–6 and ND4L) and are located in the membrane domain, while the remaining seven subunits are nuclear-encoded and belong to the peripheral domain of the enzyme.

The *Neurospora crassa* complex I is composed of at least 39 different polypeptide subunits, of which 35 are conserved in mammals. Fourteen subunits of the fungal complex represent the core subunits found in prokaryotes and the remaining 25 are entitled accessory subunits. While for the 14 central subunits, primary roles have been established, the function of most of the accessory subunits is still not clear. The 9.8 kDa and the 11.5 kDa proteins, homologues of the mammalian MWFE and 15 kDa subunits, respectively, are considered accessory subunits. Both proteins are conserved among eukaryotes and were located in the membrane domain of the fungal enzyme. The corresponding encoding genes were identified in the genome of *N. crassa* and separately inactivated by RIP (repeat-induced point-mutations). The resulting null-mutant strains were isolated and characterised. Analysis of the *N. crassa* null-mutants, lacking respectively the 9.8 kDa (MWFE) and the 11.5 kDa (15 kDa) subunits, revealed that both proteins are essential for the assembly and activity of complex I, and their absence result in the accumulation of intermediates of the enzyme. Interestingly, a similarity between the MWFE homologues and the *nqrA* subunit of bacterial Na^+ -translocating NADH:quinone oxidoreductases was found, suggesting a relationship between H^+ - and Na^+ -pumping NADH dehydrogenases.

It is recognised that the *N. crassa* complex I exists, similarly to the mammalian complex I, as a mixture of two interconvertible active (A) and de-activated (D) forms, the latter being sensitive to thiol (SH)-reagents. The cysteine-rich 15 kDa subunit of bovine complex I was considered the most likely candidate for the specific labelling by a fluorescence analogue of NEM in the de-activated form of the enzyme. Thus, the four conserved cysteine residues of the homologous 11.5 kDa subunit of *N. crassa* were separately replaced by serine residues and independently expressed in a null-mutant *Neurospora* strain, in order to investigate its role in the A/D transitions. All of the four strains, genetically altered by site-directed mutagenesis, are able to assemble complex I with kinetic properties similar to those observed in the wild-type enzyme. This indicates that none of the

cysteine residues of the 11.5 kDa protein is individually important for the A/D transition and likely the protein does not participate in the phenomenon. The main effect observed in these mutant strains was a lower amount of assembled complex I, which indicates that the cysteine residues are important for the acquisition and/or stabilization of the 11.5 kDa protein conformation and complex I assembly.

The arrangement of the *N. crassa* respiratory chain enzymes in the inner mitochondrial membrane was investigated by gentle solubilisation of the protein complexes with digitonin and subsequent separation of the solubilised extracts by 2D BN- or CN-/SDS-PAGE and 2D BN- or CN-/BN-PAGE. It was found that *Neurospora* wild-type mitochondria contain large respiratory supercomplexes comprising complexes I, III and IV (I_1IV_1 , I_1III_2 and $I_xIII_yIV_z$) as well as smaller ones (III_2IV_{1-2}). Furthermore, complex IV and half of the total ATP synthase were recovered in dimeric (IV_2 and V_2) and monomeric forms. Interestingly, the individual complex III dimer (III_2) shows a migration similar to complex V monomer, with a molecular weight of about 600 kDa, which is probable due to lipids bound to the former complex. Overall, the mitochondrial respiratory chain of *N. crassa* is organised according to the respirasome model described for mammals, in which complexes I:III:IV exist in a 1:3:6 stoichiometry.

The role of complex I in the formation of the other respiratory complexes and supercomplexes in *N. crassa* was also investigated in eight complex I mutant strains. The *Neurospora* complex I null-mutant strains nuo21, nuo29.9 and nuo51 able to assemble complex I, only lacking the respective subunit, have wild-type-like supercomplexes. The stable I-III-IV-supercomplexes in the nuo51 mutant have no NADH oxidase activity, representing the first examples of inactive respirasomes and demonstrating that supercomplexes have major non-enzymatic importance. Four of the mutants analysed (nuo9.8, nuo11.5, nuo14 and nuo20.8), lacking each a subunit of the membrane arm, and the nuo30.4 mutant, missing the 30.4 kDa subunit of the peripheral arm, all without fully assembled complex I, possess III-IV-supercomplexes (III_2IV_{1-2}). The membrane arm mutants analysed possess high-molecular-weight species of the 30.4 kDa subunit, possibly co-migrating with III-IV-supercomplexes, suggesting that these supercomplexes may be involved in assembly/stabilization of complex I. Moreover, the peripheral arm of complex I, which accumulates in the four membrane arm mutant strains, was identified by electrophoresis as a specific band with an apparent mass of 450 kDa displaying NADH dehydrogenase and deamino-NADH dehydrogenase activities. All mutant strains analysed contained similar amounts of complexes IV and V, as monomers and dimers, as well as of complex II. These results demonstrate that the absence of the holoenzyme does not influence the assembly of the other OXPHOS complexes of the mitochondrial respiratory chain of *Neurospora*. Additionally, 67 different proteins from wild-type *N. crassa* mitochondria were identified by MALDI-TOF-MS. Among them, 31 subunits belong to the five OXPHOS complexes of the

respiratory chain and the remaining 36 are non-OXPHOS proteins, such as metabolic pathways enzymes, chaperones and carriers.

RESUMO

O complexo I mitocondrial é uma grande estrutura multimérica em forma de L, com aproximadamente 1 MDa, que consiste em dois domínios perpendiculares: o braço membranar hidrofóbico e o braço periférico hidrofílico. Este complexo catalisa a transferência de dois electrões desde o NADH até à quinona, acoplada à translocação de aproximadamente quatro protões através da membrana mitocondrial interna. Na maioria das espécies procariotas, o complexo NDH-1 (NADH Desidrogenase bacteriana tipo 1) é composto por 14 subunidades distintas que representam a unidade funcional mínima da enzima, e que têm homólogos no complexo I mitocondrial de eucariotas. Sete das subunidades homólogas de eucariotas são codificadas por ADN mitocondrial (ND1–6 e ND4L) e localizam-se no domínio membranar, enquanto que as restantes sete são codificadas por ADN nuclear e pertencem ao domínio periférico da enzima.

O complexo I de *Neurospora crassa* é composto por, pelo menos, 39 subunidades distintas, 35 das quais conservadas em mamíferos. Catorze das subunidades do complexo I do fungo representam as subunidades centrais encontradas em procariotas e as restantes 25 designam-se por subunidades acessórias. Enquanto que às 14 subunidades centrais têm sido reconhecidas funções primárias, a função da maioria das subunidades acessórias continua por esclarecer. As proteínas 9.8 kDa e 11.5 kDa, homólogas respectivamente das subunidades MWFE e 15 kDa de mamíferos, são consideradas subunidades acessórias. Ambas as proteínas estão conservadas em eucariotas e foram localizadas no domínio membranar da enzima do fungo. Os genes correspondentes, que codificam para estas duas proteínas, foram identificados no genoma de *N. crassa* e separadamente inactivados por RIP (“repeat-induced point-mutations”). As estirpes mutantes resultantes deste processo, foram isoladas e caracterizadas. A análise dos mutantes nulos, sem as subunidades 9.8 kDa e 11.5 kDa, revelou que estas proteínas são essenciais para a formação e actividade do complexo I, levando a sua ausência à acumulação de intermediários da enzima. Curiosamente, foi encontrada uma similitude entre homólogos da MWFE e a subunidade nqrA da enzima bacteriana NADH:quinona oxidorreductase translocadora de Na⁺, o que sugere uma relação entre as NADH desidrogenases bombeadoras de H⁺ e as de Na⁺.

É reconhecido que o complexo I de *N. crassa*, tal como o complexo I de mamíferos, existe como uma mistura de duas formas inter-convertíveis, activo (A) e desactivado (D), sendo esta última sensível a reagentes com grupos tiol (SH). Trabalhos anteriores evidenciaram que, na forma desactivada da enzima, a subunidade de 15 kDa do complexo I de bovino, rica em cisteínas, era a melhor candidata para a marcação específica com o análogo fluorescente de NEM. Deste modo, os quatro resíduos conservados de cisteína da subunidade homóloga 11.5 kDa de *N. crassa* foram separadamente substituídos por resíduos de serina, e expressos de forma independente numa estirpe mutante nula na subunidade de 11.5 kDa de *Neurospora*, com o intuito de investigar se esta

subunidade está realmente envolvida na transição A/D. Observou-se que todas as estirpes mutantes, obtidas por mutagénesse dirigida, são capazes de formar complexo I com propriedades cinéticas semelhantes às observadas na enzima da estirpe selvagem. Isto indica que nenhum dos resíduos de cisteína da proteína 11.5 kDa é individualmente importante para a transição A/D e, portanto, a proteína não participa no fenómeno. O principal efeito observado em todas as estirpes mutantes, foi uma redução de complexo I formado. Isto sugere que os resíduos de cisteína da proteína 11.5 kDa são importantes para a aquisição e/ou estabilização da conformação proteica e, por consequência, para a formação do complexo I.

O arranjo das enzimas da cadeia respiratória de *N. crassa* na membrana mitocondrial interna foi investigado após delicada solubilização dos complexos enzimáticos com digitonina, seguida da separação dos extractos solubilizados por 2D BN- ou CN-/SDS-PAGE e 2D BN- ou CN-/BN-PAGE. Observou-se que as mitocôndrias da estirpe selvagem de *Neurospora* contêm, quer grandes supercomplexos respiratórios, formados pelos complexos I, III e IV (I_1IV_1 , I_1III_2 e $I_xIII_yIV_z$), quer supercomplexos mais pequenos (III_2IV_{1-2}). Além disso, os complexos IV e V foram recuperados sob a forma de monómeros e dímeros (IV_2 e V_2). Curiosamente, o dímero do complexo III (III_2) tem uma migração semelhante à do monómero do complexo V (peso molecular de cerca de 600 kDa), provavelmente devido a lípidos que lhe estão associados. Em suma, a cadeia respiratória mitocondrial de *N. crassa* está organizada de acordo com o modelo de respirassoma descrito em mamíferos, no qual os complexos I:III:IV estão presentes numa estequiometria de 1:3:6.

A função do complexo I na formação dos restantes complexos e supercomplexos respiratórios de *N. crassa* foi também investigada. Para tal, usaram-se oito estirpes de *Neurospora* com mutações no complexo I. Destas, as estirpes nuo21, nuo29.9 e nuo51, capazes de formar o complexo I apenas sem a respectiva subunidade, apresentam supercomplexos do tipo selvagem. Os supercomplexos estáveis do tipo I-III-IV detectados no mutante nuo51 não têm actividade de NADH desidrogenase, representando os primeiros exemplos de respirassoma inactivo e demonstrando que os supercomplexos têm outra importância que não enzimática. Os restantes cinco mutantes analisados, não são capazes de formar complexo I. Quatro deles, mutantes do braço membranar (nuo9.8, nuo11.5, nuo14 e nuo20.8) e um último mutante do braço periférico (nuo30.4), possuem supercomplexos do tipo III-IV (III_2IV_{1-2}). Observou-se também que os mutantes do braço membranar possuem espécies de elevado peso molecular da subunidade 30.4 kDa, possivelmente a co-migrar com supercomplexos do tipo III-IV, o que sugere que estes supercomplexos podem estar envolvidos na formação/estabilização do complexo I. Além disso, o braço periférico do complexo I, que se acumula nas quatro estirpes mutantes do braço membranar, foi identificado por electroforese como uma banda específica com actividade NADH e deamino-NADH desidrogenase, e apresentando uma massa aparente de 450 kDa. Todas as estirpes mutantes

analisadas contêm quantidades semelhantes dos complexos IV e V, sob a forma de monómeros e dímeros, assim como de complexo II. Estes resultados demonstram que a ausência da holoenzima não interfere com a montagem dos restantes complexos OXPHOS da cadeia respiratória mitocondrial de *Neurospora*. Adicionalmente, 67 proteínas distintas, de mitocôndrias da estirpe selvagem de *N. crassa*, foram identificadas por MALDI-TOF-MS. De entre estas proteínas, 31 pertencem aos complexos OXPHOS da cadeia respiratória e as restantes 36 proteínas são enzimas de diversas vias metabólicas, chaperones e transportadores.

RÉSUMÉ

Le complexe I mitochondrial est une grande structure multimérique en forme de L avec approchant 1 MDa, qui consiste en deux domaines perpendiculaires: le bras membranaire hydrophobique et le bras périphérique hydrophilique. Ce complexe catalyse le transfert de deux électrons de NADH à quinone, couplé à une translocation de quatre protons à travers la membrane mitochondriale interne. Chez la plupart des espèces procaryotes, NDH-1 se compose de 14 différentes sous-unités qui représentent l'unité minimale fonctionnelle, et chacune a un homologue dans le complexe I mitochondrial d'eucaryotes. Sept de ces sous-unités centrales d'eucaryotes sont codées par ADN mitochondrial (ND1-6 et ND4L) et sont localisés dans le domaine membranaire, pendant que les sept autres sont codées par ADN nucléaire et appartiennent au domaine périphérique de l'enzyme.

Le complexe I de *Neurospora crassa* est composé d'au moins 39 sous-unités polypeptidiques, dont 35 sont conservées chez les mammifères. De toutes les protéines décrites pour le complexe I de *N. crassa*, 14 sont homologues aux sous-unités centrales trouvées chez l'enzyme procaryote, et les autres 25 sont nommées sous-unités accessoires. On reconnaît des fonctions primaires aux sous-unités centrales. Pourtant, les fonctions de la majorité des sous-unités accessoires sont encore inconnues. Les protéines 9,8 kDa et 11,5 kDa du complexe I de *N. crassa* sont, respectivement, homologues à sous-unités MWFE et 15 kDa du complexe I des mammifères, étant considérées comme des sous-unités accessoires. Ces deux protéines sont conservées chez les eucaryotes et sont localisées dans le domaine membranaire de l'enzyme du mycète. Les gènes correspondants qui codifient pour les sous-unités ont été identifiés dans le génome de *N. crassa* et séparément inactivés par RIP ("repeat-induced point-mutations"). Les mutantes obtenues par ce processus ont été isolées et caractérisées. L'analyse des mutants nuls de *N. crassa*, qui sont dépourvus des sous-unités de 9.8 kDa et de 11.5 kDa, a révélé que ces protéines sont essentielles dans l'assemblage et dans l'activité du complexe I. Le résultat de cette absence est une accumulation d'intermédiaires du complexe I. De façon intéressante, une similarité a été trouvée entre les homologues de MWFE et la sous-unité nqrA de l'enzyme bactérienne NADH:quinone oxydoréductase, qui fait la translocation de Na^+ . Ceci suggère une relation entre les NADH déshydrogénase, qui pompent H^+ , et celles qui pompent Na^+ .

C'est reconnu que le complexe I de *N. crassa*, tel que celui des mammifères, existe en tant que mixture sous deux formes interconvertibles, active (A) et désactive (D), cette dernière étant sensible aux réactifs avec groupes thiol (SH). Des recherches antérieures ont considéré que, sous la forme désactivée de l'enzyme, la sous-unité 15 kDa du complexe I de bovin, riche en cystéine, était la meilleure candidate pour un marquage spécifique par fluorescence analogue de NEM. Donc, dans le but de savoir si la sous-unité est bien impliquée dans la transition A/D, les quatre résidus de

cystéine, conservés de la sous-unité 11.5 kDa de *N. crassa*, ont été séparément remplacés par des résidus de serine. Puis, chacun a été exprimé dans un mutant nul de *Neurospora*. Toutes les mutantes, obtenu par mutagenèse dirigée, sont capables d'assembler le complexe I avec les mêmes propriétés cinétiques de l'enzyme de l'espèce sauvage. Ceci nous indique qu'aucune des cystéines de la protéine 11.5 kDa n'est pas individuellement importante pour la transition A/D et que cette protéine ne participe pas au processus. L'effet le plus marquant observé en toutes les mutantes, fut la réduction de la quantité de complexe I assemblé, ce qui a montré que ces résidus sont importants pour l'acquisition et/ou la stabilisation de la conformation de la protéine 11.5 kDa, et, par conséquence, pour la formation du complexe I.

La disposition des enzymes de la chaîne respiratoire de *N. crassa*, dans la membrane mitochondriale interne, a été examinée en faisant une solubilisation douce des complexes protéiques avec de la digitonin, suivie d'une séparation de l'extrait solubilisé par 2D BN- ou CN-/SDS-PAGE et 2D BN- ou CN-/BN-PAGE. On a observé que les mitochondries de type sauvage de *N. crassa* contiennent de larges supercomplexes respiratoires, comprenant les complexes I, III, IV (I_1IV_1 , I_1III_2 et $I_xIII_yIV_z$), ainsi que des supercomplexes plus petits (III_2IV_{1-2}). De plus, les complexes IV et V ont été trouvés sous forme de monomères et dimères (IV_2 et V_2). Curieusement, le dimère du complexe (III_2) migre presque dans la même position du monomère du complexe V (poids moléculaire de 600 kDa), probablement du à l'accrochage des lipides. Bref, la chaîne respiratoire mitochondriale de *N. crassa* est organisée d'après le modèle de respirasome des mammifères, dans lequel les complexes I:III:IV existent dans la stœchiométrie 1:3:6.

Le rôle du complexe I dans la formation des autres complexes et supercomplexes respiratoires en *N. crassa* a aussi été étudié. Alors, on a utilisé huit mutantes de complexe I de *Neurospora*, trois d'entre elles capables de former des complexes. Les mutantes nuo21, nuo29.9 et nuo51, capables d'assembler le complexe I, à peine sans la respective sous-unité, présentent supercomplexes du type sauvage. Les supercomplexes stables du type I-III-IV trouvés dans nuo51 n'ont pas d'activité NADH oxydase, représentant ainsi les premiers exemples de respirasome inactif et démontrant aussi que les supercomplexes ont une autre importance qui n'est pas enzymatique. Les autres cinq mutants analysés ne sont pas capables d'assembler du complexe I. Quatre d'entre eux, mutants du bras membranaire (nuo9.8, nuo11.5, nuo14 et nuo20.8), et un dernier, mutant du bras périphérique (nuo30.4), possèdent les supercomplexes du type III-IV (III_2 et IV_2). On a observé que les mutants du bras membranaire possèdent des formes de la sous-unité 30.4 kDa avec un grand poids moléculaire, probablement co-migrant avec les supercomplexes III-IV. Ceci suggère que ces supercomplexes peuvent être impliqués dans l'assemblage et la stabilisation du complexe I. En plus, le bras périphérique du complexe I, qui s'accumule dans les quatre mutantes du bras membranaire, a été identifié comme bande spécifique par électrophorèse, avec une masse apparente de 450 kDa, ayant une activité de NADH déshydrogénase et diamine

NADH déshydrogénase. Toutes les mutantes analysées montrent des quantités similaires de complexes IV et V, sous forme de monomères et dimères, ainsi que de complexe II. Ces résultats montrent que l'absence d'un complexe I assemblé n'interfère pas avec l'assemblage d'autres complexes OXPHOS de la chaîne respiratoire mitochondriale de *Neurospora*. En plus, soixante-sept protéines ont été identifiées chez des mitochondries de *N. crassa* de type sauvage par MALDI-TOF-MS. Parmi ces protéines identifiées, 31 appartiennent aux complexes OXPHOS de la chaîne respiratoire et les autres 36 protéines participent à plusieurs fonctions, par exemple des voies métaboliques, des chaperonnes et des transports.

CHAPTER 1 – INTRODUCTION

1. Mitochondria and Respiratory Chain

The name mitochondrion derives from the Greek mitos, “thread”, and khondrion, “granule”. Mitochondria display an amazing plasticity of form and distribution. The size, shape and quantity of mitochondria vary between tissues and even among different locations within the same tissue (Stryer, 1996). According to the endosymbiotic theory, the most popular hypothesis to explain the mitochondria origin, these organelles originally arose, during biological evolution, by invasion of the cytoplasm of an anaerobic host by smaller aerobic α -proteobacteria, capable of using molecular oxygen to oxidize their nutrients within the host bacteria. Whether the host cell was prokaryotic or eukaryotic is still unclear (Gray *et al.*, 1999; Gray *et al.*, 2001).

Mitochondria are composed by a smooth outer membrane that surrounds the organelle and an inner membrane that contains invaginations called cristae, which expand the surface area enhancing the ability to generate ATP. Interestingly, mitochondria tomography studies have shown that cristae are structurally distinct compartments linked to the intermembrane space by narrow connections (Mannella *et al.*, 1998). The intermembrane space is the space between the two mitochondrial membranes, whereas the space enclosed by the inner membrane is denominated mitochondrial matrix. The matrix contains ribosomes, tRNAs and several circular double-stranded molecules of mtDNA, which make mitochondria semi-autonomous organelles (Anderson *et al.*, 1981; Attardi *et al.*, 1982). In aerobic eukaryotic organisms, mitochondria are the main source of energy required for all cellular functions. The production of energy is made through the action of the OXPHOS enzymes, consisting in the oxidation of electron-rich substrates and establishment of a proton gradient that drives ATP synthesis (Stryer, 1996; Videira, 2005).

The standard respiratory chain of almost all eukaryotes is located in the mitochondrial inner membrane and contains a series of five major membrane complexes designated by OXPHOS complexes. These include the four major respiratory complexes (I–IV) and the ATP synthase (V), together with two mobile electron carriers (quinone and cytochrome *c*) (Figure 1).

The proton translocating NADH:quinone oxidoreductase (complex I) is the largest respiratory complex, being the entry point for the major fraction of electrons into the electron transport chain. Complex I catalyses the electron transfer from NADH to quinone, through a series of redox centres coupled to proton translocation across the membrane, being one of the three energy-transducing complexes of the mitochondrial respiratory chain (Brandt, 2006).

Succinate dehydrogenase (complex II) catalyzes the oxidation of succinate to fumarate as part of the Krebs cycle, donating electrons to quinone, but not contributing to the electrochemical membrane potential. Complex II is the smallest OXPHOS enzyme comprising only 4-8 nuclear-encoded subunits plus a FAD molecule, a b-type cytochrome and non-heme iron centres as prosthetic groups (Cecchini, 2003). Ubiquinol produced by the action of complexes I and II is

oxidised by cytochrome *c* reductase (complex III or *bc1* complex), another enzyme of the mitochondrial respiratory chain where energy is conserved.

Complex III transfers the electrons to cytochrome *c*, a soluble protein localised in the intermembrane space. Mammalian complex III is composed of 11 subunits, including a minimal functional unit constituted by the cytochrome *c*₁, the Rieske iron-sulfur protein and the mitochondrial encoded cytochrome *b* (Nishikimi *et al.*, 1985; Trumpower and Gennis, 1994).

The final member of the mitochondrial electron transport chain is cytochrome *c* oxidase (complex IV), which catalyses the reduction of molecular oxygen to water coupled to proton translocation across the inner membrane. Mammalian cytochrome *c* oxidase is composed of 13 different subunits, three of them encoded by mtDNA, to which are attached four redox metal centres (cytochromes *a* and *a*₃, and two copper atoms) (Trumpower and Gennis, 1994; Yoshikawa *et al.*, 2000).

As mentioned before, the passage of electrons through complexes I, III and IV leads to the proton pumping through these complexes and originates a transmembrane electrochemical gradient. ATP synthase (Complex V or F₀F₁-ATPase) is a functionally reversible enzyme that allows protons to flow back into the mitochondrial matrix and uses the released energy to synthesize ATP from ADP and Pi (Walker, 1998). Three ATP molecules can be produced for each NADH molecule oxidised, yielding 36 or 38 ATP molecules per molecule of glucose oxidised (Stryer, 1996). Bovine complex V is composed of at least 16 different subunits, two of them encoded by mtDNA (Walker *et al.*, 1991). All complexes of the respiratory chain have dual genetic origin, except complex II that is encoded exclusively by nuclear genes.

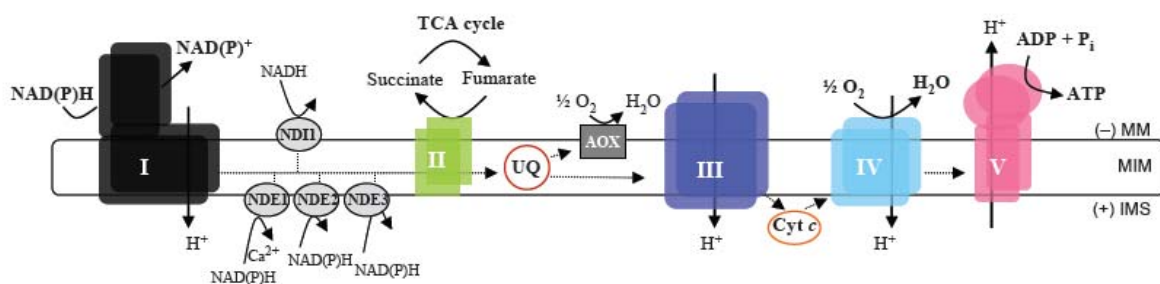


Figure 1. Scheme of the mitochondrial respiratory chain of *N. crassa*. The Roman numbers I-V stand for numbering of complexes of the respiratory chain according to the generally accepted nomenclature. The alternative NAD(P)H dehydrogenases (NDI1 and NDE1-3) and the alternative oxidase (AOX) are also shown. (MM – mitochondrial matrix; MIM – mitochondrial inner membrane; IMS – intermembrane space)

Besides the classical model of a respiratory chain, some organisms possess alternative components in their respiratory chains that are not coupled to ATP synthesis, which branch the pathway of electron transfer. The most ubiquitous and well-known of these enzymes are the type II alternative NADH dehydrogenase (NDH-2) (Melo *et al.*, 2004) and the alternative oxidase (AOX) that is sensitive to salicylhydroxamic acid (SHAM) (Joseph-Horne *et al.*, 2001). The number and specificity of the NDH-2 vary according to the organism, but to date none was described in humans. The fungus *N. crassa* possesses four NDH-2 that catalyse the same redox reaction as complex I but do not contribute to the transmembrane proton gradient (Videira and Duarte, 2002), and one AOX that carries out electron transfer from ubiquinol directly to molecular oxygen, bypassing complexes III and IV (Figure 1) (Li *et al.*, 1996; Vanlerberghe and McIntosh, 1997). The physiological role of the alternative respiratory complexes is not clear, but some evidence suggests that they are involved in the adaptation of the organisms to changeable environments such as oxidative stress, anaerobic conditions and change in food source or in temperature (Bertsova *et al.*, 2001; Duarte *et al.*, 2003).

The mammalian respiratory chain is composed of around 90 different proteins and only 13 of them are encoded by mitochondrial genes. Mitochondrial proteins encoded by nDNA are translated in the cytosol and after imported into mitochondria by translocation complexes located in the mitochondrial membranes. There are five main protein translocases that facilitate the import and correct sorting of proteins destined to mitochondria: TOM complex (Translocase of the Outer Membrane) recognizes preproteins synthesized in the cytosol and allows their passage across the mitochondrial outer membrane (Ahting *et al.*, 1999); two TIM complexes (Translocases of the Inner Membrane) allow translocation of preproteins destined either to the mitochondrial matrix (TIM23) or to the inner membrane (TIM22) (Neupert, 1997; Mokranjac and Neupert, 2005); SAM complex in the mitochondrial outer membrane assists the integration and assembling of outer membrane protein complexes (including the TOM complex); and the OXA complex that mediates insertion of preproteins into the mitochondrial inner membrane after their passage through the matrix (Dolezal *et al.*, 2006).

2. Organization of the OXPHOS Complexes

Currently there is some controversy related with the arrangement of the respiratory chain complexes in the inner mitochondrial membrane, and so two alternative models have been proposed. According to the liquid state model, all respiratory components are randomly distributed in the membrane and move freely by lateral diffusion. The electron transport is based on the random collision of the individual protein complexes and the smaller electron carriers. This model is based on the fact that OXPHOS complexes can be purified in their active state by lipid dilution experiments (Hackenbrock *et al.*, 1986). Alternatively to this respiratory chain organisation, it was originally proposed that the OXPHOS components are arranged according to a solid state model in which they may be either permanent or transiently associated forming supramolecular structures (Chance and Williams, 1955). Some evidences support this model: mutations in one respiratory complex interfere with the stability of other OXPHOS complexes (Acin-Perez *et al.*, 2004) and co-purification of more than one complex (Hatefi and Rieske, 1967).

The functional advantages of the organization of the OXPHOS complexes in supercomplexes are for instance the stabilisation of the individual complexes (Schägger, 2001; Acin-Perez *et al.*, 2004); the catalytic enhancement by substrate channelling, directing QH₂ and cyt *c* to a specific enzyme and reducing the diffusion time between enzymes (Lenaz *et al.*, 2006); and sequestration of reactive intermediate semiquinone species, preventing the generation of ROS (Schägger and Pfeiffer, 2000). Basically, electron-transfer rates should be higher in supercomplexes than on free diffusing single complexes.

These supramolecular structures were identified by gentle solubilisation of the mitochondrial protein complexes with different detergents (e.g. DIG, DDM, TX-100) and subsequent separation of the solubilized proteins by 1D non-denaturing gradient Blue Native (BN)- or Colorless Native (CN)-PAGE (Figure 2) (Krause, 2006). The mild detergent DIG was found to be the less damaging, maintaining the associations between complexes. In contrast to other native gel electrophoresis systems, in BN-PAGE the native enzymatically active OXPHOS complexes are separated according to their apparent molecular weight and independently of their isoelectric point. Protein complexes and individual proteins migrate to the anode due to their negative charge conferred by the anionic Coomassie G-250 dye, which is added to the sample shortly before the run and/or to the cathode buffer. The CN-PAGE uses the same electrophoresis conditions as BN-PAGE only without the Coomassie dye, which can disrupt labile subunits or weak interactions between protein complexes. Therefore, it is important to complement the analysis of mitochondrial protein-protein interactions by the more gentle CN-PAGE method (Krause *et al.*, 2005; Wittig and Schägger, 2005; Krause, 2006). In CN-PAGE migration of protein complexes is only due to their

intrinsic charge, which in addition to their native size and shape determines the migration distance in the gel system (Schägger *et al.*, 1994, Krause, 2006).

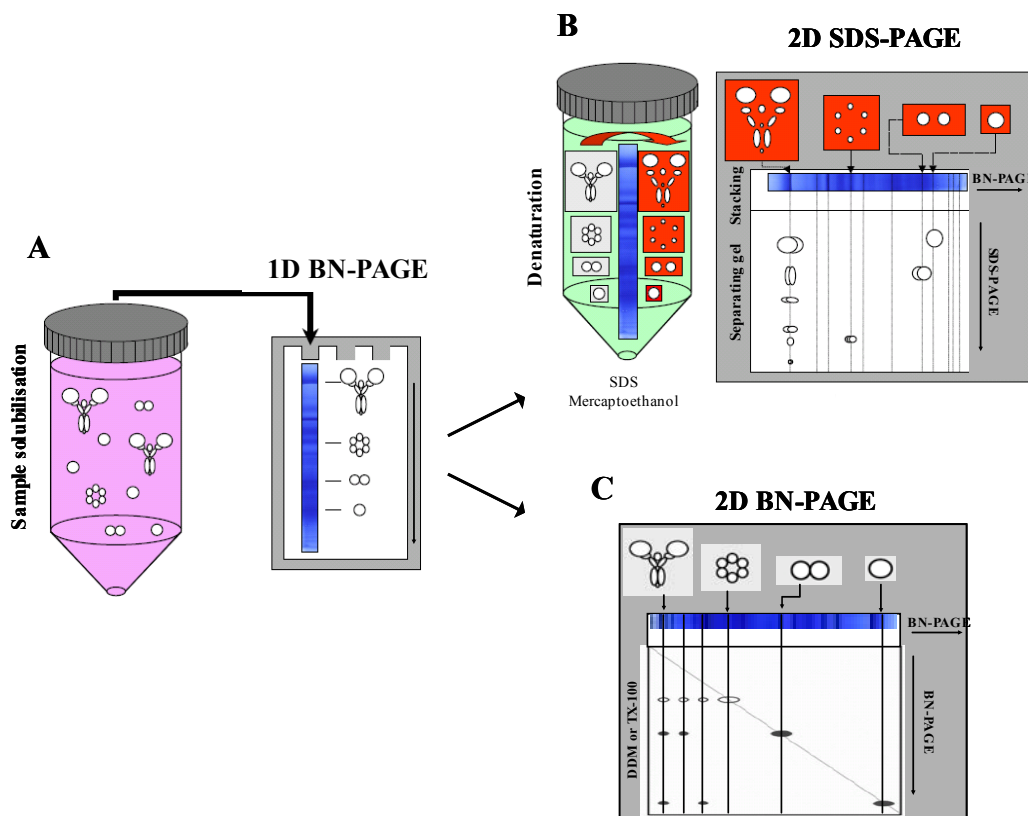


Figure 2. Protocol scheme for 1D BN-PAGE, and 2D SDS- and BN-PAGE. **A)** After solubilisation of the mitochondrial complexes, the protein complexes are separated by BN-PAGE. **B)** After the gel running, the lane of interest is cut and incubated in a denaturing solution in order to dissociate the native complexes into its constituent subunits (2D SDS-PAGE). **C)** In the 2D BN-PAGE, the OXPHOS supercomplexes can be analysed by the addition of DDM or TX-100 to the cathode buffer that lead to dissociation into individual complexes. In all gel systems the polypeptides are separated according to their molecular mass and the proteins of a complex from the 1D form a vertical row on the 2D SDS-PAGE gel. Protein complexes with the same mobility in 1D and 2D BN-PAGE migrate along a diagonal in the 2D BN-PAGE (Adapted from Eubel *et al.*, 2005).

Overall, 1D BN- and CN-PAGE represent high-resolution protein separation procedures for investigation of mitochondrial complexes in native state (Krause, 2006). Upon combination with SDS-PAGE for a second dimension gel, protein complexes separated by BN-PAGE are dissected into their subunits according to their molecular weight, which form vertical rows of spots on the resulting 2D SDS gels (Figure 2). Further characterisation of supercomplexes can be done through combination of 1D BN- and CN-PAGE with 2D BN-PAGE (Figure 2), that by the addition

of DDM or TX-100 to the cathode buffer lead to dissociation of the OXPHOS supercomplexes into its individual complexes according to their molecular mass. Combination with either in-gel staining of the respiratory enzyme activities (NADH dehydrogenase, succinate dehydrogenase, Cytochrome *c* oxidase and ATP hydrolase activities) (Zerbetto *et al.*, 1997; Sabar *et al.*, 2005) or Western blotting analysis ideally complements the identification of the mitochondrial complexes. Furthermore, identification of the subunit composition of the different mitochondrial complexes by mass spectrometry analysis is also frequently used as a way to identify, characterise and quantify the proteins present in the membrane complexes (Rais *et al.*, 2004; Krause, 2006).

The arrangement of the OXPHOS complexes into supercomplexes, seems to be well established in various organisms, such as bacteria (*e.g.* *P. denitrificans*) (Stroh *et al.*, 2004), fungi (*e.g.* *S. cerevisiae*, *Podospira anserina*) (Schägger and Pfeiffer, 2000; Krause *et al.*, 2004b), plants (*e.g.* *A. thaliana* and *Spinacia oleracea*) (Eubel *et al.*, 2004a; Eubel *et al.*, 2004b; Krause *et al.*, 2004a), and mammalian mitochondria (*e.g.* bovine, rat and human) (Schägger and Pfeiffer, 2000; Schägger *et al.*, 2004; Krause *et al.*, 2005). However, abundance and stability of the OXPHOS supercomplexes vary according to the organism.

P. denitrificans possesses supercomplexes containing complexes I, III and IV in a 1:4:4 stoichiometry (III_4IV_2 , III_4IV_4 , $\text{I}_1\text{III}_4\text{IV}_4$), and the ATP synthase was found only in monomers (Stroh *et al.*, 2004). In mitochondria of the yeast *S. cerevisiae*, which does not possess complex I, complexes III and IV are assembled into supercomplexes $\text{III}_2\text{IV}_{1-2}$ that are stabilised by the phospholipid cardiolipin, whereas the ATP synthase complex was found to exist as a mixture of monomers (V_1) and dimers (V_2) (Arnold *et al.*, 1998; Schägger and Pfeiffer, 2000; Pfeiffer *et al.*, 2003), and even higher oligomers (Paumard *et al.*, 2002). Electron microscopy analysis of ATP synthase dimers purified from *S. cerevisiae* indicated that the monomers interact through their F_O membrane domain (Dudkina *et al.*, 2006). Analysis of the subunit composition of the yeast ATP synthase dimer led to the identification of three dimer-specific subunits: e, g and k. In the absence of any of these subunits, essentially no detergent-stable complex V dimers were detected strongly suggesting that supercomplexes are not aggregation products but true association products (Arnold *et al.*, 1998). Interestingly, it was suggested that ATP synthase could be involved, through subunits e and g, in mitochondrial cristae formation by an oligomerisation process of this enzyme complex (Paumard *et al.*, 2002; Giraud *et al.*, 2002). In the fungus *P. anserina* and plants *A. thaliana* and spinach numerous types of supercomplexes were identified: including the abundant I_1III_2 supercomplex and the less abundant compositions $\text{III}_2\text{IV}_{1-2}$ and $\text{I}_1\text{III}_2\text{IV}_{1-4}$, as well as the dimeric ATP synthase (V_2) (Eubel *et al.*, 2004b; Krause *et al.*, 2004a; Krause *et al.*, 2004b). Interestingly, the alternative oxidase-dependent respiratory chain organization in COX-deficient *Podospira* strains was found to contain supercomplexes I_2 and I_2III_2 (Krause *et al.*, 2004b).

In mammalian mitochondria nearly all complex I associates into supercomplexes comprising complexes I and III, and up to four copies of complex IV ($I_1III_2IV_{0-4}$) (Schägger, 2001, 2002). These supercomplexes comprising complexes I, III and IV with varying stoichiometries ($I_xIII_yIV_z$) (Krause *et al.*, 2004b) were designated by “respirasomes”, because they can autonomously carry out respiration in the presence of the mobile carriers ubiquinone and cytochrome *c*. It was proposed that the so-called “respirasomes” consist of supercomplexes $I_1III_2IV_4$ and III_2IV_4 as building blocks in a 2:1 ratio, to fit the overall 1:3:6 stoichiometry of complexes I:III:IV (Hatefi, 1985; Schägger and Pfeiffer, 2001). It was further demonstrated the existence of larger ATP synthase oligomers besides the co-existence of monomers (V_1) and dimers (V_2) in mammalian mitochondria (Krause *et al.*, 2005; Wittig and Schägger, 2005). Hitherto, associations of complex II with the other OXPHOS complexes were not identified.

The first projection maps of respiratory supercomplexes were obtained by single particle electron microscopy. Characterisation of the I_1III_2 supercomplex from *Arabidopsis* mitochondria revealed lateral association of dimeric complex III (III_2) with the hydrophilic distal end of the membrane arm of complex I (Dudkina *et al.*, 2005). However, the precise subunit interactions responsible for formation of this supercomplex remain unclear. Additionally, monomeric complex IV was localised in the supercomplex $I_1III_2IV_1$ from bovine, most probably sharing a significant contact surface with complex I (Schäfer *et al.*, 2006). Recently, projection maps of the yeast supercomplex III_2IV_2 revealed that complex IV is specifically attached to dimeric complex III as a monomer on two opposite sides (Heinemeyer *et al.*, 2007). This supramolecular structure most likely favours the efficient electron transfer from complex III_2 to complex IV through cytochrome *c*, which is partially attached to the III_2IV_2 supercomplex. Furthermore, the structure of the dimeric ATP synthase (V_2) revealed an interaction of the monomers through their F_0 parts (Minauro-Sanmiguel *et al.*, 2005; Dudkina *et al.*, 2005). All these structures confirm the existence of assemblies and support the solid-state model.

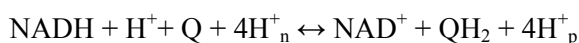
The analysis of human patients with a primary complex III deficiency, that lead to an impairment of complex III, provided evidence that the formation of “respirasomes” is essential for the assembly and/or stability of complex I, which supports the existence of supercomplexes (Acin-Perez *et al.*, 2004). On the other hand, complex III stability was not influenced by the absence of complex I (Schägger *et al.*, 2004). Interestingly, the impairment of complex III assembly in a *cyt c₁* mutant does not lead to a deficiency in complex I in *P. anserina* as reported for mammals (Sellem *et al.*, 2006), although the mitochondrial respiratory chain of *Podospora* is organised according to the “respirasome” model of mammals (Krause *et al.*, 2004b). Thus, it can be hypothesised that in *P. anserina*, complex IV alone can likewise stabilise complex I (Diaz *et al.*, 2006).

3. NADH:quinone Oxidoreductases

The NADH:quinone oxidoreductases of respiratory chains can be divided into three groups. They are the H^+ -pumping NADH:quinone oxidoreductase, the NADH:quinone oxidoreductase lacking an energy coupling site (designated NDH-2 or alternative enzymes) and the Na^+ -pumping NADH:quinone oxidoreductase (designated Na^+ -NQR). Na^+ -NQR enzymes exist in some bacteria (e.g. *Vibrio alginolyticus* and *Vibrio cholerae*) and are redox-driven pumps that couple the NADH oxidation to the translocation of Na^+ ions from the bacterial cytoplasm to the periplasmic space. NQR are complexes made of 6 subunits encoded by the *nqr* operon, and contain two FMN, one FAD and one binuclear iron-sulfur cluster as redox prosthetic groups (Steuber *et al.*, 1997; Barquera *et al.*, 2002; Barquera *et al.*, 2004). Complex I specific inhibitors like rotenone are ineffective towards Na^+ -NQR, whereas Ag^+ is a strong NQR inhibitor (Unemoto and Hayashi, 1993). The properties of the first two distinct NADH:quinone oxidoreductases will be described in more detail in the following chapters.

3.1. H^+ -Pumping NADH:quinone Oxidoreductases

NADH:quinone oxidoreductase (designated NDH-1 in bacteria or mitochondrial complex I in eukaryotes) is the largest and most complicated multisubunit enzyme of the respiratory chain involved in energy transduction. It is the first enzyme of the mitochondrial and bacterial respiratory chains, contributing to 40% of the proton-motive force across the inner mitochondrial membrane, which is used for the synthesis of ATP (Walker, 1992). Complex I uses the free energy from transferring two electrons from NADH to quinone to pump approximately four protons ($4H^+/2e^-$) from the negative side of the mitochondrial matrix (H_n^+) to the positive side of the intermembrane space (H_p^+) of the mitochondrial inner membrane, according to the following equation (Ohnishi, 1993; Galkin *et al.*, 2006):



In addition to this H^+ pumping, Steuber's group claimed that in some bacteria, such as *Klebsiella pneumoniae* and *Escherichia coli*, the NADH:quinone oxidoreductase has the capacity to pump sodium, an approach to detoxify cells from high Na^+ concentrations (Krebs *et al.*, 1999; Steuber *et al.*, 2000). Thus, this enzyme catalyses the oxidation of NADH, followed by electron transfer to quinone and Na^+ translocation across the membrane, with a stoichiometry of $2Na^+/2e^-$ (Gemperli *et al.*, 2002). However, this Na^+ pumping proposal for NDH-1 has been questioned.

Indeed, Friedrich *et al* did not find any indications for Na⁺ translocation by the *E. coli* complex I (Friedrich *et al.*, 2005). Therefore, further studies are required to decide whether complex I is capable of pumping sodium in addition to proton ions or not.

Quinones are lipid-soluble electron carriers embedded in the lipid bilayer that mediate electron transfer between protein components of respiratory chains (Lenaz, 1998). *E. coli* and other facultative bacteria like *K. pneumoniae* are able to synthesize three different types of quinones: the benzoquinone ubiquinone; and two naphthoquinones, menaquinone and demethylmenaquinone (Wallace and Young, 1977; Uden, 1988). Ubiquinone with a side chain of ten isoprenoid units (Q₁₀) is the common natural acceptor used by eukaryotes, while Q₆ is also used by yeasts and Q₈ by some bacteria. Complex I inhibitors have a molecular structure similar to quinones, with a cyclic ring corresponding to the quinone ring and a hydrophobic tail. Therefore it is safe to assume that the inhibitors act at the quinone binding site(s). Most of the inhibitors do not affect the NADH:ferricyanide activity, a non-physiological enzyme activity, but impair quinone reduction. Complex I has several specific inhibitors such as rotenone, piericidin A, capsaicin and acetogenin. Rotenone became the classical inhibitor of complex I of most organisms, but does not inhibit the NDH-1 enzyme of *E. coli* (Degli Esposti, 1998). The detergent Triton X-100, commonly used to solubilize complex I, was identified as another strong inhibitor of the NADH oxidase and the Q reductase activities of bovine heart particles (Ushakova *et al.*, 1999).

3.1.1. Origin of Complex I

Complex I is proposed to have originated by fusion of pre-existing protein assembly modules for coupled electron transfer and proton translocation (Friedrich and Weiss, 1997; Friedrich and Scheide, 2000). The homology to hydrogenases has been useful for understanding complex I evolution. The 49 kDa and PSST (19.3 kDa) subunits of bovine heart complex I are related to the large and small subunits of ancestral soluble [NiFe] hydrogenases, respectively (Albracht, 1993). This hydrogenase has expanded by addition of a dehydrogenase and a transporter modules. Thus, the electron input domain of complex I was found related to the NAD⁺-reducing hydrogenase from *Alcaligenes eutrophus*, with the 24 kDa, 51 kDa and 75 kDa (78 kDa) subunits being homologous to *hox* gene products of the hydrogenase enzyme (Pilkington *et al.*, 1991). It was also suggested that the 75 kDa (78 kDa) protein was a fusion of two proteins, since part of its C-terminal domain is homologous to a formate dehydrogenase of *Methanobacterium formicicum* (Finel, 1998). The complex I subunits 49 kDa and 30.4 kDa are similar to the ORF5 subunit of bacterial formate hydrogen lyase (Videira and Azevedo, 1994). In addition, the membrane-bound type-3 and type-4 hydrogenases of *E. coli* also contain homologues of complex I genes (Brandt *et*

al., 2003). In complex I from *E. coli* the C-terminus of the NuoC (30.4 kDa) subunit is fused with the N-terminal sequence of the NuoD (49 kDa) subunit, originating the NuoCD subunit. Furthermore, sequence similarities have suggested that the membranar subunits ND2, ND4, ND4L and ND5 have evolved from a family of Na⁺/H⁺ antiporters called Mrp and are likely to be involved in the proton translocation activity of complex I (Mathiesen and Hagerhall, 2002, Mathiesen, 2003). These results suggest that complex I evolved through the addition of different functional modules, and highlight its intricate evolution.

3.1.2. Architecture

Based on models obtained by electron microscopy of the enzyme from a variety of organisms, it is accepted that the bacterial NDH-1 and mitochondrial complex I have a characteristic L-shaped structure (Guénebaut *et al.*, 1997; Grigorieff, 1998; Guenebaut *et al.*, 1998; Djafarzadeh *et al.*, 2000). Overall, the structure is constituted by two arms arranged perpendicular to each other: the hydrophobic membrane arm embedded in the inner mitochondrial membrane (or bacterial plasma membrane), and the hydrophilic peripheral arm, protruding into the mitochondrial aqueous matrix (or bacterial cytoplasm) (Friedrich and Böttcher, 2004). Curiously, bovine complex I and *E. coli* NDH-1 structures differ slightly from *Neurospora* complex I structure, by possessing a thin stalk domain connecting the membrane and peripheral arms (Grigorieff, 1998). Some years ago, Friedrich's group claimed that the active form of *E. coli* NDH-1 has a horseshoe-like conformation with the two arms arranged side by side, and that the L-shaped structure corresponded to the inactive form of the enzyme (Böttcher *et al.*, 2002). However, this was contradicted by Sazanov's group, which showed that the active *E. coli* NDH-1 remains L-shaped and proposed that the horseshoe-appearance conformation was mistaken for dimers of complex I with lack of or inadequate staining of the peripheral arms (Sazanov *et al.*, 2003; Mamedova, 2004).

Due to its enormous complexity this is the unique enzyme of the respiratory chain for which a high resolution crystal structure is not available. Nevertheless, X-ray crystal structures of the peripheral arm of *Thermus thermophilus* (Sazanov and Hinchliffe, 2006) and of the membrane arm of *E. coli* enzymes (Baranova *et al.*, 2006) were recently solved at 3.3 and 8 angstrom resolution, respectively. Presently, one of the biggest challenges in the field is to obtain the high resolution X-ray crystal structure of the holocomplex.

Bovine complex I can be fractionated by sucrose gradient centrifugation or ion exchange chromatography in the presence of mild detergents as LDAO, allowing the isolation of four subcomplexes I λ , I α , I β and I γ (Finel *et al.*, 1994, Sazanov *et al.*, 2000). The subunit composition of these fractions was achieved by the use of three different techniques (1D SDS-PAGE, 2D

IEF/SDS-PAGE and reversed-phase HPLC), together with mass spectrometric analysis (Carroll *et al.*, 2003). Thus, 45 different polypeptides were identified in bovine complex I and allocated to its respective fraction (Figure 3) (Carroll *et al.*, 2006). The subcomplex I λ , which corresponds to the peripheral arm, is composed by 15 hydrophilic subunits including the NADH binding domain and all known redox groups. Subcomplex I α is formed by subcomplex I λ plus nine additional membrane subunits. Subcomplexes I β (13 subunits) and I γ (5 subunits) belong to the membrane arm and contain all the hydrophobic ND subunits. The subunits B15, MLRQ and SDAP have not been located, yet (Hirst *et al.*, 2003).

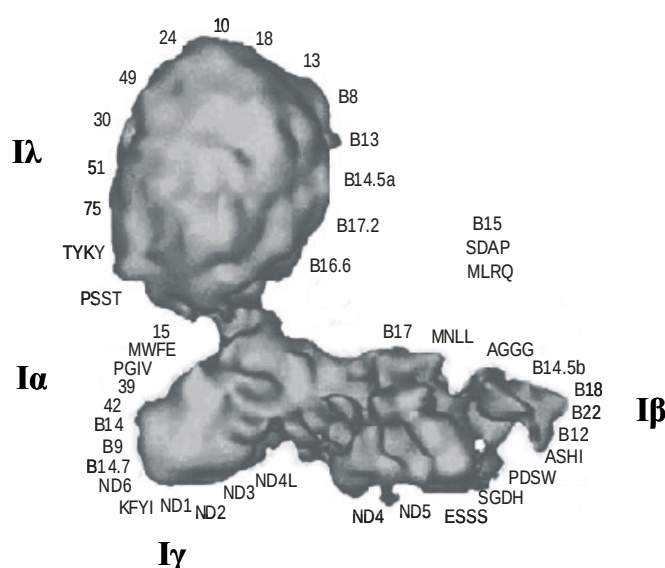


Figure 3. Subunit composition of bovine complex I grouped by the subcomplex they have been localised in (Adapted from Janssen *et al.*, 2006).

3.1.3. Subunit Composition

3.1.3.1. Core Subunits

The simplest version of the NADH:quinone oxidoreductase is the prokaryotic enzyme (NDH-1), which contains 13 or 14 dissimilar subunits and possesses all known redox components necessary to the transduction process, with a total molecular mass of about 550 kDa (Table 1). The subunits are named NuoA-N (according to *E. coli* and *Rhodobacter capsulatus* nomenclature) or Nqo1-14 (according to *T. thermophilus* and *Paracoccus denitrificans* nomenclature), and the corresponding genes are all together in the bacterial operon. The *E. coli* NDH-1 consists of only 13 different subunits, since *nuoC* and *nuoD* genes are fused and encode a single protein (Yagi and

Matsuno-Yagi, 2003). Interestingly, it was recently identified a novel NDH-1 subunit in *T. thermophilus*, designated by Nqo15, encoded in a locus separate from the *nqo* operon (Hinchliffe *et al.*, 2006). Overall, the bacterial NDH-1 is considered the minimal structural form of a proton-pumping NADH:quinone oxidoreductase required to couple the electron transfer to proton translocation (Yagi *et al.*, 1998).

The mitochondrial complex I has homologues of the 14 subunits that compose the bacterial NDH-1 (Table 1), though with dual genetic origin: seven subunits are usually encoded by mtDNA (ND1-6 and ND4L) and the other seven are nuclear encoded. The seven ND subunits are highly hydrophobic proteins with several putative transmembrane helices, being located in the membrane arm of complex I. Their function is still poorly understood, but they are most likely involved in proton translocation. This assumption came from the fact that the ND2, ND4, ND5 and ND4L subunits exhibit some sequence similarity with a family of Na^+/H^+ antiporters called Mrp (Mathiesen and Hagerhall, 2002, 2003). It was also proposed that the ND1, ND4 and ND5 subunits possess quinone binding sites (Gong *et al.*, 2003; Nakamaru-Ogiso *et al.*, 2003). Furthermore, the ND subunits were implicated in the assembly and activity of complex I in different organisms. For instance, it was demonstrated that the ND6 (Bai and Attardi, 1998) and the ND2 are essential for assembly and function of the human complex I (Ugalde *et al.*, 2006b). In *Neurospora*, a deficient mutant strain for the *nd2* and *nd3* genes assembles a subcomplex lacking the membrane arm of the enzyme (Alves and Videira, 1998). In the unicellular green alga *Chlamydomonas reinhardtii* the absence of the ND1 or ND6 subunit prevents the assembly of the holoenzyme, whereas loss of the ND4 or ND4 and ND5 leads to the formation of a smaller complex I (Cardol *et al.*, 2002). Interestingly, in *C. reinhardtii* only five subunits of complex I (ND1, ND2, ND4, ND5 and ND6) are encoded by mtDNA (Vahrenholz *et al.*, 1993). It was recently demonstrated that the ND3 and ND4L subunits, both nucleus encoded in this alga, show lower hydrophobicity compared to their mitochondrion-encoded counterparts and are equally required for assembly and activity of complex I (Cardol *et al.*, 2006).

The remaining seven subunits are nuclear gene products synthesized in the cytoplasm and after that imported into the mitochondria through translocation complexes located in the mitochondrial membranes. The nuclear encoded proteins are: 75 kDa (78 kDa), 51 kDa, 49 kDa, 30 kDa (30.4 kDa), 24 kDa, PSST (19.3 kDa) and TYKY (21.3c kDa) (Table 1). These proteins are located in the peripheral arm of complex I and bear all known prosthetic groups, namely one non-covalently bound molecule of FMN and eight or nine iron-sulfur clusters. In addition, the 51 kDa subunit possesses the NADH-binding motif (Fecke *et al.*, 1994), and the PSST (19.3 kDa) and 49 kDa subunits together with the previously referred ND1, ND4 and ND5 subunits were proposed to constitute the inhibitor- and quinone-binding pocket of complex I (Darrouzet *et al.*, 1998; Dupuis *et al.*, 2001; Prieur *et al.*, 2001; Schuler and Casida, 2001; Nakamaru-Ogiso *et al.*, 2003).

Several studies regarding the effect of these nuclear-encoded subunits in the assembly/activity of complex I have been done in *N. crassa*. By repeat-induced point-mutations or by homologous recombination, individual complex I genes were inactivated and mutant strains lacking the specific protein were analysed with respect to the assembly and activity of complex I. Hence, the peripheral arm subunits 78 kDa (75 kDa) (Harkness *et al.*, 1995), 49 kDa (Schulte and Weiss, 1995), 30.4 kDa (30 kDa) (Duarte *et al.*, 1998), 21.3c kDa (TYKY) (Duarte and Videira, 2000) and 19.3 kDa (PSST) (Duarte *et al.*, 2002) were found to be important to the assembly of *N. crassa* complex I. The 51 kDa (Fecke *et al.*, 1994) and 24 kDa (Almeida *et al.*, 1999) subunits were not found essential to the assembly of complex I but required for its activity. It is easily understood that in the nuo51 mutant, the lacking of the 51 kDa subunit led to the formation of a complex I without oxidase activity, as this protein possesses the NADH binding domain (Fecke *et al.*, 1994). Concerning the nuo24 mutant, the reason for the absence of activity was the reduced levels of the 51 kDa subunit found in the nuo24 complex I, making this mutant akin to the nuo51 mutant (Almeida *et al.*, 1999). Mutations in some of these core subunits were found to cause human mitochondrial disorders (see chapter 2.1.8.) (Greaves and Taylor, 2006).

Table 1. Core subunits and respective cofactors of complex I/NDH-1.

Bovine	<i>N. crassa</i>	<i>E. coli</i>	Location <i>Bovine / N. crassa</i>	Cofactors		Bovine Features
75 kDa	78 kDa	NuoG	I λ / p	N1b	[2Fe-2S]	Phosphorylated?
				N4	[4Fe-4S]	
				N5	[4Fe-4S]	
				N7 [#]	[4Fe-4S]	
51 kDa	51 kDa	NuoF	I λ / p	FMN		NADH binding
				N3	[4Fe-4S]	Phosphorylated?
24 kDa	24 kDa	NuoE	I λ / p	N1a	[2Fe-2S]	Phosphorylated?
49 kDa	49 kDa	NuoD*	I λ / p			Q binding?
30 kDa	30.4 kDa	NuoC*	I λ / p			
TYKY	21.3c kDa	NuoI	I λ / p	N6a	[4Fe-4S]	
				N6b	[4Fe-4S]	
PSST	19.3 kDa	NuoB	I λ / p	N2	[4Fe-4S]	Q binding?
ND1	ND1	NuoH	I γ / m			Q binding?
ND2	ND2	NuoN	I γ / m			H ⁺ translocation?
ND3	ND3	NuoA	I γ / m			
ND4	ND4	NuoM	I β / m			Q binding?
						H ⁺ translocation?
ND4L	ND4L	NuoK	I γ / m			H ⁺ translocation?
ND5	ND5	NuoL	I β / m			H ⁺ translocation?
						Q binding?
ND6	ND6	NuoJ	I α / m			

* In some bacteria (e.g. *E.coli*) NuoC and NuoD are fused, originating the NuoCD subunit.

[#] In some bacteria NuoG contains an additional [4Fe-4S] cluster (N7).

3.1.3.2. Accessory Subunits

The NADH:quinone oxidoreductase of the mitochondrial respiratory chain is one of the biggest and most complicated known membrane protein complexes. In addition to the homologues of the 14 subunits that constitute the bacterial catalytic core, the eukaryotic complex I contains many additional nuclear encoded subunits. The observed subunit composition of complex I is variable among eukaryotes, not only in terms of the number of subunits, but also in terms of specific polypeptides contained (Gabaldón *et al.*, 2005). Thus, bovine complex I comprises 45 dissimilar subunits (Table 2) with a molecular mass of about 1 MDa (Carroll *et al.*, 2006), while the complex I of the higher plant *Arabidopsis thaliana* contains around 40 subunits including some plant-specific subunits of unknown function (Heazlewood *et al.*, 2003; Cardol *et al.*, 2004) and the yeast *Y. lipolytica* no less than 37 subunits (Abdrakhmanova *et al.*, 2004).

Complex I of *N. crassa* is composed by at least 39 polypeptide subunits (Table 2), 25 of them are classified as accessory subunits (Marques *et al.*, 2005). In fact, the function of most of these accessory subunits remains unclear. It is known that they do not participate directly in electron and proton transport, but they may avoid the electrons of escaping from complex I by shielding the enzyme and thus preventing ROS generation, and can regulate the enzyme assembly and activity. Indeed, some functions were already attributed to a few of these additional subunits.

One such a function is in fatty acid biosynthesis. The 9.6 kDa/ACP (SDAP) subunit of complex I containing a phosphopantetheine group is closely related to acyl-carrier proteins involved in fatty acid biosynthesis. This suggests some biosynthetic function to complex I such as lipid synthesis and/or repair (Runswick *et al.*, 1991; Sackmann *et al.*, 1991; Schneider *et al.*, 1995). Other subunits have unclear functions, although some of their features have been identified. A number of observations indicate the presence of a NADPH-binding site in the 40 kDa (39 kDa) subunit, other than the NADH-binding site in the 51 kDa subunit. Complex I isolated from *N. crassa* contains tightly bound NADPH and experiments done with null mutants suggest the involvement of the 40 kDa (39 kDa) subunit in the enzyme biogenesis (Schulte *et al.*, 1999). The NADPH-binding activity of this subunit may be involved in intramitochondrial fatty acid synthesis. Furthermore, it was demonstrated that binding of NADPH to the homologous 39 kDa subunit in *Y. lipolytica* is not essential to complex I activity but stabilizes the enzyme (Abdrakhmanova *et al.*, 2006). Some subunits may regulate enzyme activity, as revealed by the post-translational modifications of some complex I proteins (Chen *et al.*, 2004) (see also chapter 3.1.3.3.).

Some accessory subunits were shown to be essential for the assembly/stability and function of complex I, as revealed by the phenotype of mutants lacking subunits or having altered proteins. Due to the high similarity between the fungal and mammalian respiratory chain enzymes, *N. crassa* is used as one of the main model organisms to investigate complex I (Videira, 1998). Thus, null

mutants of several *N. crassa* subunits have been produced (see also chapter 4.3.) and their effects on the assembly and activity of complex I have been investigated. The accessory subunits 21.3b (B14.7) (Nehls *et al.*, 1992), 20.9 kDa (Schulte and Weiss, 1995), 20.8 kDa (PGIV) (da Silva *et al.*, 1996), 12.3 kDa (PDSW) (Duarte *et al.*, 1995), 14 kDa (B16.6/GRIM-19) (Marques *et al.*, 2005), 11.5 (15 kDa) (Marques *et al.*, 2005) and 9.8 kDa (MWFE) (Marques *et al.*, 2003) were all found essential for complex I assembly in the *Neurospora* fungus. The bovine MWFE (9.8 kDa) is a small integral membrane protein localised in the bovine I α subcomplex of complex I (Hirst *et al.*, 2003). The MWFE protein is highly conserved, but for instance the human protein (80% identical to hamster) does not complement a null mutation in Chinese hamster cells. This may be due to a possible interaction of the MWFE protein with the ND proteins, demonstrating the need for compatibility of the nuclear and mitochondrial genomes. Indeed, it was found that the MWFE protein itself is unstable in the absence of the ND subunits (Yadava *et al.*, 2002). The human gene encoding the MWFE was mapped on chromosome X (Zhuchenko *et al.*, 1996) and mutations in the MWFE subunit of Chinese hamster cells were shown to impair complex I assembly and function (Au *et al.*, 1999; Scheffler and Yadava, 2001). Likewise, the other X-linked ESSS (11.6 kDa) subunit was found essential for assembly and activity of mammalian complex I (Scheffler *et al.*, 2004).

Another possible function for the additional subunits is in apoptosis. For example, the mammalian B16.6 subunit (14 kDa), homologue of the human GRIM-19 protein (Gene associated with Retinoid-IFN Induced Mortality) that is thought to be a cell death regulatory gene product induced by interferon and retinoic acid, is a component of mammalian complex I. It was indeed demonstrated to be released from mitochondria upon apoptosis. Thus, the B16.6/GRIM-19 is involved in the oxidative phosphorylation function as a structural component of complex I, as well as in initiation of apoptosis as a cellular component (Fearnley *et al.*, 2001). The question arises whether GRIM-19/B16.6 is involved in independent cellular processes in different subcellular compartments, or is connecting mitochondria and apoptosis (Hirst *et al.*, 2003).

Table 2. Accessory subunits of bovine and *N. crassa* complex I.

Bovine	<i>N. crassa</i>	Location Bovine / <i>N. crassa</i>	Bovine Features
39 kDa	40 kDa	I α / n.d	NADPH binding;
B13	29.9 kDa	I λ / p	
-	21.3a kDa	- / p	
B14.7	21.3b kDa	I α / m	N-Ac; Homology with Tim
18 kDa /AQDQ	21 kDa	I λ / p	Phosphorylated?
-	20.9 kDa	- / m	
PGIV	20.8 kDa	I α / m	
ASHI	20.1 kDa	I β / n.d.	
13 kDa	18.4 kDa	I λ / n.d.	N-Ac
-	17.8 kDa	- / m	
B14	14.8 kDa	I α / p	N-Ac
B16.6	14 kDa	I λ / m	N-Ac; GRIM-19 homologue
B17.2	13.4 kDa	I λ / n.d.	N-Ac
PDSW	12.3 kDa	I β / m	Phosphorylated?
B22	11.7 kDa	I β / n.d.	N-Ac
ESSS	11.6 kDa	I β / n.d.	Phosphorylated
15 kDa	11.5 kDa	I α / m	Phosphorylated?
B18	11.3 kDa	I β / n.d.	N-Myr
B12	10.6 kDa	I β / n.d.	N-Ac; Methylated
B8	10.5 kDa	I λ / n.d.	N-Ac; Similarity to
-	10.4 kDa	- / n.d.	
MWFE	9.8 kDa	I α / m	Phosphorylated
SDAP	9.6 kDa / ACP	I α and I β / p	Phosphopantetheine
B9	9.5 kDa	I α / m	N-Ac
B15	6.6 kDa	I α and I β / n.d.	N-Ac
B17	-	I β / -	N-Ac
B14.5a	-	I λ / -	N-Ac; Phosphorylated
B14.5b	-	I β / -	N-Ac
10 kDa	-	I λ / -	
42 kDa	-	I α / -	Phosphorylated
MLRQ	-	I α and I β / -	
MNLL	-	I β / -	
AGGG	-	I β / -	
SGDH	-	I β / -	
KFYI	-	I γ / -	

n.d. means not determined.

N-Ac and N-Myr: acetylation and myristylation of the N-terminal residue, respectively.

3.1.3.3. Post-Translational Modifications

Several different types of post-translational modifications have been identified for complex I subunits, including acetylation, myristylation and phosphorylation. For instance, the N-terminal methionine is removed post-translationally in several nuclear encoded subunits, while in other subunits the mitochondrial targeting sequence is only removed after import into mitochondria (Hirst *et al.*, 2003). Fourteen of the nuclear encoded bovine complex I subunits are known to have modified N-terminals. The B18 subunit (11.3 kDa) and ND5 (Plesofsky *et al.*, 2000) are N-myristylated and the remaining 13 subunits are N-acetylated (Table 2) (Carroll *et al.*, 2005; Pocsfalvi *et al.*, 2006). In addition, reversible methylation of several histidine residues takes place in the B12 (10.6 kDa) subunit, whereas the SDAP (9.6 kDa/ACP) subunit carries a phosphopantetheine prosthetic group (Carroll *et al.*, 2005).

One of the most important post-translational modifications affecting and/or regulating enzymatic activity is protein phosphorylation. In initial studies, a phosphorylated protein with 18 kDa was identified as being the 18 kDa/AQDQ (21 kDa) subunit (Papa *et al.*, 1999). However, it has been recently demonstrated that subunit ESSS (11.6 kDa), and not subunit 18 kDa/AQDQ (21 kDa), is the one that becomes phosphorylated (Chen *et al.*, 2004). Bovine complex I has additional potentially phosphorylated subunits, such as the 42 kDa (Schilling *et al.*, 2005), MWFE (9.8 kDa) (Chen *et al.*, 2004) and B14.5a (Pocsfalvi *et al.*, 2006) proteins.

The bovine 75 kDa (78 kDa), 51 kDa, 39 kDa (40 kDa), 24 kDa, TYKY (21.3c kDa), 15 kDa (11.5 kDa) and PDSW (12.3 kDa) subunits were also identified as being phosphorylated by mass spectrometry of Pro-Q Diamond stained proteins (Hopper *et al.*, 2006). However, this finding was not supported by any other experimental procedure.

3.1.4. Redox Components and Electron Transfer Pathway

Homologues of all the subunits of the bacterial NDH-1 are found in the more elaborated mitochondrial enzyme, and both contain equivalent redox components. This multisubunit enzyme possesses one FMN and 8-9 iron-sulfur clusters, making it the most elaborated iron-sulfur protein assembly known (Ohnishi *et al.*, 1998; Hinchliffe and Sazanov, 2005).

The FMN ($E_m = -336$ mV) is the entry point for electrons from NADH ($E_m = -320$ mV) and is non-covalently bounded to the 51 kDa subunit (Sled *et al.*, 1994). Depending on the origin of the enzyme, different numbers of iron sulfur clusters have been identified. Fe-S clusters identified by EPR spectroscopy include the binuclear clusters N1a and N1b and the tetranuclear clusters N2, N3, N4 and N5.

Cluster N1a has the lowest redox midpoint potential (Bovine $E_{m7} = -370$ mV) and exhibits pH dependence. This cluster is bound to the 24 kDa subunit (Yano *et al.*, 1994) and do not participate directly in the electron transfer pathway, probably functioning as an antioxidant by receiving electrons from FMN and thus preventing excessive ROS production (Sazanov and Hinchliffe, 2006). Cluster N1b (Bovine $E_{m7} = -250$ mV) could be assigned to the 75 kDa (78 kDa) subunit (Yano *et al.*, 1995). The tetranuclear cluster N3 is located in the 51 kDa subunit forming the electron input device together with FMN (Fecke *et al.*, 1994). Disruption of the gene encoding the 51 kDa subunit in *N. crassa* resulted in the loss of both FMN and iron sulfur cluster N3 (Fecke *et al.*, 1994). Clusters N4 and N5 reside in the 75 kDa (78 kDa) subunit (Yano *et al.*, 1995; Yano *et al.*, 2003). Centres N1b, N3, N4 and N5 are usually referred as the isopotential group of iron sulfur clusters of complex I (Ohnishi *et al.*, 1998). Cluster N2 is bound to the PSST (19.3 kDa) subunit (Duarte *et al.*, 2002; Flemming *et al.*, 2003) and resides at the interface between the PSST (19.3 kDa) and the 49 kDa subunits (Kerscher *et al.*, 2001). N2 is most likely located in the connection between the two perpendicular arms. It has the highest pH dependent redox potential of complex I (Bovine $E_{m7} = -150$ mV) and it is assumed that this redox centre is the immediate electron donor for quinone ($E_{m7} = +90$ mV for ubiquinone) (Yano and Ohnishi, 2001). Through sequence comparisons it was suggested that complex I contains two more EPR-silent tetranuclear clusters in subunit TYKY (21.3c kDa), called N6a and N6b (Rasmussen *et al.*, 2001). Additionally, NDH-1 from some bacteria (e.g. *E. coli*, *T. thermophilus*) contains another tetranuclear cluster (N7), which has been attributed to the 75 kDa (78 kDa) subunit and is not a component of the conserved electron transfer pathway (Friedrich, 1998; Nakamaru-Ogiso *et al.*, 2002; Nakamaru-Ogiso *et al.*, 2005).

From the peripheral arm structure of *T. thermophilus*, it is now clear that seven of the eight iron-sulfur clusters present in all versions of complex I, form a linear electron pathway through the enzymatic complex. The electron transfer from NADH to quinone, through the iron-sulfur clusters must take the following path: NADH→FMN→N3→N1b→N4→N5→N6a→N6b→N2→Q (Sazanov and Hinchliffe, 2006). Only cluster N1a and the prokaryote-specific cluster N7 are not associated with this electron transfer pathway.

3.1.5. Proton Translocation Mechanism

Mitochondrial complex I was shown to pump approximately four protons for each molecule of NADH oxidised (Ohnishi, 1993; Galkin *et al.*, 2006), but the mechanism of energy transduction is still unknown. In the literature the proton pumping is based in two main models: the direct redox driven and the indirect conformation-driven mechanisms.

In the direct redox linked proton pump model, it is assumed that the quinone-binding sites and the proton translocation components located in the membrane arm are in close contact with the redox centres of the peripheral arm, allowing direct interaction (Friedrich, 2001). However, the assumption that the antiporter-like ND4 and ND5 subunits are located at a considerable distance from the peripheral arm (Mathiesen and Hagerhall, 2002) is not consistent with a direct coupling mechanism. This model also supposed that some electron carriers involved in proton translocation were located in the membrane arm, which is not the case as established by the organisation of the iron-sulfur clusters in the peripheral arm structure from *T. thermophilus* (Sazanov and Hinchliffe, 2006).

According to the indirect conformation-driven proton pumping model, the redox centres of the peripheral arm are coupled to proton translocating components located in the membrane arm, through conformational changes. Several lines of evidence strongly favour an indirect conformational mechanism of redox-linked proton translocation (Brandt *et al.*, 2003). For instance, cross-linking studies and single particle analysis of complex I revealed that NAD(P)H binding to the 51 kDa subunit induced significant conformational changes in *E. coli* complex I, adopting a more open and expanded structure (Mamedova *et al.*, 2004). Furthermore, the distal location of the antiporter-like ND4 and ND5 subunits from the peripheral arm, which contains all known redox centres, would indicate that conformational changes are likely to play a role in the mechanism of proton pumping (Holt *et al.*, 2003). Most probably ubiquinone reduction around cluster N2 induces specific conformational changes that are then transmitted to the hydrophobic antiporter-like subunits in the membrane arm that act as ion pumps. Further evidences for a conformational proton pumping mechanism were proposed based on redox-driven conformational changes of the quinone-binding site (Ohnishi and Salerno, 2005).

3.1.6. Assembly Process

3.1.6.1. Complex I Assembly in *N. crassa*

Several studies, investigating the accumulation of assembly intermediates, have provided some insights into the assembly process of *Neurospora* and mammalian complex I. The *Neurospora* complex I is composed by a membrane arm and a peripheral arm, which are formed independently of each other before joining together as revealed by pulse-labelling experiments (Kuffner *et al.*, 1998; Schulte, 2001). Independent formation of these two arms of the enzyme can be achieved by growing *N. crassa* under special growth conditions. When *N. crassa* is grown in the presence of chloramphenicol, an inhibitor of mitochondrial protein synthesis, only the peripheral arm of complex I constituted by nuclear encoded subunits is assembled (Friedrich *et al.*, 1989). Likewise, in Mn^{2+} deprivation growth conditions only the membrane arm is able to assemble (Schmidt *et al.*, 1992). Overall, the membrane arm appears to be assembled by combining a small (~200 kDa) and a large (~350 kDa) intermediate, whereas the peripheral arm is assembled independently and is joined to the complete membrane arm in a later stage.

Two complex I assembly proteins were identified in *N. crassa*: the complex intermediate associated proteins CIA30 and CIA84 (Kuffner *et al.*, 1998). These CIA proteins are thought to chaperone the assembly of the large membrane arm intermediate by transient association with it. Knockout of either of the corresponding genes in *Neurospora* resulted in an impairment of complex I assembly, characterised by the absence of the large membrane arm intermediate and the accumulation of the small membrane arm intermediate and the peripheral arm. It has been suggested that the CIA proteins might maintain the assembly-competent state of the large membrane arm intermediate, preventing aggregation and misfolding (Schulte, 2001). These assembly chaperones have bovine homologues (Gabaldón *et al.*, 2005) and it was recently demonstrated that in human cells the *NDUFAF1* gene product acts as an assembly protein for complex I, being essential for the assembly/stability of human complex I, in agreement with the proposed function of its *N. crassa* homologue CIA30 (Janssen *et al.*, 2002; Vogel *et al.*, 2005). The predicted human homologue of the other assembly chaperone of *N. crassa* complex I, CIA84, was not functional characterised yet. Hitherto, homologues of the CIA proteins have not been detected in the prokaryotic genome.

Additionally, it was recently identified a third complex I assembly chaperone that is a paralogue of the B17.2 (13.4 kDa) subunit of the peripheral arm. The B17.2L subunit is involved in the late stage of complex I assembly, and the first mutation in this complex I assembly chaperone was described in a patient with a progressive encephalopathy (Ogilvie *et al.*, 2005). Another mitochondrial protein, the prohibitin, was found associated with a human subcomplex containing the 49 kDa, 30 kDa (30.4 kDa) and TYKY (21.3c kDa) subunits of complex I. Prohibitin is a

mitochondrial inner membrane protein that prevents protein proteolysis by *m*-AAA proteases, through protecting newly synthesized complex I subunits, thus functioning as a chaperone in complex I assembly (Bourges *et al.*, 2004).

Gene disruption experiments also confirmed independent assembly pathways of the peripheral and membrane arms of complex I. Many of the complex I subunits are required for assembly/stability and function of this enzyme, as revealed by the phenotype of *N. crassa* mutants lacking or having altered subunits. In some peripheral arm mutants, a complex I lacking only that single subunit is still able to assemble. *Neurospora* strains lacking the 21 kDa (AQDQ) (Ferreirinha *et al.*, 1999), 24 kDa (Almeida *et al.*, 1999), 29.9 kDa (B13) (Duarte *et al.*, 1995; Ushakova *et al.*, 2005), 40 kDa (39 kDa) (Schulte and Weiss, 1995) and 51 kDa (Fecke *et al.*, 1994) subunits of the peripheral arm of complex I are still able to assemble the enzyme. In *N. crassa*, the 21 kDa (18 kDa/AQDQ) protein is not essential for complex I assembly and activity (Ferreirinha *et al.*, 1999), whereas in human patients with a homozygous mutation in the AQDQ encoding gene, complex I assembly is severely affected (Scacco *et al.*, 2003). This phenotype may suggest some differences in the biogenesis of complex I between *N. crassa* and humans. In the *nuo29.9* mutant, lacking the 29.9 kDa (B13) subunit, around 20% of an active complex I is still able to assemble (Ushakova *et al.*, 2005). In the case of the *nuo51* mutant, the complex I is assembled but is not able to oxidise NADH, since the enzyme lacks the 51 kDa subunit responsible for the NADH binding, and so complex I is inactive (Fecke *et al.*, 1994).

However, in most cases the lack of single subunits of the peripheral or membrane arm can cause a complete block in the assembly pathway, leading to accumulation of intermediates of the respective arms. For instance, the mutant strains *nuo30.4* (Duarte *et al.*, 1998), *nuo19.3* (Duarte *et al.*, 2002) and *nuo21.3c* (Duarte and Videira, 2000), lacking correspondingly the peripheral arm subunits 30.4 kDa (30 kDa), 19.3 kDa (PSST) and 21.3c kDa (TYKY), are not able to assemble complex I since formation of the peripheral arm is prevented and only the membrane arm accumulates. Two of these subunits (19.3 kDa and 21.3c kDa) harbor prosthetic groups and consequently have a crucial role in electron transfer. With the exception of the 9.6 kDa/ACP (SDAP) subunit of the peripheral arm (Schneider *et al.*, 1995), loss of one peripheral arm subunit does not interfere with the membrane arm formation in agreement with the independent assembly of both arms of complex I. Accordingly, the peripheral arm of complex I is formed in all mutants disrupted for subunits of the membrane arm that were analysed until now. *Neurospora* mutant strains lacking a subunit of the membrane arm accumulate the small intermediate of the membrane arm in the *nuo20.9* (Schulte *et al.*, 1995), or the small and large membrane arm intermediates in the *nuo21.3b* (Nehls *et al.*, 1992), *nuo20.8* (da Silva *et al.*, 1996) and *nuo14* (Marques *et al.*, 2005), or even an almost complete membrane arm in *nuo12.3* (Duarte *et al.*, 1995).

3.1.6.2. Complex I assembly in Humans

Two main models for the human complex I assembly pathway were proposed. The first model based on human cells studies stated that the human membrane arm subunits are assembled into a membrane arm subcomplex before association with a peripheral arm subcomplex, resembling that of *N. crassa* (Ugalde *et al.*, 2004b). Overall, the pathway proposed involves seven distinct subcomplexes. It was proposed that the peripheral arm subunits 49 kDa, 39 kDa (40 kDa) and 30 kDa (30.4 kDa) subunits, are assembled together and associated with other intermediate composed by subunits of the peripheral arm that includes at least the 75 kDa (78 kDa), 51 kDa and 24 kDa, resulting in another assembly intermediate. Then, the partially assembled peripheral arm is coupled to a pre-assembled membrane arm intermediate assisted by assembly factors, to form a membrane-bound complex. The membrane part of the complex is formed in distinct steps like for *Neurospora*. Subsequently, more subunits associate with the peripheral and membrane arms already assembled to form the holocomplex of ~1MDa (Ugalde *et al.*, 2004b). Recently, an update of this complex I assembly pathway model was described according to which complex I assembly begins with the formation of the hydrogenase core module constituted at least by the 49 kDa, 30 kDa (30.4 kDa), PSST (19.3 kDa) and TYKY (21.3c kDa) subunits, that associates with a membrane module constituted by the mitochondrial encoded subunits and finally expanded with the addition of the NADH dehydrogenase module composed by the 75 kDa (78 kDa), 51 kDa and 24 kDa proteins. Subsequently, more subunits associate with the peripheral and membrane arms already assembled to form the holocomplex of ~1MDa (Vogel *et al.*, 2007). The difference between these two similar models is related with the moment of association of the dehydrogenase module, before or after the membrane module.

The second model, based on subcomplexes observed in complex I-deficient patients, proposes a pathway for human complex I assembly involving also seven distinct subcomplexes (Antonicka *et al.*, 2003). This model proposes the occurrence of a subcomplex containing the membrane arm subunit ND1 and the peripheral arm subunits 30 kDa (30.4 kDa), 39 kDa (40 kDa) and 49 kDa, suggesting that the peripheral and membrane arms are not assembled in independent pathways (Antonicka *et al.*, 2003). Moreover, the 24 kDa, 18 kDa/AQDQ (21 kDa) and PSST (19.3 kDa) subunits form a subcomplex without the 30 kDa (30.4 kDa) and 49 kDa subunits. This is unlikely, since it was shown that the 18 kDa/AQDQ (21 kDa) subunit interacts with the 30 kDa (30.4 kDa) and 49 kDa subunits (Yamaguchi and Hatefi, 1993). So it is more likely that these three subunits assemble together to form a subcomplex. A possible reason for these differences is that, in the patient samples used some of the proposed assembly intermediates can be breakdown products derived from complex I instability.

3.1.7. Active/De-active Transition of Complex I

In some organisms complex I exists in two slowly equilibrated forms: active (A) and de-activated (D). This A/D transition phenomenon has been described for complex I of vertebrates (carp, frog, chicken, rat and bovine), as well as for fungal mitochondrial complex I (*N. crassa* and *Y. lipolytica*) (Grivennikova *et al.*, 2001; Grivennikova *et al.*, 2003; Maklashina *et al.*, 2003). The only difference found between these two groups of organisms is that, the fungal enzyme displays A/D transitions with much lower activation barrier. The complex I of invertebrate species (earthworm, lobster and cricket) and the NDH-1 of prokaryotes (*E. coli*, *P. denitrificans* and *R. capsulatus*) do not show A/D transitions, which implicates the eukaryotic accessory subunits of the more elaborated complex I in the A/D phenomenon (Kotlyar *et al.*, 1998; Maklashina *et al.*, 2003).

The A- and D-forms of complex I differ in their catalytic and structural properties. Only the A form can catalyze the rapid rotenone-sensitive NADH:quinone oxidoreductase reaction, although the D form can catalyze the oxidation of NADH by artificial electron acceptors. The lack of the substrates like NADH and oxidised quinone or the presence of NADH and completely reduced quinone (ubiquinol) strongly shift the equilibrium to the D-form. The A to D transition (deactivation) has extremely high activation energy (~270 kJ/mol) and for that reason depends on temperature (Figure 4), not proceeding at a significant rate at temperatures below 20 °C. This high activation energy suggests that gross structural rearrangements of complex I must occur during the transition. In contrast, the D to A transition (activation) needs slow activating turnover and is not temperature dependent (~170 kJ/mol). However, the rate of D to A transition is decreased at alkaline pH and/or in the presence of bivalent cations (Ca^{2+} or Mg^{2+}) (Vinogradov and Grivennikova, 2001). Furthermore, based on affinity for the complex I inhibitors rotenone and piericidin A, and on the different sensitivities to thiol reagents, it has been shown that the A- and D-forms of complex I have different conformations. Indeed, only the D-form is specifically labelled and irreversibly inactivated by *N*-ethylmaleimide (NEM) and other sulfhydryl reagents (Figure 4) (Grivennikova *et al.*, 1997; Grivennikova *et al.*, 2001).

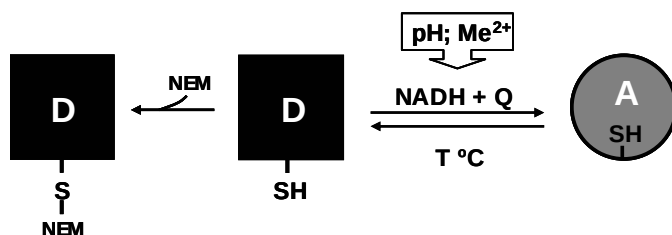


Figure 4. Illustration of the A/D transition of mitochondrial complex I (Adapted from Vinogradov and Grivennikova, 2001).

The molecular mechanism of A/D transitions and subunits involved in this process remain unclear. The difference in SH-group(s) reactivity between the A- and D-form has been employed to identify the protein that is involved in this A/D transition. Studies with a fluorescent analogue of NEM revealed that one bovine complex I subunit with a molecular mass of approximately 15 kDa is specifically labelled in the D-form of the enzyme (Gavrikova and Vinogradov, 1999). However, it is still unclear if this subunit is responsible for the structural changes occurring during the A/D transitions or if its SH-group becomes accessible to SH-reagents merely as a result of these changes. The bovine genome sequencing and proteomic approaches allowed the identification of several candidate proteins for this NEM-labelling, with a molecular weight of around 15 kDa and possessing cysteine residues. The more reasonable bovine candidates, according to the molecular weight and presence of cysteines, are the subunits 15 kDa (11.5 kDa), B14.7 (21.3b), 13 kDa (18.4 kDa), B13 (29.9 kDa), ESSS (11.6 kDa) and B18 (11.3 kDa) proteins. All of them have *N. crassa* homologues, but only the fungal homologues of the bovine 15 kDa (11.5 kDa – 4 Cys), 13 kDa (18.4 kDa – 4 Cys), ESSS (11.6 kDa – 1 Cys) and B18 (11.3 kDa – 4 Cys) subunits contain cysteine residues that can bind SH-reagents (Ushakova *et al.*, 2005; this work). Although the primary structure of this subunit and the precise position of the labelled aminoacid residue remain to be established, the bovine 15 kDa (11.5 kDa) subunit with four conserved cysteine residues was considered the most probable enzyme component involved in this A/D transition (Gavrikova and Vinogradov, 1999). In addition, it was recently demonstrated that the 29.9 kDa (B13) subunit of *N. crassa* complex I is also involved in the A/D transition. A *Neurospora* mutant lacking the 29.9 kDa subunit of complex I preserves the ability to undergo A/D transitions, though its slower de-activation process (Ushakova *et al.*, 2005). Recently, it was suggested that the sulfhydryl group of complex I sensitive to inhibition by NEM, and possibly involved in the A/D transition, is located in the inner membrane facing the matrix interface (Gostimskaya *et al.*, 2006).

This A/D transition of complex I may be physiologically important in strong hypoxic or anoxic conditions. Rapid de-activation at physiological temperature (37 °C) is expected under hypoxic or anoxic conditions, where the lack of molecular oxygen leads to high levels of ubiquinol (Maklashina *et al.*, 2002). The D to A transition of complex I is slow in the presence of divalent cations, and during hypoxia the concentration of Mg^{2+} and Ca^{2+} in the mitochondrial matrix is high, which prevents the activation of complex I. However, the deactivated complex I is still capable of oxygen reduction with production of ROS, which happens if the de-activated complex I remained in D-form for some time after reoxygenation (Vinogradov and Grivennikova, 2001). Thus, regulation of complex I activity may have important consequences for the production of ROS.

3.1.8. Diseases Associated with Complex I

A number of devastating neurodegenerative disorders have been associated with defects of one or more enzyme complexes of the OXPHOS system. Oxidative phosphorylation disorders make a contribution of 1 per 10,000 live births in man (Rftig *et al.*, 2004). One of the ways in which malfunctioning of the respiratory complexes may lead to mitochondrial disorders is through the production of reactive oxygen species, and complexes I and III are considered the main sites of ROS production in mammalian (Robinson, 1998). Indeed, it was recently demonstrated that superoxide production is increased in inherited complex I deficiency (Verkaart *et al.*, 2007). With such an important role, it is not surprising that a number of severe human mitochondrial disorders, including the most frequently encountered, are associated with complex I deficiency. These mitochondrial diseases are associated with a wide variety of clinical signs and symptoms that frequently result in early childhood death (DiMauro, 2004). Moreover, complex I deficiency was also found in late onset diseases such as Parkinson's (Schapira *et al.*, 1990), Huntington's (Arenas *et al.*, 1998) and Alzheimer's disease (Kim *et al.*, 2001), as well as in HIV infection (Ladha *et al.*, 2005) and cancer (Sanchez-Cespedes *et al.*, 2001).

Complex I deficiency is genetically heterogeneous due to the particular genetic system of mitochondria. In some cases, point mutations in the mtDNA genes coding for the ND1, ND4 and ND6 subunits have been described as causing complex I deficiency and constitute the primary genetic defect in Leber's hereditary optic neuropathy (LHON) (Howell *et al.*, 1991; Degli Esposti *et al.*, 1994; Carelli *et al.*, 2004). The LHON pathology is characterised by central vision loss and optic atrophy. In addition, mutated ND2, ND3, ND4 and ND5 subunits were also found to cause Leigh syndrome (Greaves and Taylor, 2006; Ugalde *et al.*, 2006b; Sarzi *et al.*, 2007). However, not only mutations in the *ND* genes but also in mitochondrial genes coding for rRNAs and tRNAs, as well as in those encoding mitochondrial aminoacyl-tRNA synthetases and tRNA modification enzymes, affect the correct assembly and normal function of complex I. For example, mutations in mitochondrial genes *tRNA^{Leu}* and *tRNA^{Lys}* were found to cause MELAS and MERRF syndromes, respectively (DiMauro, 2004).

Although mutations in mtDNA have been described as causing complex I deficiency, they only account for a small percentage of the total number of complex I deficient patients. In fact, in the majority of these patients, mutations in nuclear genes are expected. Most nDNA mutations identified in patients with isolated complex I deficiency are mainly associated with Leigh syndrome or Leigh-like diseases characterised by motor and intellectual developmental delay. During the past years, DNA sequence analysis in complex I-deficient patients have revealed specific point mutations in nuclear genes coding for the following polypeptides: 75 kDa (78 kDa) (Bénit *et al.*, 2001; Martin *et al.*, 2005), 51 kDa (Schuelke *et al.*, 1999; Bénit *et al.*, 2001), 49 kDa

(Loeffen *et al.*, 2001), 30 kDa (30.4 kDa) (Bénit *et al.*, 2004), 24 kDa (Bénit *et al.*, 2003), 18 kDa/AQDQ (21 kDa) (Loeffen *et al.*, 1998a), PSST (19.3 kDa) (Triepels *et al.*, 1999; Lebon *et al.*, 2007), TYKY (21.3c kDa) (Loeffen *et al.*, 1998b; Procaccio and Wallace, 2004), 13 kDa (18.4 kDa) (Kirby *et al.*, 2004) and MWFE (9.8 kDa) (Fernandez-Moreira *et al.*, 2007) polypeptides of complex I. Recessive X-linked hereditary defects of complex I are suggested by the high male-to-female ratio among patients. The human *NDUFA1* and *NDUFB11* genes, coding for respectively the MWFE (9.8 kDa) and ESSS (11.6 kDa) subunits, are the only complex I genes mapped on the human X chromosome so far (Zhuchenko *et al.*, 1996; Scheffler *et al.*, 2004). Hitherto, only mutations in the *NDUFA1* gene encoding the MWFE (9.8 kDa) polypeptide were found in patients with mitochondrial complex I deficiency (Ugalde *et al.*, 2006a; Fernandez-Moreira *et al.*, 2007).

Neurospora strains harboring some of the mitochondrial disease-associated mutations in the 19.3 kDa (PSST), 21.3c kDa (TYKY) and 51 kDa subunits are able to assemble a functional complex I, though in diminished amounts (Duarte *et al.*, 2005), suggesting that the reduce formation and stability of complex I are the major reasons for complex I deficiency in humans.

Complex I mutations can also affect the stability of other mitochondrial complexes. Indeed, in patients with mutations in the complex I genes *NDUFS2* and *NDUFS4* encoding respectively the 49 kDa and 18kDa/AQDQ (21 kDa) subunits, it was detected diminished amounts of fully assembled complex III, which may indicate a physical interaction between both complexes (Scacco *et al.*, 2003; Ugalde *et al.*, 2004a).

3.2. Non-Pumping NAD(P)H:quinone Oxidoreductases

Besides the NADH:quinone oxidoreductase capable of both NADH oxidation and proton translocation, there are alternative enzymes that carry out the same redox reaction but do not contribute to the proton gradient across the membrane. These enzymes lacking an energy-coupling site are known as NDH-2 or alternative NADH dehydrogenases.

NDH-2 are usually single polypeptides with a molecular weight of approximately 50 kDa, contain one or two sequence motifs for presumably binding NAD(P)H and FAD or FMN, and are insensitive to classical complex I inhibitors such as rotenone and piericidin A. In eukaryotes they are localised in the mitochondrial inner membrane, with their NAD(P)H reaction site facing either the mitochondrial matrix (internal enzymes or NDI) or the intermembrane space (external enzymes or NDE). Some of these proteins accept NADPH instead of NADH as substrate, and some require calcium for activity (Kerscher, 2000; Melo *et al.*, 2004). Despite that these NDH-2 enzymes have been widely described, their specific function and the mechanisms of their physiological role are still not clear. They may regulate the reduction state of the cytoplasm and mitochondrial matrix

pools, bypassing the common electron transport chain. In this manner, the alternative dehydrogenases could function to avoid ROS production by complex I.

They are present in a variety of organisms such as bacteria (Melo *et al.*, 2004) and archaea (Bandeiras *et al.*, 2003), as well as in mitochondria of fungi and plants (Videira, 1998; Kerscher, 2000), but they vary widely in number and specificity, suggesting that they may have organism-specific functions. The yeast *Saccharomyces cerevisiae* possesses three NDH-2 enzymes: the internal NDI1 for oxidation of matrix NAD(P)H (Velazquez and Pardo, 2001), and two external NDE1 and NDE2 to oxidise cytosolic NAD(P)H (Marres *et al.*, 1991; Luttik *et al.*, 1998; Small and McAlister-Henn, 1998). These enzymes have a special importance in *S. cerevisiae* whose respiratory chain is devoid of complex I, making them the main entry point of electrons of the respiratory chain. The fungus *N. crassa* contains in addition to complex I, four alternative NADH:quinone oxidoreductases: the internal NDI1 facing the mitochondrial matrix (Duarte *et al.*, 2003), and three external enzymes (NDE1, NDE2 and NDE3) capable to oxidise the cytosolic NAD(P)H (Figure 1) (Melo *et al.*, 2001; Carneiro *et al.*, 2004, 2007). *N. crassa* strains lacking a functional complex I are viable, since this organism contains the NDI1 capable to oxidize the matrix NADH. Up to seven genes of Arabidopsis coding for alternative NAD(P)H dehydrogenase were described (Michalecka *et al.*, 2003), but not thoroughly characterized. Of the ones already characterised two are internal enzymes facing the mitochondrial matrix and two are external (Rasmusson *et al.*, 2004). Mammalian respiratory chains are thought to lack these non-pumping NAD(P)H:quinone oxidoreductases. However, the human mitochondrion-associated inducer of death (AMID), homologue of the apoptosis-inducing factor (AIF), has considerable similarity to NDH-2 enzymes, including the presence of the two typical ADP-binding motifs, suggesting that it could have NAD(P)H dehydrogenase activity (Wu *et al.*, 2002). In fact, the NADH oxidase activity was detected in the mitochondrial AIF that upon apoptosis translocates from mitochondria to the nucleus where it participates in apoptotic chromatinolysis (Susin *et al.*, 1999; Miramar *et al.*, 2001).

The alternative enzymes may possibly be used therapeutically in cases of complex I failure, by introducing a NDH-2 enzyme in patient cells to restore the NADH dehydrogenase function in their mitochondria. A successful approach showed that the expression of the NDI1 of *S. cerevisiae* in human cells lacking a functional complex I, completely restores the NADH dehydrogenase activity. The expressed yeast NDI1 protein was correctly targeted to the matrix side of the inner mitochondrial membrane and was fully functional (Bai *et al.*, 2001). Moreover, it was recently demonstrated that the yeast NDI1 improves oxidative phosphorylation ability and increases protection against oxidative stress and cell death in cells carrying a Leber's hereditary optic neuropathy mutation in the *ND4* gene (Park *et al.*, 2007). This demonstrates the potential of the alternative NDH-2 enzymes to overcome human complex I deficiency.

4. Use and Choice of *N. crassa* as a Model System

The fungus *N. crassa* is a multicellular haploid eukaryote included in the kingdom Fungi, class Ascomycetes and genus *Neurospora*. This non-pathogenic filamentous fungus was first described in 1843 as the causative agent of an orange mold infestation in French bakeries (Payen, 1843) and in the 1920s developed as an experimental organism (Shear and Dodge, 1927). Later in 1941, Beadle and Tatum took advantage of *N. crassa* and defined the “one gene, one enzyme” hypothesis that states that specific genes code for specific proteins, for which they won the Nobel Prize in 1958 (Beadle and Tatum, 1941). Additionally, *Neurospora* is known for its ability to germinate after forest fires; being one of the first colonists in burned-vegetation areas, which reflects the activation of ascospores, the products of the sexual life cycle, by the heat and the well-developed capability of *Neurospora* to use cellulosic materials (Jacobson *et al.*, 2006).

This filamentous fungus is considered a model organism because of its simplicity, rapid and economical growth, haploid short life cycle and amount of genetic information available (Davis and Perkins, 2002). This and the high similarity between the fungal and mammalian respiratory chain enzymes, allowed *N. crassa* to be used as one of the main model organisms to investigate complex I (Videira, 1998). Indeed, *Neurospora* mutant strains lacking particular subunits of complex I or expressing mutated proteins can be constructed, and any mutation should be easily detected since mutations will not be masked by another allele (Videira and Duarte, 2002). Thus, the recreation of mutations in *N. crassa* is one way to clarify mitochondrial disorders and overcome the limitation of human patient samples. In addition, investigation of many other biological processes such as mitochondrial protein import, circadian rhythms and mitochondrial biogenesis has been done using *N. crassa*.

4.1. Life Cycle

Neurospora has a haploid life cycle composed by a sexual and an asexual phase. Depending on environmental factors including nutrient, light, and temperature, the vegetative mycelium can undergo asexual sporulation processes of macroconidiation and microconidiation or it can enter the sexual cycle by forming protoperithecia; which upon fertilization, can begin development leading to the production of ascospores (Springer, 1993). In the asexual part of this cycle, germination and growth of a haploid asexual spore (conidium) results in a mass of hyphae. Hyphae have no cross walls, so is essentially a single cell containing many haploid nuclei. Aerial hyphae bud off millions more conidia, multinucleate macroconidia and uninucleate microconidia, and these disperse and repeat the asexual cycle if they land on a suitable substrate. In the sexual

phase, when colonies of different mating types come into contact, their cell walls and nuclei fuse resulting in many transient diploid nuclei inside perithecia. Each diploid nucleus undergoes meiosis originating four haploid products that stay in an ascus. Then, each of the four products of meiosis undergoes a mitotic division, resulting in eight ascospores within each ascus. Ascospores germinate and produce hyphae exactly like those produced by asexual spores (Davis, 2000).

Interestingly, it was found that complex I is not essential for *N. crassa* vegetative growth, because of the presence of alternative NADH dehydrogenases (see also chapter 3.2.). However, it is required for normal development of the *N. crassa* sexual life cycle and genetic crosses between strains carrying homozygous mutations in complex I genes are blocked at early stages of development (Duarte and Videira, 2000).

4.2. Genome

The nuclear genome of *N. crassa* was reported as completely sequenced in the year of 2003. Its size is about 40 megabases distributed among seven chromosomes. A total of 10,082 protein-coding genes were predicted, and 41% of the predicted proteins lack significant similarity to known proteins (Galagan *et al.*, 2003). Interestingly, there is a project underway to produce *N. crassa* mutant knockouts for every gene (Dunlap *et al.*, 2007).

In addition to nuclear DNA, *Neurospora* contains multiple copies of a 64,840 bp circular mitochondrial DNA coding for two mitochondrial rRNAs, at least 27 tRNAs, the S5 ribosomal protein and 14 subunits of OXPHOS complexes (Borkovich *et al.*, 2004).

4.3. Inactivation of Nuclear Genes in *N. crassa* by RIP

Protein characterisation is usually achieved by disruption of the encoding genes and subsequent analysis of the produced mutants. The best studied phenomenon of gene-silencing in *Neurospora* is called repeat-induced point mutation (RIP) (Selker and Garrett, 1988; Selker, 2002). RIP is a process that efficiently detects duplicated DNA sequences longer than 400 bp with more than 80% nucleotide identity, and riddles both copies with GC-to-AT mutations and methylation, mostly in cytosine residues, causing gene silencing in *N. crassa* (Galagan *et al.*, 2003). Duplications that occur during the vegetative phase of the *Neurospora* life cycle are detected and inactivated by RIP during the sexual cycle. Thus, RIP functions as a fungi defence mechanism that helps to destroy foreign DNA (Borkovich *et al.*, 2004). Indeed, the *Neurospora* genome analysis revealed that RIP had a profound impact on genome evolution, by slowing the creation of new genes through genomic duplication (Galagan *et al.*, 2003).

Overall, taking advantage of this process it is theoretically possible to inactivate any nuclear gene, through the introduction of a cloned gene into a *Neurospora* strain by transformation, in order to duplicate the gene. Then, crossing this transformed strain with another *Neurospora* strain of opposite mating type, RIP will be induced and the duplicated gene will be inactivated with a certain frequency. The resulting ascospores produced carrying the mutation can be selected by direct analysis of the DNA or by checking the presence of the corresponding protein.

CHAPTER 2 – AIMS

AIMS

Research on complex I is of great significance since many human mitochondrial diseases involve structural and functional defects of this enzyme. Despite the importance of complex I, many aspects about this enzyme remain to be elucidated. As such, the identification of complex I subunits and their assignment into the different parts of the mature enzyme is essential. Moreover, it is crucial to understand complex I mechanisms and enzymatic properties, both as isolated enzyme and in association with the other OXPHOS complexes. Unravelling the intricate mechanisms underlying the functioning of this membrane-bound enzyme will help the development of new diagnostic procedures and therapeutic strategies.

In this context, the specific aims achieved throughout this project thesis are described below:

- a. Identification of the subunit composition of mitochondrial complex I in the filamentous fungus *N. crassa*.
- b. Determination of the functional role of the 9.8 kDa and the 11.5 kDa accessory subunits in the assembly and activity of *N. crassa* complex I.
- c. Elucidation of the role of the conserved cysteine residues of the 11.5 kDa subunit in the active/de-active transition of complex I.
- d. Investigation of the organisation of *N. crassa* respiratory chain complexes and subsequent identification of mitochondrial proteins by MALDI-TOF-MS.

The work performed with *N. crassa* was expected to contribute to a better knowledge of complex I of the mitochondrial respiratory chain.

CHAPTER 3 – RESEARCH WORK

1. The 9.8 kDa subunit of complex I, related to bacterial Na⁺-translocating NADH dehydrogenases, is required for enzyme assembly and function in *Neurospora crassa*

Isabel Marques, Margarida Duarte and Arnaldo Videira

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The 9.8 kDa Subunit of Complex I, Related to Bacterial Na⁺-translocating NADH Dehydrogenases, is Required for Enzyme Assembly and Function in *Neurospora crassa*

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A nuclear gene encoding a 9.8 kDa subunit of complex I, the homologue of mammalian MWFE protein, was identified in the genome of *Neurospora crassa*. The gene was cloned and inactivated *in vivo* by the generation of repeat-induced point mutations. Fungal mutant strains lacking the 9.8 kDa polypeptide were subsequently isolated. Analyses of mitochondrial proteins from mutant nuo9.8 indicate that the membrane and peripheral arms of complex I fail to assemble. Respiration of mutant mitochondria on matrix NADH is rotenone-insensitive, confirming that the 9.8 kDa protein is required for the assembly and activity of complex I. We found a similarity between the MWFE homologues and the C-terminal part of the nqrA subunit of bacterial Na⁺-translocating NADH:quinone oxidoreductases (Na⁺-NQR), suggesting a link between proton-pumping and sodium-pumping NADH dehydrogenases.

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Keywords: mitochondria; NADH dehydrogenases; repeat-induced point-mutations; sodium translocation; *Neurospora crassa*

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Introduction

Mitochondrial NADH:ubiquinone oxidoreductase (complex I, EC 1.6.5.3) transfers electrons from NADH to ubiquinone and links this process with translocation of protons across the inner membrane, thereby contributing to the establishment of a transmembrane proton gradient needed for the synthesis of ATP.¹ Experiments with the fungus *Neurospora crassa* indicated that the enzyme is composed of two distinct subcomplexes, arranged perpendicularly to each other in an L-shaped structure, that undergo independent assembly. The membrane arm is embedded in the mitochondrial membrane, while the peripheral arm is mainly protruding into the mitochondrial matrix.² Mammalian NADH:ubiquinone oxidoreductase might contain 46 subunits,³ seven of which are encoded by mitochondrial DNA, and contains non-covalently bound FMN and six to eight iron–sulphur clusters as prosthetic groups.⁴

Bacteria also possess enzymes equivalent to eukaryotic complex I, with a similar constitution

of prosthetic groups but with much fewer proteins, only 13–14 subunits. The prokaryotic enzymes contain homologues of the seven proteins encoded by mitochondria and homologues of seven proteins encoded by the nucleus in fungi or mammals. These core subunits are involved in the known complex I activity, electron transport coupled to proton translocation across a membrane.^{5,6} The many additional (“accessory”) subunits of mitochondrial complex I are likely involved in other functions of the enzyme (e.g. regulation or biosynthetic activities) but their precise role remains largely unknown. Besides complex I, two other types of NADH:quinone oxidoreductases can be found in respiratory chains, the alternative NADH dehydrogenases (NDH-2) and the bacterial Na⁺-translocating NADH dehydrogenases (Na⁺-NQR). One or more NDH-2 are present in different organisms, as single polypeptides or dimers, working without an energy coupling site.⁷ Na⁺-NQR are complexes of six independent subunits that couple electron transfer to the pumping of sodium ions across the bacterial membrane. Based on comparison of protein sequences, no relationship between the three types of respiratory chain NADH dehydrogenases has been found so far.^{8,9}

Mutations in both nuclear and mitochondrial

Abbreviations used: CI, complex I.
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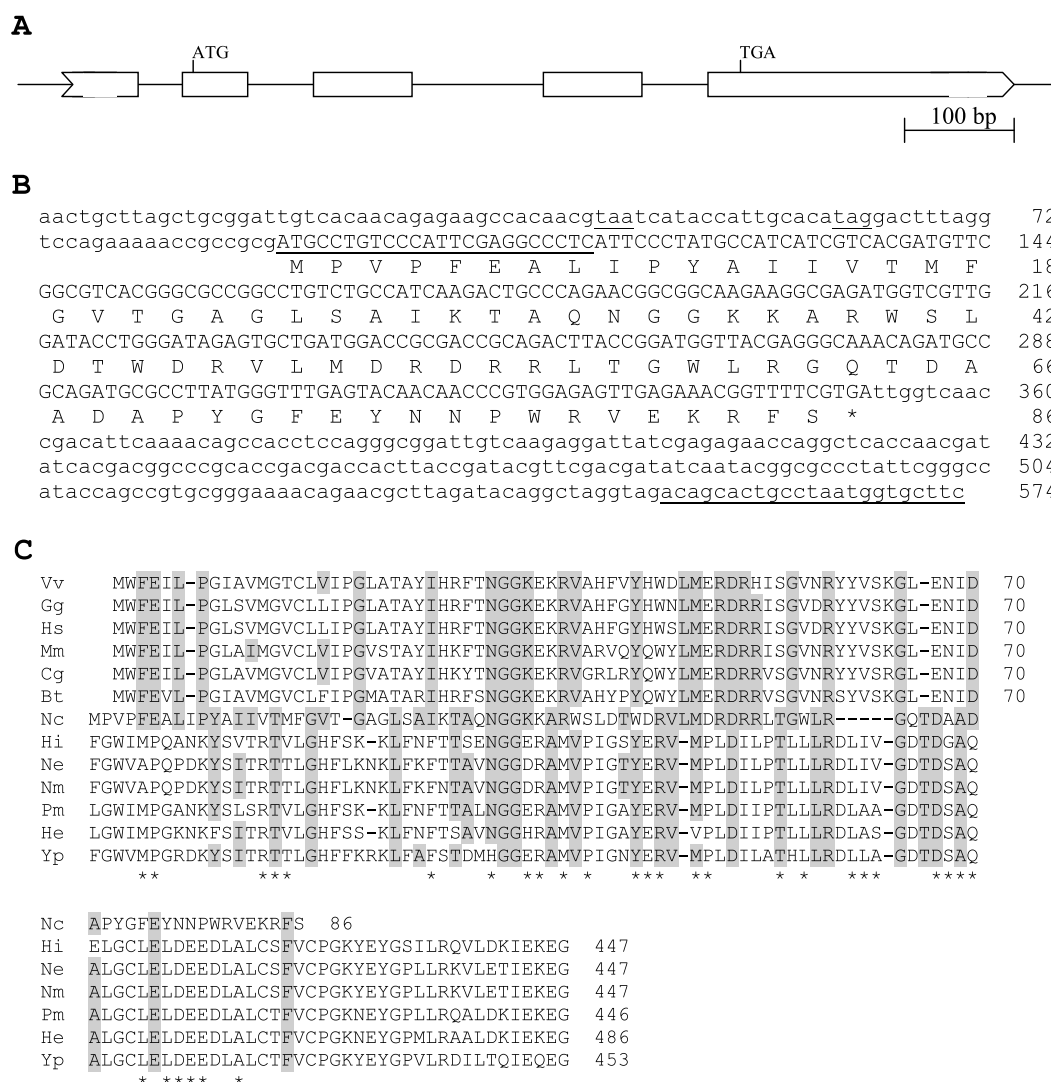


Figure 1. Characteristics of the *N. crassa* MWFE homologue. (A) Genomic DNA structure. The boxes represent sequences present in cDNA clones. (B) cDNA sequence and deduced primary structure of the 9.8 kDa polypeptide. Stop codons preceding in-frame the initiation codon as well as sequences used for primer design are underlined. The determined cDNA sequence was combined with EST sequences (upstream of the initiation codon, EMBL accession BG280238). (C) Alignment of the MWFE homologues from *Varecia variegata* (Vv), *Gorilla gorilla* (Gg), human (Hs), *Mus musculus* (Mm), hamster (Cg), *Bos taurus* (Bt), and *N. crassa* (Nc) with C-terminal residues of the nqrA subunits of Na⁺-translocating NADH dehydrogenase from *H. influenzae* (Hi), *Neisseria meningitidis* Z2491 (Ne), *N. meningitidis* MC58 (Nm), *Pasteurella multocida* (Pm), *Haemophilus somnus* (He) and *Yersinia pestis* KIM (Yp). Amino acid residues identical to *N. crassa* or present in at least seven sequences are shadowed. The asterisks below the sequences mark conservative amino acid substitutions.

genes of complex I, including those encoding accessory subunits, account for mitochondrial disease.^{10,11} Recently, it was found that mutant forms of the MWFE subunit, including a conservative R50K substitution, rendered complex I inactive in Chinese hamster cells interfering with complex I assembly.^{12,13} The corresponding human NDUF1 gene, located on chromosome Xq24 and consisting of three exons, codes for a polypeptide of 70 amino acid residues and is highly expressed in cardiac and skeletal muscle.¹⁴ The protein is imported into mitochondria without proteolytic processing⁴ and is likely inserted in the mitochondrial internal membrane.¹³ The MWFE poly-

peptide is highly conserved among animals,¹³ but no specific function was yet assigned to this protein.

This prompted us to look for and characterise the MWFE homologue of *N. crassa*, since the fungus has been used as a powerful genetic system.¹⁵ Complex I of *N. crassa* contains at least 37 subunits, including an homologue of the mammalian MWFE polypeptide. Overall, the fungal enzyme is quite similar to that of mammals. Up to now, only four of the fungal subunits could not be related to mammalian subunits of complex I^{7,16,17} (unpublished data). Here, we report the identification and characterisation of the 9.8 kDa MWFE-homologue

of *N. crassa*. Our results indicate that, as in hamster cells, the protein is required for complex I assembly and function. Apparently, lack of the protein prevents assembly of the peripheral and membrane domains of the enzyme. We also found that MWFE is similar to the C-terminal part of the *nqrA* subunit of bacterial Na^+ -translocating NADH dehydrogenases. Thus, for the first time, a link between proton-pumping and sodium-pumping NADH:quinone oxidoreductases of respiratory chains is recognized.

Results

Identification of the fungal MWFE homologue and its relationships

A computer search in the *N. crassa* genome, available from the *Neurospora* Sequencing Project, Whitehead Institute/MIT Center for Genome Research, led to the identification of a gene potentially encoding the fungal homologue of the MWFE subunit of mammalian complex I. The gene structure is not easy to predict because the fungal protein is not highly homologous to their mammalian counterparts (Figure 1). Therefore, we cloned and fully sequenced both strands of the corresponding cDNA. Two in-frame stop codons preceding the presumed ATG initiation codon confirmed that the latter is correct. The single-copy gene is located on linkage group II of the *N. crassa* genome and comprises four exons. The protein has 86 amino acid residues, a molecular mass of 9750 Da, without a predicted cleavable signal sequence.

An interesting finding was the similarity between the MWFE protein and the C-terminal part of the *nqrA* subunit of bacterial Na^+ -translocating NADH:quinone oxidoreductases (Figure 1(C)). In the region of similarity, 21 amino acid residues are conserved between *nqrA* and the *N. crassa* 9.8 kDa subunit of complex I (24% identity, or 55% similarity when conservative amino acid substitutions are considered). This similarity is comparable to the homology between the fungal 9.8 kDa polypeptide and mammalian MWFE. Also only 21 amino acid residues are conserved between the fungal and hamster subunits of complex I (30% identity). In addition, some very conserved residues between the fungal and mammalian proteins are also present in the Na^+ -NQR A subunit. It should also be noticed that while some amino acid residues of *nqrA* are conserved in the fungal subunit of complex I and not in the mammalian proteins, some other residues are conserved in the mammalian and not in the fungal protein. Combined with the fact that both MWFE and *nqrA* are subunits of (energy-coupling) NADH:quinone oxidoreductases, their relationship can be considered significant.

Since the peripheral and membrane arms of complex I assemble independently of each other,

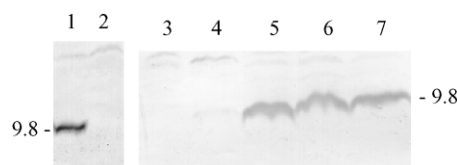


Figure 2. Presence of the 9.8 kDa polypeptide in mitochondria from complex I mutants. Total mitochondrial proteins (100 μg) from wild-type (1 and 7) and mutants *nuo9.8* (2), *nuo20.8* (3), *nuo12.3* (4) *nuo21.3c* (5) and *nuo24* (6) were analysed by Western blotting with an antiserum against the 9.8 kDa protein.

most null-mutants in subunits of one arm do not affect assembly of the other arm of the enzyme. Therefore, we checked the presence of the 9.8 kDa polypeptide in different mutants of complex I. Mutant mitochondria were analysed by Western blotting with antiserum against the 9.8 kDa protein (Figure 2). The polypeptide is clearly visible in wild-type mitochondria as well as in mitochondria from the peripheral arm mutants *nuo21.3c*¹⁸ and *nuo24*.¹⁹ In contrast, the 9.8 kDa polypeptide is only faintly visible or not detected, respectively, in the membrane arm mutants *nuo12.3*²⁰ and *nuo20.8*.²¹ These results indicate that the 9.8 kDa subunit is part of the membrane arm of complex I. They are also in agreement with the findings that the protein is rather unstable when it is not assembled.¹³

The 9.8 kDa protein is required for complex I assembly and function

We obtained a null-mutant of the 9.8 kDa subunit of complex I through the generation of repeat-induced point mutations (methylations and GC to AT transitions) in the respective gene. We took advantage of the fact that duplicated sequences in the genome of *N. crassa* are frequently inactivated by repeat-induced point mutations when passed through a genetic cross.²² Thus, we cloned a 1.7 kb DNA fragment containing the *nuo-9.8* gene and duplicated it in the genome of *N. crassa* by transformation. The transformant was crossed with a wild-type strain of the opposite mating type and individual ascospore progeny were isolated. Among them, the mutant strain *nuo9.8*, lacking the 9.8 kDa subunit of complex I, was identified by immunoblotting analysis of mitochondrial proteins with antiserum against the protein (Figure 2).

The state of assembly of complex I in mutant *nuo9.8* was evaluated by gradient centrifugation analysis of mitochondria solubilised with a non-denaturing detergent. Fractions of the gradients were analysed for NADH:hexa-amminoruthenium oxidoreductase activity, a typical artificial activity of complex I or its peripheral arm alone.²³ The gradient fractions were also checked for the presence of complex I subunits by Western blotting analysis of aliquots with antiserum against specific proteins

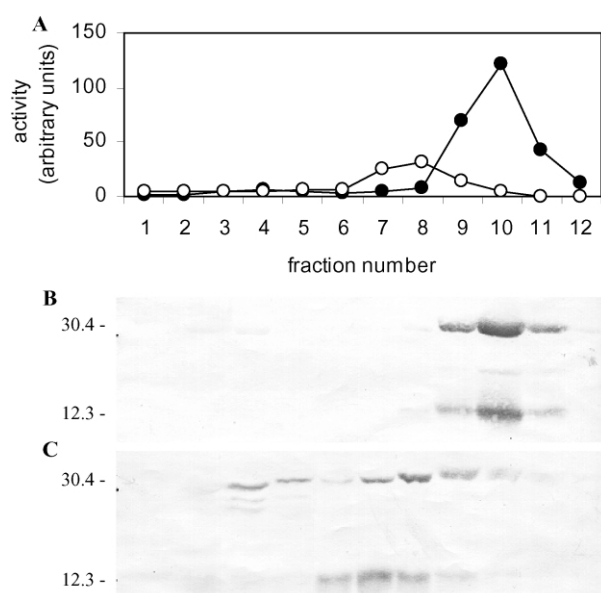


Figure 3. Complex I assembly in mutant *nuo9.8*. Mitochondria were isolated, solubilised with Triton X-100 and centrifuged in sucrose gradients. Fractions of the gradients (numbered 1–12 from top to bottom) were collected and analysed for NADH:hexa-amminoruthenium oxidoreductase activity, shown in (A) (filled circles, wild-type; open circles, mutant *nuo9.8*). The same fractions from the wild-type strain (B) and mutant *nuo9.8* (C) were analysed by Western blotting with a mixture of individual antisera against the subunits of complex I indicated at the left.

of the peripheral and membrane arms of the enzyme (Figure 3). In the wild-type strain, most of the oxidoreductase activity elutes in fractions 9–11 (Figure 3(A)), in agreement with the elution profile of the proteins analysed by Western blotting (Figure 3(B)), reflecting the presence of complex I in these fractions. In the mutant strain *nuo9.8*, both the oxidoreductase activity and the 30.4 kDa subunit of the peripheral arm of complex I²⁴ elute with a peak in fraction 8 of the gradient (Figure 3(A) and (C), respectively). This material likely represents the peripheral arm of complex I. In fact, the total NADH:ferricyanide reductase activities of *nuo9.8* mitochondria are about 40% of the wild-type values (not shown), a characteristic of mutants able to form the peripheral arm of complex I.²³ Most of the 12.3 kDa subunit of the membrane arm of complex I²⁵ is detected in fractions 6–8 of the sucrose gradients, with a peak in fraction 7. This material might represent an almost complete membrane arm of complex I or an intermediate of it. This phenotype resembles that of the *nuo12.3* mutant itself.²⁰ We conclude that the peripheral and membrane arms of complex I fail to assemble in the *nuo9.8* mutant.

Since the activities measured in the sucrose gradients do not reflect the physiological activity of complex I, we wanted to know if electrons could still be accepted from NADH by *nuo9.8* complex and passed on to the rest of the respiratory chain.

Table 1. Measurements of the respiratory activities of mitochondria. The activities are expressed in nmol of O₂/mg protein/min. Percent inhibition is indicated between brackets

Mitochondria	Wild-type	<i>nuo9.8</i>
Malate	60.2	58.2
+ Rotenone	31.7(47)	50.0(14)
+ Antimycin A	0(100)	0(100)
NADH	90.6	288.6
+ Antimycin A	0(100)	0(100)

Therefore, we determined the effect of the complex I inhibitor rotenone upon the respiration of isolated mutant mitochondria (Table 1). The oxygen uptake of wild-type mitochondria respiring on pyruvate/malate, which generates NADH in the matrix, was inhibited by about 50% with rotenone. While mitochondria of the mutant *nuo9.8* displayed similar levels of respiration, they were mostly resistant to rotenone inhibition (about 15% inhibition). This level of rotenone inhibition is also obtained with other non-functional mutants of complex I, like *nuo51*²⁶ and *nuo30.4*,²⁷ and can be considered background inhibition due to some unspecific effect of the drug (not shown). These results suggest that mutant complex I is not active and respiration is carried out by an internal alternative NADH dehydrogenase, in agreement with the results obtained with the sucrose gradients. Interestingly, we found that the respiratory activity of mutant mitochondria is about three times higher than that of wild-type when NADH is used directly as substrate (Table 1). This was somehow unexpected because this NADH oxidation reflects the activities of external alternative NADH dehydrogenases that oxidise cytosolic NADH.²⁸

Independent evidence that complex I is not assembled in the *nuo9.8* strain came from analysis of proteins from mutant mitochondria in non-denaturing gels. In contrast to wild-type, no high molecular weight complex I can be detected when mutant mitochondria are subjected to Blue Native electrophoresis (Figure 4). Complex I (CI) was

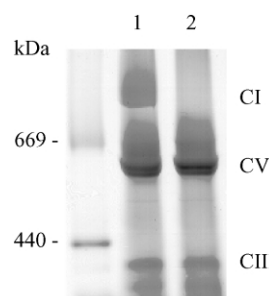


Figure 4. Analysis of mitochondrial complexes by Blue Native electrophoresis. Mitochondrial proteins (200 µg) from wild-type (1) and mutant *nuo9.8* (2) were solubilised with dodecyl maltoside and subjected to Blue Native electrophoresis. Complexes I (CI), III (CIII) and V (CV) are indicated.

Table 2. Number of spores resulting from crosses between the *ndi1* strain and complex I mutants

Cross	nuo	ndi1	Progeny		Total
			Wild-type	nuo, <i>ndi1</i>	
nuo9.8 × <i>ndi1</i>	6	16	11	0	33
nuo21.3a × <i>ndi1</i>	3	6	6	3	18

identified by Western blotting with antisera against specific subunits (not shown). Complex III (CIII) and complex V (CV) were tentatively assigned in the Figure by comparison with the patterns obtained with bovine mitochondria.²⁹ Results from crosses involving the nuo9.8 mutant further support the conclusion that complex I is not active in this strain. Homozygous crosses with nuo9.8 mutants resulted in the formation of barren perithecia, failing to produce sexual spores, as shown with homozygous crosses between mutants carrying a non-functional complex I.^{18,27} In addition, we recently identified and disrupted an internal alternative NADH dehydrogenase of *N. crassa* mitochondria, the NDI1 protein. We found that double mutants from crosses between *ndi1* mutants and mutants without a functional complex I are not viable (unpublished results). Similarly, no double mutants were obtained from a cross between the nuo9.8 and the *ndi1* mutant, while wild-type recombinants were readily isolated (Table 2). As a positive control, the cross between the *ndi1* mutant and the complex I nuo21.3a mutant (which carries a functional enzyme³⁰) yielded double mutants.

Discussion

Here, we described the identification and characterisation of the 9.8 kDa subunit of complex I from *N. crassa*, which is the fungal homologue of the mammalian MWFE protein. This polypeptide is present in eukaryotic but not in prokaryotic complex I. MWFE is highly conserved among animals and a small region of variability of about eight amino acid residues (residues 39–46) seems to be involved in species-specific compatibility.¹³ The human and *Xenopus* proteins are also very conserved (70% identity¹³). With cloning of the *Neurospora* homologue, we now show that the polypeptide sequence can deviate considerably. Only two small regions (surrounding that region of variability in animals) and a few scattered amino acid residues are conserved between the fungal and animal proteins.

We have isolated an *N. crassa* mutant strain devoid of the 9.8 kDa polypeptide after the generation of repeat-induced point mutations in the respective gene, *in vivo*. Analysis of complex I from the null-mutant indicated that the 9.8 kDa polypeptide is required for the assembly and function of the enzyme. By the analysis of mitochondrial proteins in sucrose gradients or in native

gels, we could not detect the formation of a full complex in the mutant. In addition, respiration of nuo9.8 mitochondria on matrix-generated NADH is insensitive to inhibition by the complex I inhibitor rotenone. Independent support of that conclusion was obtained from mating experiments. As described for other non-functional complex I mutants of *N. crassa*,¹⁸ it was not possible to cross nuo9.8 with itself and obtain viable progeny (homozygous crosses) and it was not possible to obtain double mutants from crosses between nuo9.8 and *ndi1* mutants (which lack an internal alternative NADH dehydrogenase). These results are in agreement with previous findings in hamster cell mutants. It was shown that truncated forms or specific amino acid alterations in the MWFE protein interfere with complex I formation and render the enzyme inactive.^{12,13} We extended these observations showing that the peripheral and membrane arms of complex I fail to assemble.

An interesting finding in the nuo9.8 mutant was that the activities of alternative NADH dehydrogenases were raised as compared with the wild-type strain, especially, the observation that the respiratory activity of mutant mitochondria is much higher when NADH is used directly as substrate (Table 1). This reflects the activities of external alternative NADH dehydrogenases, which oxidise cytosolic NADH, and it is not clear how a deficiency in matrix NADH oxidation due to the complex I mutation would have an effect on these external enzymes. An enhancement in external (and internal) NADH oxidase activities was also noticed in plant mitochondrial mutants with complex I deficiency.^{31,32} Preliminary results with specific antibodies did not reveal higher levels of external alternative NADH dehydrogenases in nuo9.8 mitochondria as compared with the wild-type strain (not shown). It is possible that the existence of pathways for exchanging matrix/cytosolic NADH explain these observations. On the other hand, they might suggest a more direct regulation of alternative NADH dehydrogenase enzymes by complex I. Post-transcriptional regulation and direct enzyme regulation control the activity of alternative oxidase, a protein that transfers electrons from ubiquinol to oxygen and is thus a bypass of the cytochrome pathway.^{33–35}

A major finding of this work was the similarity between the MWFE homologues and the C-terminal part of the *nqrA* subunit of bacterial Na⁺-translocating NADH:quinone oxidoreductases.⁸ Despite the fact that the *nqrA* protein is much bigger (447 amino acid residues in *Haemophilus influenzae*) than MWFE, there are other examples of relationships between complex I subunits and bigger bacterial proteins. For instance, the α -subunit of the NAD⁺ hydrogenase of *Alkaligenes eutrophus* represents a fusion between the 51 kDa and 24 kDa subunits of complex I³⁶ and the ORF5 subunit of formate hydrogenlyase of *Escherichia coli* represents a fusion between the 49 kDa and 30.4 kDa subunits of complex I.³⁷ The conclusion

that MWFE and *nqrA* are related is based on sequence as well as on functional similarity, since both polypeptides belong to (energy-coupling) NADH:quinone oxidoreductases. Contrary to what is currently believed, this similarity suggests a relationship between proton-pumping and sodium-pumping NADH dehydrogenases and might be reminiscent of a common origin of both complexes. On the other hand, MWFE homologues are not present in bacterial complex I.⁵ This might indicate the existence of a specific function of mitochondrial complex I that is shared by bacterial Na⁺-NQR. Clearly, a more precise definition of the roles of MWFE and *nqrA* in their respective complexes is required.

Material and Methods

Molecular cloning and generation of antisera

Standard cloning techniques were employed.^{38,39} The plasmid vectors pGEM-T easy (Promega) and pCSN44⁴⁰ were used for bacterial subcloning and fungal transformation, respectively. A fragment of *N. crassa* genomic DNA of 1693 bp, containing the *nuo-9.8* gene, was amplified by PCR using the two specific primers 5'-CGCT TTGTTCTGAGCTCCCGGC-3' and 5'-GAAGCACCAT T AGGCAGTGCTGT-3'. The first (upstream) primer has a restriction site for *Sac*I (bolded) that was introduced by changing two bases (underlined). The amplified 1.7 kb fragment was cloned in the pGEM-T easy vector in *E. coli* DH5 α . Then, it was excised from the recombinant vector with the enzymes *Sac*I and *Eco*RV and cloned in the pCSN44 vector, originating pCSN-15SE.

In order to express the 9.8 kDa protein, *N. crassa* cDNA was amplified from the mycelial library M-1 cloned in Uni-Zap XR (obtained from the Fungal Genetics Stock Center) by PCR using the primer 5'-ATG CCTGTCCCATTCGAGGCCCTC-3' that hybridises on the initial ATG codon (bold). The second (downstream) primer was the same used in the amplification of genomic DNA. The PCR product was cloned in the pGEM-T easy vector. Then, the segment with the gene was excised from the recombinant plasmid with the enzymes *Sph*I and *Pst*I (which cut in pGEM-T) and cloned in the pQE31 vector (Qiagen) in *E. coli* M15. The resulting fusion protein, consisting of the 9.8 kDa protein and 26 extra amino acid residues at the N terminus, was induced with IPTG and purified in nickel columns followed by SDS-PAGE. Rabbits were immunised as described.⁴¹

Mutant isolation

N. crassa wild-type strains 74-OR23-1A and 74-OR8-1a were grown and handled according to standard procedures.⁴² Spheroplasts were prepared from seven-day old conidia of the 74-OR8-1a strain and transformed with the recombinant plasmid pCSN-15SE. The transformants were selected in plates containing hygromycin B (100 μ g/ml) and purified by successive asexual transfers in slants of Vogel's minimal media containing 50 μ g/ml of the same drug.²⁰ In order to select single-copy transformants, genomic DNA of individual transformants was isolated from 20 ml mycelial cultures²⁵ and analysed by Southern blotting, using DNA encoding the 9.8 kDa

protein as a probe. A single-copy transformant was separately crossed with the 74-OR23-1A wild-type strain. The mutant strain *nuo9.8* was identified among spore progeny by immunoblotting analysis of mitochondrial proteins with antiserum against the 9.8 kDa subunit of complex I.

Oxygen consumption

Purified mitochondria were prepared as before.²⁸ To assess their quality, the activities of cytochrome *c* oxidase (EC 1.9.3.1) and malate dehydrogenase (EC 1.1.1.37) were measured in the presence and in the absence of Triton X-100⁴³ to calculate the latent activities of the enzymes. Oxygen uptake was measured polarographically at 25 °C with a Clark-type oxygen electrode (Hansatech), in a total volume of 1 ml. The NADH assays were started with the addition of 1 mM NADH to the reaction medium (0.3–0.5 mg protein, 0.3 M sucrose, 10 mM potassium phosphate (pH 7.2), 5 mM MgCl₂, 1 mM EGTA, 10 mM KCl, 4 μ M carbonyl cyanide *m*-chlorophenylhydrazone and 0.02% (w/v) bovine serum albumin). The malate assays were started with the addition of 10 mM malate to reaction medium containing 1 mM NAD⁺ and 10 mM pyruvate. Rotenone and antimycin A were added to final concentrations of 40 μ M and 0.2 μ g/ml, respectively.

Protein analysis and enzymatic activities

The techniques used for protein determination,⁴⁴ SDS-PAGE,⁴⁵ blotting and incubation of blots with antisera,^{41,46} detection of alkaline phosphatase-conjugated second antibodies,⁴⁷ sucrose gradient centrifugation analysis of detergent solubilised mitochondrial proteins,³⁰ NADH:hexa-amminoruthenium (III)⁴⁸ and NADH:ferricyanide reductase activities⁴⁹ and Blue Native gel electrophoresis of respiratory complexes solubilised with 1.7% dodecyl- β -D-maltoside²⁹ have been published. Web-based computer programs such as Blast and ClustalW (and the Blosum 45 matrix to identify conservative amino acid alterations) were used for bioinformatics.

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Composition of complex I from *Neurospora crassa* and disruption of two “accessory” subunits

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Abstract

Respiratory chain complex I of the fungus *Neurospora crassa* contains at least 39 polypeptide subunits, of which 35 are conserved in mammals. The 11.5 kDa and 14 kDa proteins, homologues of bovine IP15 and B16.6, respectively, are conserved among eukaryotes and belong to the membrane domain of the fungal enzyme. The corresponding genes were separately inactivated by repeat-induced point-mutations, and null-mutant strains of the fungus were isolated. The lack of either subunit leads to the accumulation of distinct intermediates of the membrane arm of complex I. In addition, the peripheral arm of the enzyme seems to be formed in mutant nuo14 but, interestingly, not in mutant nuo11.5. These results and the analysis of enzymatic activities of mutant mitochondria indicate that both polypeptides are required for complex I assembly and function.

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Keywords: Mitochondria; Respiratory chain; Complex I; NADH dehydrogenase; Mutant; *Neurospora crassa*

1. Introduction

Respiratory chain complex I (NADH:ubiquinone oxidoreductase, EC 1.6.5.3) exists in a variety of organisms and catalyses the oxidation of NADH by ubiquinone with the formation of a transmembrane proton gradient, necessary for ATP synthesis. Both the eukaryotic and bacterial complex I contain 8–9 iron–sulfur clusters and a non-covalently bound FMN molecule [1,2]. According to electron microscopy studies, the enzyme is L-shaped, with one arm located in the membrane and the other arm protruding into the mitochondrial matrix. The first is composed by the more hydrophobic

subunits and contains the ubiquinone-binding site, whereas the second, named peripheral arm, is made of hydrophilic proteins and contains all known prosthetic groups. These two arms are formed independently of each other, before joining together. The membrane arm is formed through the assembly of a large and a small intermediate in *Neurospora crassa*, with the association of two CIA proteins to the former intermediate that are not present in the mature complex I [3]. In mammals, 46 polypeptides with a total molecular mass of about 1 MDa were proposed to be subunits of the enzyme [4]. Bacterial complex I is much simpler, consisting of only 14 subunits [5,6], considered the “minimal” structure required to couple electron transfer to transmembrane proton translocation [2]. Mitochondrial complex I have homologues of all these 14 proteins, including the seven subunits encoded by mtDNA. The role of the many additional “accessory” subunits of the mitochondrial enzyme is largely unknown [7,8].

The fungus *N. crassa* has been invaluable in complex I research. The specific disruption of many subunits and their

Abbreviations: BN-PAGE, Blue native electrophoresis; dNADH, deamino-NADH; HAR, hexaammineruthenium (III); Q₁, 2,3-dimethoxy-5-methyl-6-isoprenyl-1,4-benzoquinone

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effect on complex I phenotype confirmed that they are bona fide constituents of the enzyme [3,7,9,10]. We have identified and studied two of the *N. crassa* nuclear-coded “accessory” subunits of complex I, the 11.5 kDa and 14 kDa polypeptides. The cysteine-rich 11.5 kDa protein is homologous to the bovine IP15 protein, suggested to be involved in complex I active/de-active transitions. These transitions result likely from structural alterations between an active (A) and a de-activated (D) form of the enzyme, which have different sensitivity to SH-reagents. Studies with a fluorescent analogue of *N*-ethyl maleimide identified a specifically labelled subunit of complex I with a molecular mass of 15 kDa [11]. The 14 kDa polypeptide is the fungal equivalent of the bovine B16.6 subunit of complex I. This protein is 83% identical to the human GRIM-19 protein, which is believed to be involved in interferon- β and all-*trans*-retinoic acid pathway of apoptosis induction [12]. In fact, the cell-death regulator GRIM-19 is primarily localized in the mitochondria in a variety of cells, as a component of complex I, and plays a role in complex I assembly and function [13]. Here, we analyse the polypeptide constitution of mitochondrial complex I and present the characterization of *N. crassa* null-mutants, lacking the 11.5 kDa and 14 kDa subunits. Both proteins are essential for enzyme assembly and activity and their absence results in the accumulation of distinct complex I intermediates.

2. Materials and methods

2.1. Strains and plasmids

The *N. crassa* wild type strains 74-OR23-1A (74A) and 74-OR8-1a (74a) were grown and handled according to standard procedures [14]. A fungal cDNA library cloned in Uni-ZAP XR was obtained from the Fungal Genetics Stock Center. The plasmid vectors pGEM-T Easy (Promega), pCSN44 [15] and pQE31 (Qiagen) were used for intermediate cloning, fungal transformation and protein expression in bacterial systems, respectively. *Escherichia coli* strains DH5 α and M15 were used as hosts in intermediate cloning and protein expression experiments, respectively.

2.2. Molecular cloning and protein expression

The *nuo-11.5* gene was amplified by PCR from genomic DNA using primers 5'-**GATATCGTACTCATGTGTCGCGGTACAG**-3' and 5'-**GATATCCACACTTGTTCTCTTTCATCG**-3'. These primers introduce the restriction sites for *EcoRV* (in bold). The amplified 1606 bp fragment of genomic DNA was cloned in pGEM-T [16]. Then a smaller 1430 bp fragment was excised from the recombinant vector with the enzyme *SacI* and cloned in pCSN44 for fungal transformation. The cDNA encoding the 11.5 kDa protein was amplified by PCR using primers 5'-ATGTCGTCCGGC-TATGGCATG-3' and 5'-TTATTTCGACCCCAACACCGC

-3' and cloned in pGEM-T. The sequence of both strands of the cDNA was determined. Then, it was excised from the recombinant vector with the enzymes *SphI* and *PstI* and cloned in pQE31 for protein expression.

The *nuo-14* gene was amplified by PCR from genomic DNA using primers 5'-**AAGCTTGGCATGGAACGGGG-AATGGAGG**-3' and 5'-**AAGCTTCAGCCAACCGCCA-ACAACCAGC**-3'. Sequences in bold were added to create *HindIII* restriction sites. The amplified 2083 bp fragment of genomic DNA was cloned in pGEM-T. The fragment was excised with *HindIII* and cloned in pCSN44 for fungal transformation. The cDNA encoding the 14 kDa protein was amplified by PCR using primers 5'-ATGCCTCAGGATAT-GCCCCC-3' and 5'-**AAGCTTCAGCCAACCGCCAAC-AACCAGC**-3'. The 465 bp fragment obtained was cloned in pGEM-T, both strands were sequenced, then excised with *SphI* and *PstI* and also cloned in pQE31 for protein expression.

Bacterial strains containing pQE31 recombinant plasmids were induced with IPTG in order to express the fungal proteins. In this way, the 11.5 kDa polypeptide and the 14 kDa polypeptide were separately expressed as fusion proteins containing 26 extra residues at the N-terminus and including a six histidine-tag. The fusion proteins were purified in nickel columns and used to immunize rabbits as previously described [17]. Specific polyclonal antisera against the polypeptides were thus obtained.

2.3. Isolation of fungal mutants

Spheroplasts were prepared from 7 day old wild type conidia from the 74a strain and transformed with pCSN44 recombinant plasmids [18]. The transformants were selected in plates containing hygromycin B (100 μ g/ml) and purified by successive asexual transfers in slants of Vogel's minimal media containing 50 μ g/ml of the same drug. Genomic DNA of individual strains was isolated from 20 ml mycelial cultures and analysed by Southern blotting [16], using selected restriction enzymes and DNA probes, in order to identify single-copy transformants. These transformants were separately crossed with the 74A wild type strain. Random progeny of the genetic crosses were germinated on minimal media and used for the isolation of mitochondria. The mutant strains *nuo11.5* and *nuo14* were identified by immunoblotting analysis of the organelle proteins with the specific antisera against the respective proteins.

2.4. Oxygen consumption and enzymatic activities

Oxygen uptake in purified mitochondria [19] was measured polarographically at 25 °C with a Clark-type oxygen electrode (Hansatech) in a total volume of 1 ml. The malate assays were started with the addition of 10 mM malate to a mitochondrial suspension (0.3–0.5 mg) in 0.3 M sucrose, 10 mM potassium phosphate pH 7.2, 5 mM MgCl₂, 1 mM EGTA, 10 mM KCl, 4 μ M carbonyl cyanide *m*-chlorophe-

nylhydrazone, 0.02% bovine serum albumin, 1 mM NAD⁺ and 10 mM pyruvate. Rotenone and antimycin A were added to final concentrations of 40 μ M and 0.2 μ g/ml, respectively.

The oxidation of (d)NADH in inside-out sub-mitochondrial particles [19] was measured photometrically (ϵ_{340} =6.22 mM) in a standard assay mixture (50 mM Tris/HCl, 0.25 M sucrose, 0.2 mM EDTA, pH 8.0). When (d)NADH:Q₁ reductase activity was assayed, 2 mM KCN, 100 μ M (d)NADH and 60 μ M Q₁ were added to the reaction mixture. NADH:hexaammineruthenium III (HAR) reductase activity was measured photometrically by the oxidation of NADH (ϵ_{380} =1.25 mM) in the presence of 2 mM KCN, 120 μ M NADH and 2 mM HAR. NADH:ferricyanide oxidoreductase activity was measured photometrically (ϵ_{420} =1.05 mM) in the standard assay mixture containing 2 mM KCN, 120 μ M NADH and 0.5 mM ferricyanide.

2.5. Other techniques

The techniques used for sub-cellular fractionation of *N. crassa* mycelia [20], protein determination [21], SDS-PAGE [22], blotting and incubation of blots with antisera [23], the detection of anti-rabbit horseradish peroxidase (Amersham) or alkaline phosphatase conjugated second antibodies [24], the sucrose gradient centrifugation analysis of detergent solubilized mitochondrial proteins [25] and BN-PAGE of respiratory complexes solubilized with dodecyl maltoside [26] have been published before. Web-based computer programs were used for bioinformatics.

3. Results

3.1. Composition of complex I from *N. crassa*

In the last years, we have been characterizing polypeptide subunits of complex I from *N. crassa* mitochondria. The screening of the fungal genome with homologues from other organisms has identified novel subunits, some of which are shown in Fig. 1. The Neurospora Sequencing

Project of the Whitehead Institute/MIT Center for Genome Research (<http://www.genome.wi.mit.edu>), and data contained therein, was essential to this work and to build Table 1. Fungal complex I is composed of at least 39 dissimilar subunits, including seven polypeptides encoded by mtDNA. With a few exceptions, the 32 nuclear genes that code for complex I proteins contain 1–3 short intronic sequences. Seventeen of these fungal subunits have predicted mitochondrial targeting cleavable sequences (presequences) as the corresponding bovine homologues [8], except the B22 protein. With a few exceptions, most of the peripheral arm subunits possess and most of the membrane arm polypeptides lack presequences. Eleven fungal subunits (15 polypeptides in bovine) contain predicted transmembrane helices. These transmembrane domains are conserved in the 8 bovine homologues of the 11 fungal membrane-spanning proteins. In agreement with their location, none of the subunits of the peripheral arm of complex I displays predictable transmembrane domains. Most subunits can be located either in the peripheral or membrane arms of complex I, especially when mutants are available. The location of the 40 kDa subunit is still unclear, but it has likely a peripheral location since the nuo40 mutant assembles a complex I without NADH:Q₁ activity [27], and, to date, only the disruption of peripheral arm subunits still allows enzyme assembly. Mutant strains for 19 of the nuclear-coded proteins have been generated [7,28] (this work), and their characterization allows the identification of these polypeptides as genuine complex I components.

3.2. Disruption of the 11.5 kDa subunit of complex I

The single copy gene encoding the 11.5 kDa subunit of *N. crassa* complex I (bovine 15 kDa homologue) is located on linkage group V of the fungal genome. The corresponding protein has 105 amino acid residues and a molecular mass of 11,518 Da. Since the peripheral and membrane arms of complex I assemble independently of each other, most null-mutants in the subunits of one arm do not affect the assembly of the other arm of the enzyme [10,28].

14 kDa

MPQDMPPAGG YDAVQYKRN L PPSAFKPKTL LAFGGLIMVY GWYHLFHGIR 50
EQRELAREKM WSRIHLIPAL QAEEDRDLVR RHLADVQREK ELLGDKAVKA 100
YHSDRYVRPT FAITPGKIIK

11.7 kDa

MSTDRAVL LSL YRRSLKLSLD WAVNRQLWRG QALYIRSLFE KNRDVHDP RQ 50
LRVLLRETEK LLEHWKHPDP FVPLSPGGS KYERNLSAHV LDPPPPLRF

11.3 kDa

MASDSSITTA RRNSPSQSTL PDATREEMRD AKLPLAYRDS CAHLLIPLNR 50
CRYDTYYLPW KCEDERHTYE KCQYVEFKKR VAKMDELRAA KGGARSN

Fig. 1. Primary structure of subunits of *N. crassa* complex I. The 14 kDa sequence was deduced from the nucleotide sequence of a cDNA clone and contains 5 amino acid residues (underlined) not present in the Whitehead database sequence. The 11.7 kDa sequence was deduced from an EST sequence (contig 161). The 11.3 kDa sequence was predicted from genomic DNA by comparison with homologous proteins.

Table 1
Polypeptide composition of *N. crassa* complex I

<i>N. crassa</i> accession no.	Bovine	Features	Location/Presequence ^a	Mutant	TMH ^b /introns
78 NCU01765.1	75	[2Fe2S] N1b; [4Fe4S] N4, N5	p/+	yes	0/5
51 NCU04044.1	51	NADH-binding; FMN; [4Fe4S] N3	p/+	yes	0/2
49 NCU02534.1	49	Quinone binding?	p/+	yes	0/1
30.4 NCU04074.1	30		p/+	yes	0/1
24 NCU01169.1	24	[2Fe2S] N1a	p/+	yes	0/2
21.3c NCU05009.1	TYKY	[4Fe4S] N6a, N6b	p/+	yes	0/2
19.3 NCU03953.1	PSST	[4Fe4S] N2	p/+	yes	0/2
40 NCU02373.1	39	NADPH	nd/+	yes	0/7
29.9 NCU05299.1	B13		p/+	yes	0/2
21.3a NCU08930.1	–		p/–	yes	0/2
21.3b NCU02280.1	B14.7		m/–	yes	3/3
21 NCU05221.1	AQDQ	Phosphorylation	p/+	yes	0/2
20.9 NCU01859.1	–		m/–	yes	1/4
20.8 NCU02472.1	PGIV		m/–	yes	0/3
20.1 NCU09460.1	ASHI		nd/+		1/3
18.4 NCU00484.1	IP13		nd/+		0/2
17.8 NCU00969.1	–		m/+		1/2
14.8 NCU00418.1	B14		p/–		0/3
14 NCU09299.1	B16.6	GRIM-19 homologue	m/–	yes	1/2
13.4 NCU01142.1	B17.2		nd/–		0/3
12.3 NCU03093.1	PDSW		m/–	yes	0/1
11.7	B22		nd/+		0/4
11.6 NCU04753.1	ESSS	Phosphorylation	nd/+		1/1
11.5 NCU01360.1	IP15		m/–	yes	0/2
11.3	B18	Myristoylation	nd/–		0/2
10.6 NCU09002.1	B12		nd/–		1/2
10.5 NCU03156.1	B8		nd/–		0/3
10.4 NCU01467.1	–		nd/+		1/1
9.8 ^c	MWFE	Phosphorylation	m/–	yes	1/3
9.6 NCU05008.1	SDAP	Phosphophantetheine	p/+	yes	0/3
9.5 NCU00670.1	B9		m/–		1/1
6.6 NCU00160.1	B15		nd/–		1/1
ND1	ND1	Quinone binding?	m	*	10/mtDNA
ND2	ND2	Proton translocation?	m	*	12/mtDNA
ND3	ND3		m	*	3/mtDNA
ND4	ND4	Proton translocation?	m	*	14/mtDNA
ND4L	ND4L		m	*	3/mtDNA
ND5	ND5	Proton translocation?/myristoylation	m	*	16/mtDNA
ND6	ND6		m	*	5/mtDNA

p and m, peripheral and membrane arms of complex I, respectively; nd, not determined.; *some stopper mutants affected in one or more mtDNA genes are available [51].

^a Predicted with MITOPROT (<http://ihg.gsf.de/ihg/mitoprot.html>).

^b Transmembrane helices (TMH) were predicted with the TMHMM server v.2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>).

^c Protein sequence from Ref. [32].

Therefore, we checked the presence of the 11.5 kDa protein in Western blots of different complex I mutants. The protein is visible in wild type mitochondria, as well as in mitochondria from the peripheral arm mutants nuo51 [29], nuo30.4 [30] and nuo19.3 [31]. In contrast, it was faintly detected in the mitochondria from the membrane arm mutants nuo9.8 [32] and nuo12.3 [18], indicating that the 11.5 kDa subunit belongs to the membrane arm of complex I (data not shown).

In order to obtain a null mutant of the 11.5 kDa complex I subunit, we used the RIPing method, which consists in the generation of repeat-induced point-mutations (methylations and GC to AT transitions) in the respective gene. Duplicated sequences in the genome of *N. crassa* are frequently

inactivated by RIPing when passed through a genetic cross [33]. Thus, we cloned a 1.4 kb DNA fragment containing the *nuo-11.5* gene and duplicated it in the genome of *N. crassa* by transformation. The transformant was crossed with a wild type strain of the opposite mating type, and an individual ascospore progeny was isolated. The mutant strain nuo11.5, lacking the 11.5 kDa subunit of complex I, was identified by immunoblotting analysis of mitochondrial proteins with antiserum against the protein (Fig. 2). An analysis of mutant mitochondria by BN-PAGE revealed the absence of complex I in the nuo11.5 strain (Fig. 3).

The state of assembly of complex I in mutant nuo11.5 was further evaluated by sucrose gradient centrifugation analysis of mitochondrial proteins solubilized with Triton X-100. The

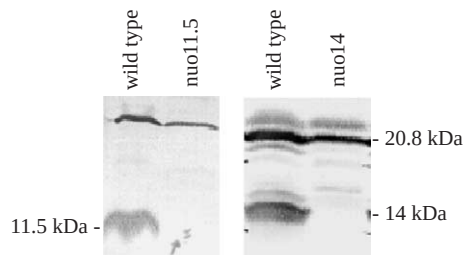


Fig. 2. Identification of complex I mutant strains. Total mitochondrial proteins (100 μ g) from wild type and mutants nuo11.5 and nuo14 were analysed by Western blotting with antisera against the indicated subunits of complex I.

gradients were fractionated and aliquots were analysed for NADH:ferricyanide reductase activity (and NADH:HAR reductase activity), a typical artificial activity of complex I, and for the presence of complex I subunits by Western blotting (Fig. 4). In the wild type strain, most of the oxidoreductase activity elutes in fractions 9–11 (Fig. 4A), in agreement with the elution profile of the proteins (Fig. 4B), reflecting the behaviour of complex I. In mutant nuo11.5, the elution profile of the 20.8 kDa polypeptide [34] displays a peak in fractions 8–9, while the 12.3 kDa polypeptide [35] is mainly detected in fractions 6–8 (Fig. 4C). Since both proteins are members of the membrane arm of complex I, these results indicate that two distinct intermediates of the membrane arm of complex I accumulate in nuo11.5, though in small amounts. The 14 and 20.9 kDa [36] subunits of the membrane arm of complex I elute in the same fractions of the 20.8 kDa subunit (not shown). The 51 [37] and 30.4 kDa [38] subunits of the peripheral arm of complex I are predominantly detected in fractions 3 and 4 of the gradients, likely representing free subunits or smaller subcomplexes. This and the fact that NADH:ferricyanide reductase activity could not be detected in the gradients indicate that neither complex I nor its peripheral arm is present in nuo11.5 mitochondria. Subunits

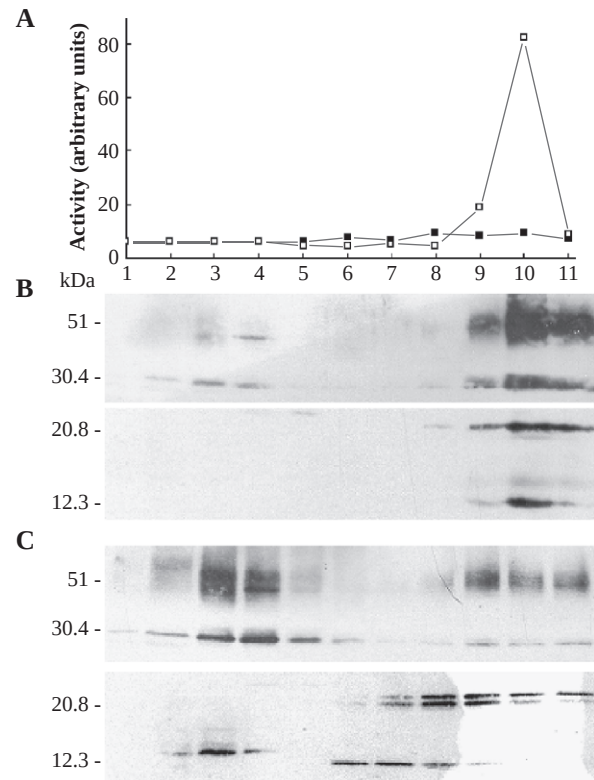


Fig. 4. Analysis of mitochondrial proteins from nuo11.5 by sucrose gradient centrifugation. Mitochondria were isolated, solubilized with Triton X-100 and centrifuged in sucrose gradients. Fractions of the gradients (numbered 1–11 from top to bottom) were collected and analysed for NADH:ferricyanide reductase activity, shown in panel A ($\square$, wild type strain; $\blacksquare$, mutant nuo11.5). The same fractions from the wild type strain (B) and mutant nuo11.5 (C) were analysed by Western blotting with antisera against the indicated subunits of complex I.

of both the peripheral and membrane arms of complex I can also be seen in the high molecular weight region of the gradients (Fig. 4C). However, since, based on the determination of enzymatic activities, we have no evidence for the presence of either complex I or its peripheral arm (see also below), we attribute this pattern to protein aggregation.

Next, we determined the mitochondrial respiratory activities of the mutant using pyruvate/malate as substrates, which generate NADH inside the organelles (Table 2). The oxygen uptake of wild type mitochondria was inhibited about 71% with the specific complex I inhibitor rotenone. The respiration of nuo11.5 is completely insensitive to rotenone. It is likely due to the activity of the single internal alternative NADH dehydrogenase [39] and further indicates that complex I is defective in mutant mitochondria. Antimycin A completely inhibits the respiratory activities

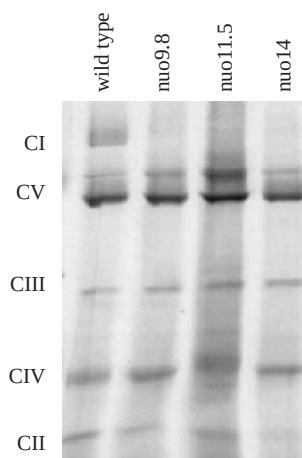


Fig. 3. Analysis of mitochondrial complexes by BN-PAGE. Mitochondrial proteins (150 μ g) from wild type and mutants nuo9.8 (negative control), nuo11.5 and nuo14 were solubilized with dodecyl maltoside (1.5 g/g protein). The respiratory complexes were tentatively identified by comparison with the electrophoretic pattern of bovine complexes.

Table 2

Respiratory activities of wild type and nuo11.5 mutant mitochondria

Activity nmol O ₂ /min per mg	Wild type	nuo11.5
Plus malate	32	30
Plus rotenone (% inhibition)	9 (71)	30 (0)
Plus antimycin A (% inhibition)	0 (100)	0 (100)

of both wild type and nuo11.5. We also examined the different oxidoreductase activities of inside-out sub-mitochondrial particles (Table 3). In these experiments, dNADH was employed as electron donor to determine more specifically the activity of complex I, since alternative NADH dehydrogenases are able to oxidise NADH but not dNADH [40]. It is evident that nuo11.5 mutant membranes lack the rotenone-sensitive NADH:Q₁ or dNADH:Q₁ reductase activities and that the NADH:Q₁ reductase activity is higher than the dNADH:Q₁ reductase activity, again reflecting the action of the internal alternative NADH dehydrogenase. Furthermore, the NADH:HAR reductase activity, which can be used to estimate the amount of complex I or its peripheral arm [41], is also highly defective in this mutant (<10% of the wild type value). Whether employing dNADH or NADH, the values of HAR reduction were similar, and therefore, only the NADH:HAR reductase activities are reported. Taken together, these results support our conclusion that the nuo11.5 mutant does assemble neither complex I nor even its peripheral arm.

3.3. Disruption of the 14 kDa subunit of complex I

A single gene encodes the 14 kDa polypeptide, a 121 amino acid residue protein homologous to bovine B16.6, which contains a putative transmembrane domain at the N-terminus (residues 29–46, Fig. 1) conserved in homologues from other organisms. The protein is clearly visible in the Western blots of mitochondria from complex I mutants in peripheral arm subunits (nuo51, nuo30.4 and nuo29.9 [18]), but less visible in the mitochondria from the membrane arm nuo9.8 mutant. Furthermore, it was found associated with the membrane arm of complex I in Western blots following sucrose gradient centrifugation of mitochondrial proteins from mutant nuo21.3c [42] (not shown). Thus, we conclude that the 14 kDa subunit belongs to the membrane arm of *N. crassa* complex I and it is probably connected to the inner mitochondrial membrane by its putative transmembrane segment. Since this protein may be involved in cell death, we also investigated its sub-cellular location. Mycelia from wild type *N. crassa* was fractionated into cytoplasmic, mitochondrial and nuclear portions and analysed by Western blotting. The 14 kDa protein, as well as the control 30.4 kDa subunit of complex I, was detected exclusively in the mitochondrial fraction (not shown).

A fungal null-mutant in the 14 kDa protein was generated by RIPing (Fig. 2), as described for the 11.5 kDa polypep-

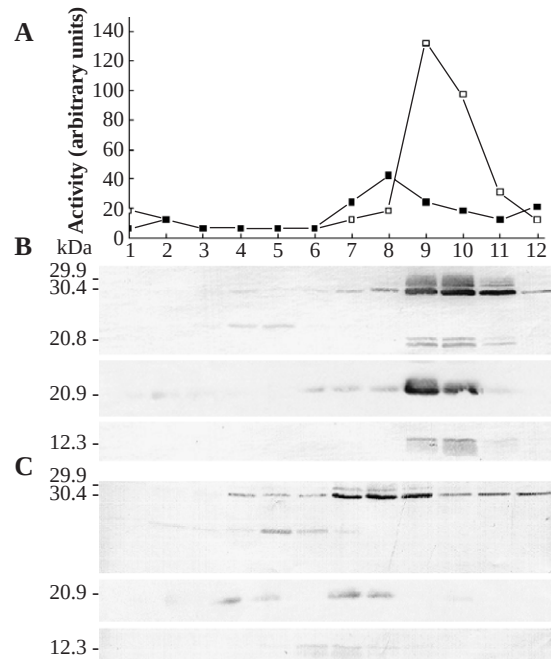


Fig. 5. Analysis of mitochondrial proteins from nuo14 by sucrose gradient centrifugation. The experiment was performed as described in the legend of Fig. 4. NADH:ferricyanide reductase activities are shown in panel A (□, wild type strain; ■, mutant nuo14), and Western blots from wild type and mutant nuo14 are depicted in Panels B and C, respectively.

tide. An analysis of mutant mitochondria by BN-PAGE revealed the absence of complex I in the nuo14 strain (Fig. 3). The assembly of complex I in nuo14 was further assessed by sucrose gradient centrifugation of mitochondrial membrane proteins (Fig. 5), as described above for nuo11.5. In the wild type strain, complex I is present mostly in fractions 9 and 10, as judged by the elution profile of the NADH:ferricyanide reductase activity (and NADH:HAR reductase activity) and of enzyme subunits (Fig. 5A and B, respectively). In the nuo14 mutant, both the NADH:ferricyanide reductase activity and the 30.4 and 29.9 kDa [43] peripheral subunits elute mainly in fractions 7–9, representing an assembled peripheral arm of complex I. The membrane arm subunits 12.3 kDa and 20.9 kDa elute mainly in fractions 6–7 and 7–8, respectively, identifying two distinct intermediates of the membrane arm of complex I. We conclude that complex I assembly is prevented, but the peripheral domain and two intermediates of the membrane arm of the enzyme are formed in nuo14 mitochondria.

This conclusion is supported by the determination of enzymatic activities of mutant mitochondria. The NADH:-

Table 3
Catalytic properties of sub-mitochondrial particles from wild type and the complex I mutant strains

Assay	Wild type	nuo11.5	nuo14
NADH:Q ₁ reductase activity $\mu\text{mol NADH/min per mg}$ (sensitivity to 15 μM rotenone)	0.324 $\pm$ 0.023 (82%)	0.104 $\pm$ 0.001 (0%)	0.104 $\pm$ 0.003 (0%)
dNADH:Q ₁ reductase activity $\mu\text{mol dNADH/min per mg}$ (sensitivity to 15 μM rotenone)	0.311 $\pm$ 0.019 (94%)	0.043 $\pm$ 0.004 (25%)	0.020 $\pm$ 0.008 (0%)
NADH:HAR reductase activity $\mu\text{mol NADH/min per mg}$	1.288 $\pm$ 0.041	0.118 $\pm$ 0.032	0.475 $\pm$ 0.052

ferricyanide reductase activity in the mitochondria of mutant *nuo14* solubilized with Triton X-100 is about 30% of the wild type activity, characteristic of complex I mutants that assemble the peripheral arm of the enzyme [44]. Mutant sub-mitochondrial particles lack the rotenone-sensitive NADH:Q₁ or dNADH:Q₁ reductase activities (Table 3) and thus lack active complex I. Therefore, the NADH:HAR reductase activity shown in Table 3 (37% of the wild type value) is a rough estimation of the amount of peripheral arm of complex I present in *nuo14* mutant mitochondria.

4. Discussion

The filamentous fungus *N. crassa* has been long used as a model eukaryote to characterize the genetic, assembly, structural and functional properties of respiratory chain complex I [7]. While the first cloning of a nuclear-coded subunit of the fungal enzyme was carried out about 15 years ago [34], the recent sequencing of the genome [45] allowed significant advances regarding the definition of the polypeptide composition of *N. crassa* complex I. In addition, extensive gene disruption experiments confirmed most polypeptides as true subunits [7,28]. We found that *N. crassa* complex I contains at least 39 proteins, including the 14 bacterial homologues that constitute the “minimal” form of the enzyme, and 25 “accessory” subunits. Considering each subunit present only once, the total molecular mass of the complex approaches 1 MDa, being almost as large as the mammalian enzyme (about 1.1 MDa).

Thirty five of the fungal subunits are conserved in mammals, pointing to the high similarity between the enzyme from these two types of organisms. Homologues of the remaining 4 proteins (21.3a, 20.9, 17.8 and 10.4 kDa) can be found in the genomes of other species. The NUWM subunit of *Yarrowia lipolytica* complex I (37 identified subunits) was considered as fungal-specific and, based on predicted structural characteristics, suggested to be related to *N. crassa* 17.8 kDa [46]. Rather, it seems that NUWM is equivalent to *N. crassa* 11.6 kDa and bovine ESSS proteins, as also pointed out recently [47]. A plant homologue of the fungal 20.9 kDa protein was noticed before [10]. Homologues of the *N. crassa* 17.8 kDa and 10.4 kDa were found neither in *Y. lipolytica* [46] nor in plants [48], but they are present in the genomes of some fungi and might be unique to this group of organisms. Recently, the polypeptide composition of complex I from plants was analysed, and 41 subunits were described, including several plant-specific proteins [48]. Analysis of the similar complex from the unicellular alga *Chlamydomonas reinhardtii* revealed that it is likely composed of 42 proteins, and 31 of them are conserved among eukaryotes. Interestingly, a series of ferripyochelin-binding protein-like proteins were found among 12 subunits considered plant-specific components [47,48]. We found several sequences related to this type of proteins in the genome of *N. crassa*, which, however, do not contain clear mitochondrial pre-

sequences. Without any further experimental evidence, they cannot be considered as complex I subunits in the fungus. We did not find *N. crassa* homologues of any of the other plant- and alga-specific proteins. Mammalian complex I contains 46 subunits [8], 35 of which have fungal counterparts as referred above. Conversely, 10 out of the 11 remaining subunits (one was not yet sequenced) seem to be present only in mammals and might be unique and fulfil special roles in complex I of higher eukaryotes.

In order to further characterize the roles of the “accessory” subunits of complex I, we have separately disrupted the nuclear genes encoding the 11.5 and 14 kDa subunits of the membrane arm of the enzyme. The results obtained with a combination of different techniques indicate that both proteins are required for complex I assembly and function. The 11.5 kDa polypeptide was suggested to participate in complex I active/de-active transitions, because its bovine homologue is among a few candidate proteins that might have been labelled with SH-reagents in the de-active form of the enzyme [11]. In fact, the phenomenon has been described in *N. crassa* [49,50], and the 11.5 kDa polypeptide as well as homologues from different organisms contain four highly conserved cysteine residues that can possibly react with these reagents. The disruption of the fungal protein leads to the accumulation of two intermediates of the membrane arm of complex I. Interestingly, we were unable to detect the formation of an active peripheral arm of the enzyme. Up to now, the assembly of this extrinsic domain of complex I was observed in all mutants lacking the subunits of the membrane domain [7], including mutants affected in mtDNA-encoded polypeptides [51]. Indeed, with the exception of the ACP mutant [52], mutants in one arm of the enzyme are able to assemble the complementary arm, supporting the idea that both domains of complex I are assembled independently [53]. This is the first description that the assembly of the peripheral arm of complex I is affected by the absence of a membrane subunit.

The 11.5 kDa protein is located in the membrane domain of *N. crassa* complex I. The bovine homologue is likely in fragment I α [8] and was also found associated with an iron-sulfur protein fragment obtained by chaotropic resolution of complex I [54], suggesting that it lies in the boundary between the extrinsic and membrane domains. We would like to speculate that the protein participates in the connection of the peripheral and membrane arms of complex I. In its absence, peripheral arm subunits might be degraded or aggregated, and this would explain the high molecular mass complexes observed in sucrose gradients (which contained peripheral arm subunits without oxidoreductase activity). These results are not completely compatible with results from studies in cultured human cells, suggesting that the 11.5 kDa homologue is added to complex I at a later stage, after the connection between an hydrophilic and a membrane fragment took place [55]. On the other hand, it is not clear if the specific contributions of orthologues from different organisms to the assembly/stability/function of

complex I have changed during evolution. For instance, the AQDQ-type subunit was found essential for complex I assembly and function in human cells [56], but not in *Neurospora* [44], though later, a high molecular mass subcomplex was identified in mutant human cells [57]. It appears that the fungal protein is not required for complex I function [44], while its phosphorylation regulates the activity of the mammalian enzyme [58].

A homologue of the 14 kDa subunit of *N. crassa* complex I was first identified in bovine mitochondria (B16.6 polypeptide) and found to be identical to the cell-death regulator GRIM-19 [12]. The protein is located in the membrane arm of fungal complex I and in subcomplex I λ of the bovine enzyme [8], also suggesting that it lies in the boundary between the extrinsic and membrane domains. Recent studies established that homologous deletion of GRIM-19 prevents the assembly and function of complex I and causes embryonic lethality, showing that the enzyme is required for mouse development [13] as previously noticed in other species, like *N. crassa* [30,42], *Drosophila melanogaster* [59] and *Caenorhabditis elegans* [60]. We extended these observations by disrupting the fungal homologue of GRIM-19. The lack of the 14 kDa protein in fungal mitochondria leads to the accumulation of the peripheral arm and two intermediates of the membrane arm of complex I. Based on Western blots of fractionated cells from wild type *N. crassa*, we also found the 14 kDa protein exclusively in the mitochondrial fraction, in agreement with the previous report that the GRIM-19 polypeptide is primarily localized in the mitochondria in a variety of mouse cells and is associated with the native form of complex I [13]. Further work is required to unravel the role of the polypeptide in cell death and the possible link between this phenomenon and bioenergetics.

The assembly of complex I is still a puzzling issue. The process has been mainly studied in *N. crassa*, but it has been recently analysed in other systems [61–64]. It is clear that complex I formation does not proceed by the accretion of individual subunits at a time but rather by the independent assembly of subcomplexes that later come together. Pioneer work with the fungus provided the current model for enzyme assembly. Following findings that the peripheral and membrane arms of complex I assemble independently of each other [53], it was found that the latter arm results from the joining of a small and a large intermediate, which binds transiently two chaperones [3,65]. Our own results using genetic approaches largely support this model. The peripheral or the membrane arm is usually formed in mutants lacking subunits of the complementary arm. In addition, most mutants in the membrane arm subunits (nuo21.3b [65], nuo20.8 [66], nuo14, nuo11.5 and nuo9.8 [32]) appear to accumulate two intermediates of this hydrophobic domain of complex I. Mutants nuo20.9 [28] and nuo12.3 [18] appear to accumulate only one intermediate, respectively, the small and large intermediates. The analysis of complex I assembly in human cells led to

conflicting results, either supporting [55] or contradicting [67] the *Neurospora* model. These questions deserve further scrutiny, and, at present, it can also not be excluded that different organisms have evolved different complex I assembly pathways.

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3. Role of the conserved cysteine residues of the 11.5 kDa subunit in complex I catalytic properties

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Role of the Conserved Cysteine Residues of the 11.5 kDa Subunit in Complex I Catalytic Properties

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Mitochondrial complex I exists as a mixture of two inter-convertible forms: active (A) and de-activated (D), the latter being sensitive to SH-modifying compounds. To investigate if the conserved cysteine-rich 11.5 kDa subunit of *Neurospora crassa* complex I is involved in this process, we subjected the corresponding genomic DNA to site-directed mutagenesis. The four cysteine residues of the subunit were separately substituted with serine residues and the resulting proteins were independently expressed in a null-mutant strain. All of the obtained mutant strains were able to assemble a complex I with similar kinetic properties to those observed in the wild-type enzyme, indicating that none of the cysteine residues of the 11.5 kDa protein is individually relevant for the A/D transition process. Diminished amounts of assembled complex I seem to be the major effect of these specific mutations. The cysteine residues are likely important to the acquisition and stabilization of the correct 11.5 kDa protein conformation and this is reflected in the assembly/stability of complex I.

Key words: active/de-active transition, complex I, mitochondria, *Neurospora crassa*, site-directed mutagenesis.

The mitochondrial NADH: ubiquinone oxidoreductase (complex I) couples the oxidation of NADH and the reduction of ubiquinone to the generation of a trans-membrane proton gradient, which is then used for ATP synthesis. The minimal structural elements common to the mitochondrial and the bacterial complex are 14 polypeptides arranged in an L-shaped structure, one non-covalently bound FMN group and 8–9 iron–sulphur clusters (1–3). Mitochondrial complex I contains many additional accessory subunits for which the function is largely unknown. The bovine complex I contains 45 different polypeptides, while the enzyme from the filamentous fungus *N. crassa* is composed of at least 39 mostly conserved subunits (4–7).

The mammalian mitochondrial complex I exists in two distinct inter-convertible forms, the active (A) and the de-activated (D) forms (8, 9). Only the A form of the enzyme is able to catalyse a rapid oxidation of NADH and the $\Delta\mu\text{H}^+$ -dependent reaction of reverse electron transport. The D form can be fully reduced by NADH and oxidized by artificial electron acceptors, but is unable to transfer electrons to ubiquinone. De-activation occurs if complex I activity is limited due to the lack of one of the substrates of the enzyme. The D form can be transformed into the A form by incubation with NADH under conditions where the enzyme turnover occurs. The rate of enzyme activation decreases at alkaline pH or in the presence of bivalent cations (10, 11). The A form is

spontaneously converted in the D form in a reaction extremely temperature dependent. The enzyme de-activates during incubation without substrates at high temperatures. Both forms differ in their affinity for the complex I inhibitors rotenone and piericidin A and in their accessibility to thiol reagents (12). The active form of complex I is not sensitive to sulphhydryl reagents, whereas the D form is irreversibly inhibited by *N*-ethylmaleimide (NEM) (13).

This process is known in mammals for a long time, and was recently described in other organisms, including the fungi *N. crassa* and *Yarrowia lipolytica* (10, 14). The fungal enzymes display A/D transitions with much lower activation barrier compared to the bovine enzyme. It is believed that A/D transitions are exclusive to eucaryotes, although nothing is known about the catalytic properties of the plant enzyme so far. The molecular mechanism of the A/D transition and the complex I subunits implicated in the process are still unknown. We have recently demonstrated that a 29.9 kDa protein in *N. crassa* is involved in the active/de-active transition. A mutant lacking the 29.9 kDa subunit of complex I retains the capability to undergo A/D transitions, though with a slower de-activation process (15). Another small subunit of the bovine complex I, with an apparent molecular weight of 15 kDa, was specifically labelled by the fluorescent NEM analogue in the D form of the enzyme. The bovine IP-15 subunit of complex I was a likely candidate for this specific labelling, since it has around 15 kDa and contains four highly conserved cysteine residues that can possibly interact with the thiol reagent (13). Thus, we decided to study the catalytic

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properties of *N. crassa* mutants in the 11.5 kDa subunit, the homologue of bovine IP-15. The 11.5 kDa subunit appears to be located in the membrane arm of the *N. crassa* enzyme, and its lack leads to the accumulation of intermediates of the membrane arm and to the impairment of the peripheral arm assembly, which prevents any kinetic measurements (7). To circumvent this result, we performed independently site-directed mutagenesis of the four conserved cysteines of the protein, and analysed the assembly and function of complex I in the resulting *Neurospora* strains. Our results indicate that none of the cysteine residues of the 11.5 kDa subunit is individually important for the A/D transition, but they are important to the assembly and stability of complex I.

MATERIALS AND METHODS

Handling of Strains and Plasmids—The *N. crassa* strains were grown and handled according to the standard procedures (16). We used the *N. crassa* strain 74-OR23-1A and the *nuo11.5* mutant strain obtained previously by repeat-induced point mutations in the *nuo11.5* gene. This mutant strain lacks the 11.5 kDa subunit of complex I and has no detectable enzyme assembly or activity (7). Spheroplasts were prepared from 7 days old conidia from the *nuo11.5* strain, transformed with the recombinant plasmids derived from pMYX2 and selected on plates containing the antibiotic benomyl (0.5 µg/ml). Expression of the genomic DNA was induced by the addition of 10 mM quinic acid.

Cloning and Generation of Point Mutations—The genomic DNA coding for the 11.5 kDa subunit was amplified by PCR using the primers 5'-**CCCGGG** CAGTATGTGCGTATCATCA-3' and 5'-**CCCGGGGTGG** TATGGTATAGGTACAA-3' that contain the restriction site for *Sma*I (in bold). The amplified 740 bp fragment was cloned in the TOPO TA Cloning vector (Invitrogen). Then, it was excised from the recombinant vector and cloned in the *Sma*I site of the expression vector pMYX2, downstream of the *qa-2* promoter. Site-directed mutagenesis was done using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). The pMYX2 recombinant vector and pairs of synthetic complementary oligonucleotide primers containing the desired mutation were used in PCR reactions to create the mutated plasmids. The mutagenic primers were: C14S, 5'-CATCCCGTTCCTTTCCCTTTTGG-3'; C24S, 5'-TGCT CGCCTCCTACGTGGTC-3'; C40, 5'-GCAGAAAGAAGTC CTCGCCGATG-3'; C50S, 5'-ACTACGAGTCCTTGC ACCACAAGAAGG-3'. The mutagenic oligonucleotide primers were designed in order to change four independent codons in the genomic DNA (underlined nucleotides) that resulted respectively in the replacement of cysteines C14, C24, C40 and C50 by serine residues in the 11.5 kDa subunit. The mutagenesis was confirmed by complete sequencing of the PCR products.

Enzymatic Activities—Mitochondria were isolated from *N. crassa* strains (17) and then used for the preparation of sub-mitochondrial particles (SMP). Mitochondria were suspended in a buffer containing 50 mM Tris-HCl (pH 8.0), 0.25 M sucrose and 0.2 mM EDTA to a final

protein concentration of 10 mg/ml and the pH was adjusted to 8.6 with 1 M NH₄OH. The mitochondrial suspension was then subjected to ultrasonic treatment (five 30-s pulses with 1-min intervals) and centrifuged at 30,000g for 15 min. The supernatant was then centrifuged at 120,000g for 1 h. The pellet was washed, suspended in the same buffer and stored at -80°C.

Oxidation of deamino NADH (dNADH) was measured photometrically ($\epsilon_{340\text{ nm}} = 6.22\text{ mM}^{-1}\text{ cm}^{-1}$) in a standard assay mixture containing 50 mM Tris-HCl (pH 8.0), 0.25 M sucrose, 0.2 mM EDTA and 1 mg/ml BSA. The activities were measured after addition of SMP (~50 µg/ml) to the standard medium. In the dNADH:Q₁ reductase activity assay, 2 mM KCN, 100 µM dNADH and 60–100 µM Q₁ were added to the reaction mixture. The activity was also measured in the presence of 15 µM of the complex I inhibitor rotenone.

The fully activated enzyme was obtained by pre-incubation of SMP in the reaction mixture with 10–20 µM of dNADH, until complete oxidation of the substrate, in order to eliminate the lag phase in the onset of the catalytic activity. Then, the dNADH:Q₁ reductase activity was monitored in the presence or absence of 2 mM NEM. For thermal de-activation, SMP were incubated 15 min at 30°C in the presence or absence of 2 mM NEM and their activity was determined in the same way.

NADH:hexaammineruthenium III (HAR) reductase activity was measured photometrically by the oxidation of NADH ($\epsilon_{380\text{ nm}} = 1.25\text{ mM}^{-1}\text{ cm}^{-1}$) in the presence of 2 mM KCN, 100 µM NADH, 2 mM HAR and 1 mg/ml BSA.

Protein Analysis—Blue-native polyacrylamide gel electrophoresis (BN-PAGE) was performed according to the standard procedures (18). One-hundred and fifty micrograms of total mitochondrial proteins were solubilized using 3.0 g of *n*-dodecyl- β -D-maltoside (DDM)/g of protein, and the resulting solubilized complexes were separated on a 3–13% gradient gel. The NADH dehydrogenase activity of complex I was detected in the gel (19).

The techniques used for development of the rabbit antiserum directed to the 11.5 kDa subunit of the complex I (7), determination of protein concentration (20), SDS-polyacrylamide gel electrophoresis (21), blotting and detection of alkaline phosphatase-conjugated secondary antibody (22), have been described before.

RESULTS AND DISCUSSION

Complex I Assembly in the *nuo11.5* Mutants—The *nuo11.5* mutant strain lacking the 11.5 kDa subunit accumulates intermediates of the membrane arm, preventing the further characterization of complex I activity. Since this subunit was a possible candidate for the SH-labelling related with the A/D transitions, we decided to change independently the four conserved cysteine amino acids for serine residues (C14S, C24S, C40S and C50S). The altered genes as well as the intact gene were expressed in the null-mutant strain *nuo11.5* under the control of a heterologous promoter. The presence of the 11.5 kDa subunit on the various strains was checked by western blotting and the strains expressing the highest amount of protein were chosen for further analysis. As shown in Fig. 1, the 11.5 kDa protein is present in all

site-directed mutant strains, although in lower amounts than in wild-type. The electrophoretic migration behaviour of the 11.5 kDa protein in all the strains is the same in the presence and absence of the disulphide reducing agent mercaptoethanol (5% v/v), indicating that the polypeptide does not form intramolecular disulphide bonds (data not shown).

To characterize complex I assembly in the strains harbouring the point mutations, we solubilized the mitochondrial proteins with the non-ionic detergent DDM and separated them by BN-PAGE. As depicted in Fig. 2A, complex I is present in all of the site-directed mutant strains though in different relative amounts. It is evident that the C40S and C50S mutations have the most severe effect in complex I assembly. The in-gel NADH dehydrogenase activity revealed the presence of complex I activity in all four site-directed mutants, as well as the existence of a smaller complex with a

molecular weight of ~500 kDa, likely representing the peripheral arm of complex I (Fig. 2B). These mutant strains accumulate peripheral arm, possibly due to the limiting amount of properly assembled membrane arm. This suggests that the four conserved cysteine residues are required for the acquisition and stabilization of the protein structure and consequently to the correct assembly of the membrane arm. The involvement of this subunit in the assembly of the membrane arm was already observed in the null-mutant strain (7). This strain also assembles tiny amounts of peripheral arm, not previously observable because it is only detectable by in-gel NADH dehydrogenase activity (Fig. 2B).

Enzymatic Characteristics of the Mutants—We investigated complex I activities with different electron donors/acceptors in SMP from the wild-type and the mutant strains. The NADH:HAR reductase activity, which can be used to estimate the amount of complex I or its peripheral arm, is residual in *nuo11.5* due to the small amounts of assembled peripheral arm in this strain (Fig. 2). The null-mutant rescued by wild-type genomic DNA strain has roughly 50% of the wild-type activity. In the strains harbouring the site-specific mutations C14S, C24S, C40S and C50S, the NADH:HAR activity is 52, 31, 22 and 26%, respectively, which is in agreement with the results obtained by the BN-PAGE. The mutation C14S is the less harmful for complex I assembly, resulting in a complex I activity similar to wild-type rescued enzyme.

Then, we measured the dNADH:Q₁ reductase activity in order to determine more specifically the complex I activity, since *N. crassa* has multiple alternative NADH dehydrogenases capable of oxidizing NADH but not dNADH (6). The rotenone sensitive dNADH:Q₁ reductase activity is lower in the site-directed mutants than in the wild-type, and the *nuo11.5* has no detectable rotenone sensitive dNADH:Q₁ reductase activity (Table 1). The null-mutant rescued by wild-type genomic DNA and the C14S strains display about 40% of wild-type activity and the C24S, the

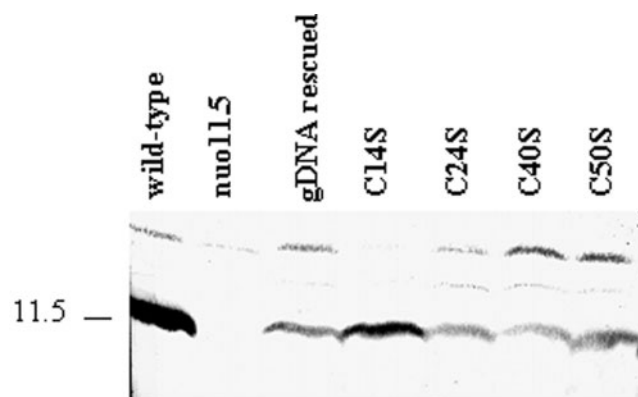


Fig. 1. Western blotting analysis of mitochondrial proteins from the site-directed mutant strains. Total mitochondrial proteins from wild-type and mutant strains were analysed by western blotting with an antiserum against the 11.5 kDa subunit of complex I.

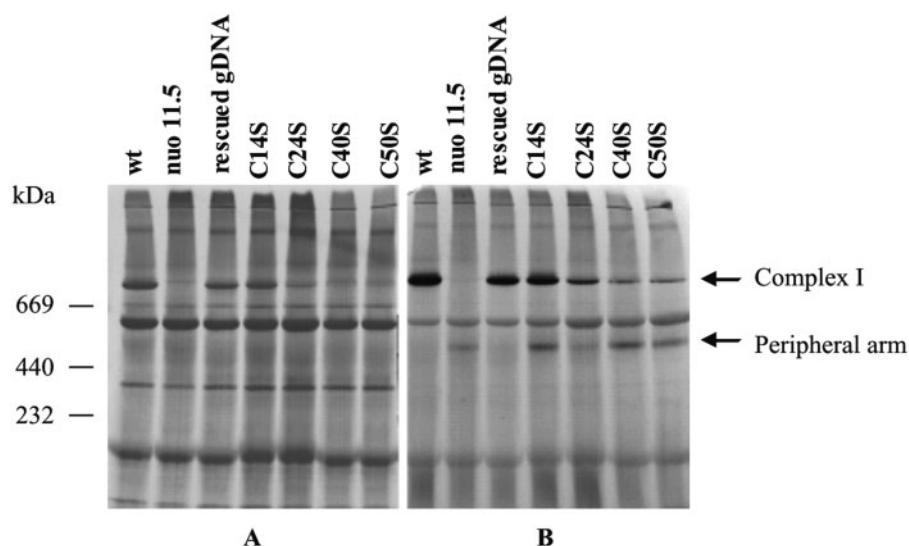


Fig. 2. Analysis of the mitochondrial respiratory chain complexes from different *N. crassa* strains by BN-PAGE. One-hundred and fifty micrograms of total mitochondrial

proteins were solubilized with 3 g DDM/g of protein and applied on a 3–13% gradient gel. (A) Coomassie Brilliant Blue stained gel; (B) in-gel NADH dehydrogenase activity.

Table 1. Catalytic properties of SMP from different strains at 25°C.

<i>N. crassa</i> strain	Rotenone sensitive dNADH:Q ₁ reductase activity ^a	NADH:HAR reductase activity
Wild-type	0.336 ± 0.020	1.093 ± 0.108
nuo11.5	0.003 ± 0.001	0.154 ± 0.012
Rescued gDNA	0.142 ± 0.016	0.524 ± 0.064
C14S	0.122 ± 0.007	0.565 ± 0.059
C24S	0.062 ± 0.003	0.335 ± 0.014
C40S	0.050 ± 0.015	0.239 ± 0.014
C50S	0.043 ± 0.002	0.283 ± 0.002

μmol (d)NADH/min per mg of protein.

^a15 μM rotenone was added.

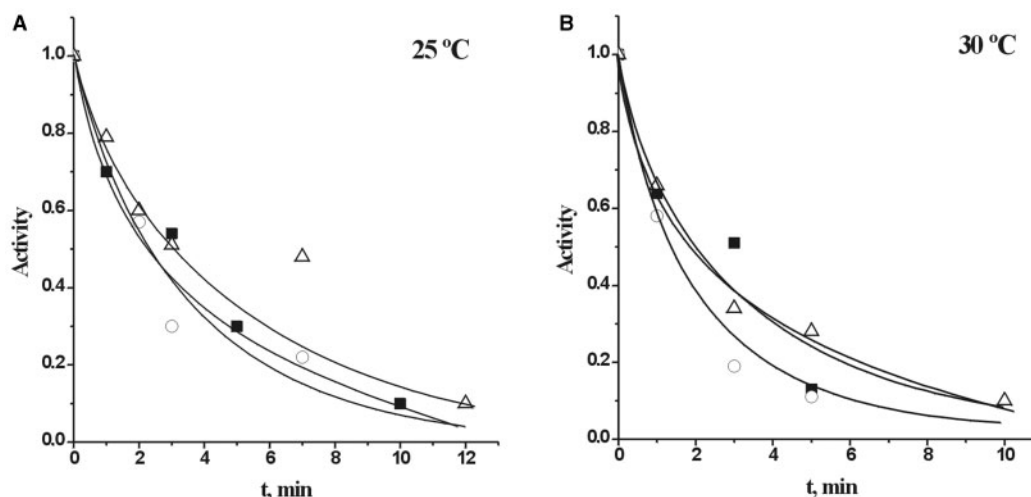


Fig. 3. De-activation of complex I in *N. crassa* strains at two different temperatures. Fifty micrograms per millilitre of SMP from wild-type (filled square), C14S (open circle) and C40S (open triangle) were pre-pulsed aerobically with 10–20 μM dNADH in a spectrophotometric cuvette at 25°C (A) and 30°C (B). After complete depletion of dNADH the incubation was continued for the times indicated on the abscissa and the initial rates of rotenone sensitive dNADH:Q₁ reductase activity were

determined in the presence of 2 mM MgCl₂ at 25 or 30°C. Two millimoles of KCN and 100 μM Q₁ were added and the reaction was started by the addition of 100 μM dNADH. The initial activities for wild-type, C14S and C40S were 0.1503, 0.0774 and 0.0368 μmol dNADH/min per mg of protein at 25°C; and 0.1594, 0.0964 and 0.0398 μmol dNADH/min per mg of protein at 30°C, respectively.

C40S and the C50S strains only around 15%. The lower activities of the C24S, C40S and C50S strains reflect the small amount of assembled complex I. Moreover, the ratios between the quinone and HAR activities show that the complex I present in the mutant strains has no enzyme activity alteration, indicating that the mutations interfere with the assembly but not with the complex I activity.

A/D Transitions in the Mutant Strains—The rates of complex I de-activation of the cysteine mutants were analysed at two different temperatures. This was checked by measuring the initial dNADH:Q₁ reductase rates catalysed by SMP, in the presence of 2 mM MgCl₂, after their incubation for different times at a given temperature. Complex I de-activates faster at higher temperatures and thus the *t*_{1/2} at 30°C was approximately half of the *t*_{1/2} at 25°C. No significant differences were observed between the mutants and the wild-type enzyme concerning the kinetic parameters of complex I de-activation. The de-activation curves obtained for wild-type, C14S and C40S strains are shown in Fig. 3.

The de-activated form of complex I from different organisms is irreversibly inhibited by SH-modifying reagents such as NEM, whereas the active form is not sensitive to these inhibitors (13). This is a powerful diagnostic tool to study the amount of de-activated complex I in an enzymatic preparation. Inhibition by NEM was seen for the thermally de-activated SMP from all different preparations. As shown in Table 2, the dNADH:Q₁ reductase activities of all strains were not altered by the addition of 2 mM NEM when SMP were pre-pulsed with dNADH before the addition of the inhibitor, demonstrating that the active form is resistant to NEM inhibition. The dNADH:Q₁ reductase activity from all strains was inhibited around 60% by NEM, when SMP were deactivated by incubation at 30°C for 15 min. Thus, complex I from all mutant strains is able to bind NEM similarly to the wild-type enzyme, indicating that none of the cysteine residues of the 11.5 kDa protein is involved in the NEM binding and inhibition process.

It is not clear whether the complex I subunit labelled with NEM is also involved in the structural changes of the A/D transition phenomenon. However, it was

Table 2. Effect of NEM on active and thermally de-activated SMP from different strains at 30°C.

<i>N. crassa</i> strain	dNADH:Q1 reductase activity			
	Active SMP		De-activated SMP	
NEM treatment	–	+	–	+
Wild-type	0.524	0.502 (96%)	0.467 (89%)	0.240 (46%)
Rescued gDNA	0.193	0.171 (88%)	0.146 (76%)	0.180 (47%)
C14S	0.186	0.168 (91%)	0.162 (87%)	0.079 (42%)
C24S	0.115	0.113 (98%)	0.115 (100%)	0.051 (44%)
C40S	0.065	0.059 (90%)	0.050 (77%)	0.025 (39%)
C50S	0.083	0.078 (94%)	0.069 (83%)	0.036 (44%)

μmol dNADH/min per mg of protein.

In brackets % of activity related to the activity of active SMP without NEM.

recently suggested that the sulphhydryl group of complex I sensitive to inhibition by NEM, and possibly involved in the active/de-active process, is located in the inner membrane facing the matrix interface (23). Our results suggest that the 11.5 kDa subunit of *N. crassa* is neither involved in the NEM inhibition nor in the A/D transition process, but it is important for an efficient assembly and stability of complex I.

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4. Supramolecular organization of the respiratory chain in *Neurospora crassa* mitochondria

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Supramolecular organization of the respiratory chain in *Neurospora crassa* mitochondria

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Running title: Inactive respiratory supercomplexes in nuo51 mutant

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Abstract

The existence of specific respiratory supercomplexes in mitochondria of most organisms has gained much momentum. However, its functional significance is still poorly understood. The availability of many deletion mutants in complex I (NADH:ubiquinone oxidoreductase) of *Neurospora crassa*, distinctly affected in the assembly process, offers unique opportunities to analyze the biogenesis of respiratory supercomplexes. Herein, we describe the role of complex I in assembly of respiratory complexes and supercomplexes as suggested by blue- and colorless-native PAGE and mass spectrometry analyzes of mildly solubilized mitochondria from both wild-type and eight deletion mutants. As an important refinement of the fungal respirasome model, we found that the standard respiratory chain of *N. crassa* comprises putative complex I dimers in addition to I-III-IV and III-IV-supercomplexes. Three *Neurospora* mutants able to assemble a complete complex I, only lacking the disrupted subunit, have respiratory supercomplexes, in particular I-III-IV-supercomplexes and complex I dimers, like the wild-type strain. Furthermore, we were able to detect the I-III-IV-supercomplexes in the nuo51 mutant with no overall enzymatic activity, representing the first example of inactive respirasomes. In addition, III-IV-supercomplexes were also present in strains lacking an assembled complex I, namely in four membrane arm subunit mutants as well as in the peripheral arm nuo30.4 mutant. In membrane arm mutants, high-molecular-weight species of the 30.4 kDa peripheral arm subunit comigrating with III-IV-supercomplexes and/or the prohibitin complex were detected. The data presented herein suggest that the biogenesis of complex I is linked with its assembly into supercomplexes.

Introduction

The filamentous fungus *Neurospora crassa* with a sequenced 40 Mb genome (31, 52) is an outstanding eukaryote model for all aspects of biosciences, with numerous molecular genetics tools available (19). The so-called RIPing (repeat-induced point mutation), which is an effective defence against duplicated sequences, is commonly used to achieve gene disruption (43, 74). Overall, *N. crassa* has been vastly used in mitochondria research such as the protein import machinery (e.g., 2, 8, 75).

The standard oxidative phosphorylation (OXPHOS) system is composed of the four major respiratory chain complexes and the F_0F_1 -ATP synthase (complex V), and is responsible for most of the cellular ATP production. Research on complex I (NADH:ubiquinone oxidoreductase), the largest and most intricate OXPHOS component, has taken on greater significance since many human mitochondrial diseases involve structural and functional defects of this enzyme complex (reviewed in 30, 42). Contradicting the predominant view (35), the respirasome model was introduced based on the results by blue-native (BN)-PAGE of efficient but mildly solubilized bovine heart mitochondria that led to the separation of high yields of stoichiometric respiratory supercomplexes and of ATP synthase dimers (67, 68). In line with the ancient “solid-state model” (13) it postulates the quantitative assembly of the I, III, and IV respiratory complexes into two different supercomplexes $I_1III_2IV_4$ and III_2IV_4 , occurring in a 2:1 ratio. Furthermore, the complexes I, III, and IV are present in a 1:3:6 stoichiometry (37, 68). In fact, the analysis of digitonin-solubilized mitochondria, isolated from fresh bovine heart, by a particular gentle colorless-native (CN)-PAGE recovered nearly all of the I, III, and IV complexes as supercomplexes and most of ATP synthases as dimers and higher oligomers (46, 48). Likewise, specific respiratory supercomplexes were detected by similar approaches in mitochondria of various eukaryotes (15, 24, 26, 45, 47, 61, 62, 64, 71, 94, 97, 98), including the filamentous fungus *Podospora anserina* (45), as well as in bacteria (79). Recently, the first single particle structures of respiratory supercomplexes were determined (24, 39, 66) and several other important studies by the bioenergetics/mitochondrial community have supported the respirasome model (1, 6, 10, 18, 21, 49, 56, 62, 85, 86, 88, 96). However, the functional significance of respiratory supercomplexes, including its enzymatic advantages, is still poorly documented. Since these supercomplexes represent assemblies of sequential enzymes, substrate channeling has been proposed (67), which is plausible in theory but difficult to prove experimentally (77). Accumulating evidence from the investigation of mutants and human patients, with specific assembly defects of a single respiratory complex, suggests that complexes III and IV are involved to various extents in the assembly/stabilization of complex I in mammals (1, 7, 18, 21, 56, 71). In contrast, the absence of complex I in mitochondria of human patients (71) and plants (60, 62) was found not to reduce the

abundance of the other OXPHOS complexes. However, site-directed mutations of the NDUFS2 (49 kDa subunit) and NDUFS4 (18 kDa/AQDQ, homologue of the 21 kDa subunit of *N. crassa*) genes in human patients (85) and the gene encoding for the 51 kDa subunit homologue in *Caenorhabditis elegans* (32, 33) were reported to reduce the amounts of both assembled complex I and of complex III (85) or complex IV (32, 33), respectively.

The molecular details of complex I biogenesis are far from being elucidated. However, and in contrast to human complex I (3, 86, 91), a modular assembly pathway of the peripheral and membrane arms has been described in *N. crassa* (84). This is at least partially due to the existence of a comprehensive collection of complex I mutants in *N. crassa* (reviewed in 55, 89), only possible to exist because the fungus possess several alternative NADH dehydrogenases (e.g., 12, 55, 89). To unravel the molecular details underlying the formation of respiratory supercomplexes and their functional significance we analyzed the steady-state supramolecular organization of the OXPHOS machinery of wild-type and eight complex I deletion mutants of *N. crassa* using BN- and CN-PAGE. For the first time, putative complex I dimers could be detected as components of the standard respiratory chain in a filamentous fungus. In addition, we provide evidence showing that I-III-IV-supercomplexes have non-respiratory relevance, since the inactive complex I of *nuo51* is assembled into supercomplexes like those found in wild-type mitochondria as well as in the *nuo21* and *nuo29.9* mutants. Furthermore, digitonin-stable ATP synthase dimers and III-IV-supercomplexes were also present in all strains studied. In 2D BN/SDS gels of complex-I-deletion mutants lacking a membrane arm subunit, considerable amounts of total 30.4 kDa subunit comigrated with III-IV-supercomplexes and/or the prohibitin complex, suggesting a putative interaction with them or other non-identified assembly factors.

Materials and Methods

Strains and isolation of crude mitochondria.

The wild-type 74-OR23-1A (74A) and eight mutant strains of *N. crassa* (*nuo* 9.8, *nuo*11.5, *nuo*14, *nuo*20.8, *nuo*21, *nuo*29.9, *nuo*30.4 and *nuo*51) were investigated in this study. Crude mitochondria of these strains were prepared as described (93) using slight alterations. Mycelium was grown in 1× liquid Vogel's medium supplemented with 1.5% of sucrose for 16 to 18 h, whereas some mutant strains required 20 to 24 h of growth at 26 °C with shaking. The mycelium was separated from the culture medium by filtration through a layer of cheesecloth. All steps described afterwards were done at 4 °C. The mycelium was disrupted mechanically in a buffer containing 0.44 M sucrose, 30 mM Tris/HCl (pH 7.4), 2 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. Then, cell debris, nuclei, and mycelium were removed by

centrifuging twice at 2,000 g for 5 min. The pellet was discarded, while the supernatant was centrifuged at 20,000 g for 45 min. The crude mitochondrial pellet obtained was suspended in the same washing buffer and centrifuged at 31,000 g for 20 min. Finally the mitochondrial pellet was suspended in the same buffer. After isolation, the crude mitochondria were frozen as aliquots in liquid nitrogen and stored at -80 °C until use.

Electrophoretic techniques.

Digitonin and n-dodecylmaltoside (DDM) were of high purity grade from Calbiochem Merck Biosciences GmbH, and Triton X-100 of high purity grade was purchased from Roche Diagnostics GmbH.

Mitochondria were thawed on ice and centrifuged at 10,000 g for 5 min. The pellet was suspended in the solubilization buffer containing 50 mM NaCl, 50 mM imidazole/HCl (pH 7.0), 10% glycerol and 5 mM 6-aminocaproic acid (final concentration). Mitochondria from *N. crassa* strains were solubilized with digitonin using a detergent/protein ratio of 3.5 g/g or with Triton X-100 or DDM using a detergent/protein ratio of 1.5-3 g/g at a final detergent concentration of 1% by adding a freshly prepared 10% detergent solution. The samples were incubated 30 min on ice followed by centrifugation at 20,800 g for 10 min. Each lane was loaded with the extract from mitochondria containing 150 μ g (for in-gel activity), 300 μ g (for 2D SDS-PAGE) or 400 μ g (for 2D BN-PAGE) of protein before solubilization assessed by Bradford assay. For BN-PAGE and CN-PAGE, linear 3–13% gradient gels overlaid with a 3% stacking gel were used in a Hoefer SE 600 system (18 x 16 x 0.15 cm³) (40, 69, 72). CN-PAGE was performed according to Schägger *et al.* (72) omitting Coomassie Blue dye in the cathode buffer but without the addition of detergent in the gel (46, 61). The apparent molecular masses of the OXPHOS complexes and their supercomplexes were calibrated by digitonin-solubilized bovine heart mitochondria (67) applied to the same first dimension BN gel as earlier described (45, 47, 64). The supercomplexes were assigned according to their subunit compositions and apparent molecular masses. Lanes from the first dimension BN-PAGE or CN-PAGE were then excised and used for a second dimension 13% SDS-PAGE (64) or alternatively, for a second dimension 3–20% BN-PAGE with addition of 0.02% (w/v) DDM to the cathode buffer (67). Strips used for second dimension SDS-PAGE were incubated in a solution containing 1% SDS and 1% mercaptoethanol for 2 h at room temperature. The first dimension gels and 2D BN-PAGE were Coomassie Blue R-250 stained, whereas the 2D SDS-PAGE were stained with silver nitrate (64) or SYPRO Ruby (Bio-Rad), respectively.

In-gel activity assays were performed as described elsewhere (45, 47, 48) using the addition of potassium cyanide as specific inhibitor to detect stained bands specific of complex IV,

as well as comparative deamino-NADH dehydrogenase in-gel activity specific of complex I in parallel BN gels.

In-gel Digestion, MALDI-TOF-MS and Bioinformatics.

Proteins were excised from second dimension SDS gels and subjected to in-gel digestion by overnight trypsin (Promega) incubation at 37 °C. Desalination was achieved using μ -C18 Zip-Tips (Millipore) by peptide elution from the tips onto a matrix-assisted laser desorption/ionization (MALDI) sample target using 5 mg/mL 4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid. Peptide samples were analysed by MALDI-TOF-MS peptide mass fingerprint (Applied Biosystems Voyager-DE PRO) as described (64, 65).

The peptide mass lists were matched against the fungi database subset from the NCBI database using the MASCOT search engine (www.matrixscience.com). The search included one possible missing cleavage site as well as possible methionine oxidation and cysteine derivatization by acrylamide (Cys-S- α -propionamide) with a maximal MS error tolerance of 30-90 ppm. The probability score calculated by the software was set to be higher than 58 ($p < 0.05$) as criterion for correct identification. Furthermore, the spot position and its apparent mass in the second dimension gel had to be in line with the calculated physical data of the protein found in the database and literature search. Prediction of transmembrane domains was according to the TMHMM (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>) program, and the molar mass, pI and the grand average of hydropathicity (GRAVY) were calculated using the Protparam tool (us.expasy.org/tools/protparam.html) as described elsewhere (64).

Western Blot Technique.

After 2D BN/SDS-PAGE, proteins were electrotransferred onto a polyvinylidene difluoride membrane purchased from Bio-Rad using the semi-dry method. The Whatman paper was soaked in blotting buffer (anode: 300 mM Tris, 100 mM Tricine, pH ~8.7; cathode: 300 mM 6-aminocaproic acid, 30 mM Tris 0.05% SDS, pH ~9.2). The membranes were pre-incubated in methanol and then in anode blotting buffer, and the gels were washed in cathode blotting buffer. 2D SDS gels were blotted for 2 hours at maximal settings of 400 mA and 25 V. After transfer, membranes were treated according to Towbin *et al.* (83) and incubated over night with polyclonal antibodies against the 30.4, 20.8, 14, 11.5 and 9.8 kDa subunits of *N. crassa* complex I. Proteins were detected by alkaline phosphatase conjugated second antibodies.

Results

Supramolecular organization of *N. crassa* wild-type respiratory chain complexes.

To analyze the role of complex I in the supramolecular organization of the OXPHOS system in *N. crassa*, proteins from crude wild-type mitochondria were solubilized with 3.5 g digitonin/g protein and further separated using 2D BN/SDS-PAGE and BN/BN-PAGE. These analyses revealed characteristic subunit patterns of protein complexes and the individual complexes that form the supercomplexes, respectively (Fig. 1A) in line with in-gel activity stainings of BN gels like that of cytochrome *c* oxidase (Fig. 1A, see also below). In addition, 31 subunits of all five OXPHOS complexes in 2D BN/SDS gels were identified by MALDI-TOF-MS (Fig. 2, Table 1), corroborating the assignment of OXPHOS complexes and supercomplexes as described below.

In line with results from mammalian, plant and *P. anserina* mitochondria (45-48, 67, 68), high amounts of large respiratory supercomplexes comprising complexes I, III, and IV (I_1IV_1 , I_1III_2 , $I_xIII_yIV_z$) as well as the smaller ones (III_2IV_1 and III_2IV_2) were found (Figure 1). The supercomplex I_1IV_1 has essentially the same apparent mass as dimeric ATP synthase (V_2 , ~1,250 kDa) whereas the supercomplex III_2IV_2 runs slightly faster. The small supercomplex I_1IV_1 , indicating a direct complex-I-IV-interaction, has only been previously found in bovine heart mitochondria by native electrophoresis (46, 48, 67). The two largest I-III-IV-supercomplexes had mobilities similar to that of the ketoglutarate dehydrogenase complex (~2,800 kDa) (Fig. 2, Table 1), suggesting compositions like $I_1III_2IV_{5-6}$. Comparable supercomplexes with molecular masses up to 3300 kDa were also observed in 2D BN/SDS gels of digitonin-solubilized mitochondria isolated from fresh bovine heart (40, 46, 48). About half of the total ATP synthase was recovered as dimers (V_2) as for digitonin-solubilized *Podospora* wild-type mitochondria (45). Another observation was the reduced mobility of individual dimeric complex III (III_2) migrating nearly at the same position as monomeric ATP synthase with an apparent molecular mass of ~600 kDa, confirming the results described for *Podospora* (45). This is probably due to boundary lipids (45), known to be important for the stability and function of complex III (e.g., 51). Similarly, the mobilities of individual monomeric complex IV (IV_1) and its dimers (IV_2) were slightly reduced when compared to those of bovine heart, likewise due to tightly bound lipids (73), apparently removed by solubilization with Triton X-100 and DDM (see below).

Although at a slightly reduced resolution, the proportion of preserved supercomplexes could be enhanced with the gentler CN-PAGE method (Fig. 1B) as seen in other organisms (45, 46, 48).

Identification of protein complexes from wild-type mitochondria by MALDI-TOF-MS.

Overall, 2D BN/SDS-PAGE of mitochondrial detergent-extracts followed by MALDI-TOF-MS enables a rather mitoproteome analysis of protein complexes (11, 40, 46, 64). Importantly, the simultaneous separation of various protein complexes (11, 40, 46, 64), in particular if most of the complexes are already known, mutually supports their physiological significance arguing against artificial aggregation (64).

Through MALDI-TOF-MS analysis, 67 different proteins were identified, among which 31 subunits are components of the five OXPHOS complexes, both in individual complexes or supercomplexes. Interestingly, a protein spot that could only be detected as a subunit of the ATP synthase dimer but not of the monomer was identified as subunit g, (Fig. 2, spot 35) also known to be dimer-specific in yeast (4). This suggests a close structural relationship between yeast and filamentous fungal ATP synthase dimerization.

The 36 non-OXPHOS proteins identified represent a variety of functions, such as metabolic pathways, chaperones and transporters of which most are constituents of known complexes. Two such examples are the pyruvate dehydrogenase complex (~8,000–10,000 kDa) and the ketoglutarate dehydrogenase complex (~2,800 kDa), both mitochondrial multienzyme assemblies, as well as their subcomplexes, also identified in mammalian mitochondria (40, 64, 69). The mitochondrial import receptor Tom40 (Figure 2, spot 42) was also identified as a subunit of the TOM holo or core complex (2, 63, 75, 76, 82), and found to comigrate with monomeric complex IV and heterooligomeric NAD⁺-dependent isocitrate dehydrogenase (Figure 2, spots 40 and 41). Tom40 is the main component of the TOM complex and essential for viability of *N. crassa* (2, 82).

In the chaperone category we found the heat shock protein 60 (HSP60) (Fig. 2, spot 36) that is required for the assembly of proteins into oligomeric complexes and for the stabilization of preexisting proteins under stress conditions (14, 41). The apparent mass in the first dimension was ~650 kDa suggesting a tetradecameric assembly built by two stacked rings of seven monomers each, in contrast to the mammalian HSP60 complex that displays an apparent size of ~420 kDa in BN gels (64). Another group of chaperones are the highly conserved prohibitins, which stabilize newly synthesized subunits of mitochondrial respiratory chain complexes (9, 57). The prohibitin complex (apparent mass of 1,200 kDa) comprising the two homologues Phb1 (Fig. 2, spot 8) and Phb2 (Fig. 2, spot 7) was detected as it has been for yeast (57), plant (38), nematode (5) and mammalian (57, 64) mitochondria. Additionally, we also observed that ≥ 50% of the prohibitins migrated as an even heavier complex with a molecular mass greater than 2,500 kDa. This large complex was visualized in BN gels of digitonin-solubilized yeast mitochondria and assigned as a supercomplex of the prohibitin complex with the *m*-AAA protease (81) that was previously detected by coimmunoprecipitation and gel-filtration analysis (78). In fact, the only *Neurospora*

homologue of the two yeast *m*-AAA protease subunits, MAP-1 (44), was found comigrating with this heavier prohibitin complex (Fig. 2, spot 2) and displaying the same band shape, which strongly suggests that this low-mobility species is a prohibitin/MAP-1 supercomplex.

In the inner mitochondrial membrane there are two AAA proteases, which form independent oligomeric complexes involved in quality control by degrading misfolded membrane proteins. The *m*-AAA protease (MAP-1) has its catalytic site facing the matrix, whereas that of the *i*-AAA protease is exposed to the mitochondrial intermembrane space. The activity of the *m*-AAA protease is modulated by the prohibitin complex as suggested by their physical interaction (78, 81). The *N. crassa* homologue of the *i*-AAA protease, IAP-1 (Fig. 2, spot 3), was identified for the first time in a putative supercomplex with an apparent molecular mass of ~4,000-6,000 kDa much higher than that reported based on gel-filtration (44). Supporting its high molecular weight organization, we identified a comigrating protein homologous to the human stomatin-like 2 protein (Figure 2, spot 6), a mitochondrial inner membrane polypeptide (16) with an apparent mass of ~1,800 kDa (64), which is widely expressed in different mammalian tissues (59, 92). Recently, an interaction of stomatin-like 2 protein with mitofusin 2 in HeLa cells was found by co-immunoprecipitation (36). As such, and based on their mobility in BN-PAGE we suggest that IAP-1 and stomatin-like 2 protein form a large supercomplex in the inner membrane of *N. crassa* mitochondria.

Other interesting protein complexes (Fig. 2, Table 1), of which some are putative novel ones, will be described elsewhere.

Putative complex I dimers in *N. crassa* wild-type mitochondria.

To complement the analysis of OXPHOS supercomplexes we utilised the less mild detergents Triton X-100 and DDM for solubilization of wild-type mitochondria, known to disrupt OXPHOS supercomplexes and other mitochondrial protein complexes more easily than digitonin (8, 46, 67). Interestingly, using either Triton X-100 or DDM at a detergent/protein ratio of 1.5 g/g, minor amounts of low-mobility species of complex I were observed, presumably containing no additional proteins (Fig. 3A, B) which is further supported by 2D BN/PAGE (Fig. 3C). Its apparent molecular mass (~1900 kDa) was consistent with that of the dimeric complex I previously detected in COX-deficient strains of *P. anserina* (45). An increase on the detergent/protein ratio to 3 g/g resulted in the dissociation of the putative complex I dimers and the ATP synthase dimers into the respective monomers (not shown). In addition, ATP synthase monomers (complex V) migrate a little faster (Fig. 3A) or at the same position as complex III (Fig. 3B) when solubilized with Triton X-100 or DDM, respectively. Likewise, complex IV monomers migrated with higher mobility than those obtained upon digitonin solubilization (Fig. 1A, see above).

Comparative analysis of the OXPHOS system from eight complex I mutants by in-gel activity assays after BN-PAGE.

The availability of a nearly complete set of *N. crassa* complex I mutants (55, 89) offers a unique opportunity to investigate not only the assembly process of complex I but also its role in the formation of OXPHOS supercomplexes. We selected eight deletion mutants (nuo9.8, nuo11.5, nuo14, nuo20.8, nuo21, nuo29.9, nuo30.4 and nuo51) representing the whole range of the various reported defects in complex I biogenesis. Their mitochondrial detergent-extracts were analyzed in the same manner as those of the wild-type through native gel electrophoresis. The mutants nuo21, nuo29.9 and nuo51 are able to assemble an intact complex I lacking the respective peripheral arm subunit, although the nuo29.9 mutant assembles reduced amounts (approx. 20%) of complex I (23, 28, 29, 87). The fourth peripheral arm mutant used in this study, nuo30.4, cannot assemble a peripheral arm and accumulates membrane arm intermediates (22). The other four mutants investigated (nuo9.8, nuo11.5, nuo14 and nuo20.8) each lack a membrane arm subunit of complex I and are able to assemble the peripheral arm and fragments of the membrane arm (17, 54, 55).

For direct comparison of the OXPHOS system in the wild-type and the eight mutants, their digitonin-solubilized mitochondrial proteins were separated by BN-PAGE and probed for in-gel activity of NADH dehydrogenase (complex I), cytochrome *c* oxidase (complex IV), ATP hydrolase (complex V), and succinate dehydrogenase (complex II) giving a qualitative indication of these four OXPHOS complexes (Fig. 4). The NADH dehydrogenase (NADH-DH) bands attributed to complex I were assigned by comparative gels tested for deamino-NADH dehydrogenase activity, specific for complex I (not shown). The specificity of cytochrome *c* oxidase (COX) activity of complex IV was confirmed by inhibition with cyanide in parallel experiments (not shown).

All nine strains contained similar amounts of complex V (ATP synthase) in about equal proportion (~50%) of monomers and dimers (Fig. 4C), as well as of active complex IV monomers (Fig. 4B) and complex II (Fig. 4D). Furthermore, in all strains two prominent low-molecular-weight bands with NADH-DH activity were visualized, which were not detected in bovine heart mitochondria. Additionally, these two bands were almost undetectable when deamino-NADH was used instead of NADH, suggesting that they may correspond either to alternative NADH dehydrogenases or to non-OXPHOS enzymes (Fig. 4A). The differences in the staining intensities are partially due to deviations in the loaded amounts.

In wild-type, the in-gel NADH-DH activity staining revealed bands corresponding to individual complex I as well as to its supercomplexes with complex III and IV like I_1III_2 , I_1IV_1 , and $I_1III_2IV_X$ (Fig. 4A). In addition, monomeric complex IV, its dimer (IV_2), the smaller supercomplexes III_2IV_1 and III_2IV_2 , as well as complex I supercomplexes containing one or more copies of complex IV displayed cyanide-sensitive COX activity in wild-type (Fig. 4B). The same pattern of active supercomplexes containing complexes I and/or IV found in wild-type was also

detected in the mutant nuo21 (Fig. 4A and B), indicating that the loss of the 21kDa subunit does not impair the formation and stability of III-IV- and I-III-IV-supercomplexes.

In contrast, the nuo51 mutant displayed no complex I NADH-DH activity (Fig. 4A) but rather a wild-type-like pattern of high-molecular-weight species with COX activity (Fig. 4B), suggesting that these active bands correspond to the III-IV- and I-III-IV-supercomplexes observed in the wild-type and mutant nuo21 strains. In fact, this mutant strain does not possess any rotenone-sensitive NADH:ubiquinone oxidoreductase activity of complex I due to the absence of the highly conserved 51 kDa subunit that binds NADH (28).

The mutant nuo29.9 showed no NADH-DH activity related to complex I possibly due to assay sensitivity (Fig. 4A). Moreso, hardly detectable high-molecular-weight COX bands at the positions of the wild-type I-III-IV-supercomplexes could be noticed whereas the bands of the small supercomplexes III₂IV₁ and III₂IV₂ were clearly identified after COX activity staining (Fig. 4B).

Strikingly, the four membrane arm mutant strains, nuo9.8, nuo11.5, nuo14 and nuo20.8, displayed a specific single band with both NADH-DH and deamino-NADH-DH activity with an apparent molecular mass of ~450 kDa that seemingly corresponded to the peripheral arm domain (Fig. 4A). Accordingly, the NADH-DH activity 450 kDa band could not be detected in either the nuo30.4 mutant or the wild-type and nuo21 strains (Fig. 4A). The four membrane arm mutants displayed both monomeric complex IV and high-molecular-weight complexes with cyanide-sensitive COX activity which corresponded to IV₂, III₂IV₁ and III₂IV₂ as found in the other strains, and in addition some weakly stained bands with an apparent mass comparable to those of the wild-type complex I supercomplexes (Fig. 4D). Overall, the absence of an assembled complex I does not influence the assembly of the other four OXPHOS complexes.

Respiratory supercomplexes are assembled even with an inactive complex I.

We further investigated the supramolecular organization of the OXPHOS complexes in the mutants nuo21, nuo29.9 and nuo51 by 2D BN/SDS-PAGE. Supporting the results obtained by the in-gel-activity assays (Fig. 4), the second-dimension gel of nuo21 (Fig. 5A) and nuo 51 (Fig. 5B) mitochondria revealed a wild-type-like distribution of individual OXPHOS complexes and supercomplexes, in particular III₂IV₁, III₂IV₂, and I-III-IV-supercomplexes.

The wild-type-like I-III-IV-supercomplexes in the nuo51 mutant are the first examples of respirasomes without overall enzymatic activity (NADH oxidase, I+III+IV) only able to oxidize ubiquinol by molecular oxygen (ubiquinol oxidase, III+IV). This finding is the first direct demonstration that the assembly of the complexes I, III and IV into supercomplexes is independent of complex I activity and does not represent a transient-like interaction required for efficient electron transfer from complex I to III. In fact, the biogenesis of supercomplexes is an elaborate

and energy-consuming process, and complex III and IV of these “inactive respirasomes” rely on alternative NADH dehydrogenases or FADH₂-linked enzymes to deliver electrons to ubiquinol to be operable. Taken together these results suggest that it is conceivable that III-IV-supercomplexes play a role in the assembly/stability of complex I.

High-molecular-weight species of the 30.4 kDa subunit comigrating with III-IV-supercomplexes and the prohibitin complex in the membrane arm mutants.

To achieve a sensitive detection of any complex I species in mutants nuo9.8, nuo11.5, nuo14, nuo20.8, nuo30.4, and nuo29.9 we used immunoblots of 2D BN/SDS gels from digitonin-solubilized mitochondria probed with a set of polyclonal antibodies each raised against a particular subunit of complex I from *N. crassa*. We used antibodies against the 30.4 kDa subunit of the peripheral arm and either the 9.8 kDa, the 11.5 kDa, the 14 kDa or the 20.8 kDa subunits of the membrane arm. In particular, the screening for the 30.4 kDa subunit was performed due to its crucial role in the assembly of the peripheral arm and thus of intact complex I (22, 86, 90).

In wild type mitochondria, the corresponding subunits of individual complex I as well as its supercomplexes were decorated with the four employed antibodies indicating the presence of fully assembled complex I (Fig. 6A, lower panel). Note that the 30.4 kDa subunit of individual complex I was also identified by MALDI-TOF-MS (Fig. 2 and Table 1). The only other significant signals arose from a cross-reaction with the β -subunit of both monomeric and dimeric ATP synthase (complex V) which could actually be used as a helpful marker to align the blots with parallel silver-stained 2D SDS gels (Fig. 6A-F) and 1D BN gels probed for in-gel COX activity (Fig. 6D-F, upper panels). The corresponding immunoblot of nuo29.9 mitochondria (Fig. 6B, lower panel) displayed a result similar to that of the wild-type (Fig. 6A, lower panel). This corroborates the presence of low amounts of assembled complex I separated as individual complex I and its supercomplexes also detected in silver-stained 2D gels (Fig. 6B, lower panel). Notably, the signal of the 30.4 kDa subunit was rather weak since this protein, located in close vicinity to the 29.9 kDa subunit, is strongly reduced in the mutant nuo29.9 (87). No subcomplexes of complex I could be immunodetected, indicating that in the nuo29.9 mutant essentially all assembled peripheral arm joins with the membrane arm, occurring in surplus quantity, to form “complete” complex I. Additionally, it is likely that the excess hydrophobic membrane arm forms low-mobility membrane aggregates contributing to the immunosignals in Fig. 6B (see below).

In the nuo30.4 mutant a smear-like panel of high-molecular weight species of the 20.8 kDa subunit was immunodetected (Fig. 6C, lower panel), where the most intense ones were near the top of the first dimension gel. Such high-molecular-weight species of membrane subunits were previously observed after density gradient centrifugation of Triton X-100 extracts from

mitochondria of peripheral arm mutants like nuo30.4 (22) but also from nuo29.9 (87), which generates a surplus of the membrane assembly intermediate (84) as mentioned above. Most probably, the largest 20.8 kDa species represented unspecific aggregates of partially assembled hydrophobic membrane subunits as previously suggested (e.g., 22, 23). However, it cannot be ruled out that there might be assembly intermediates of the membrane arm bound to “assembly factors” among those high-molecular weight species.

The 2D BN/SDS gels and the corresponding representative immunoblots of the investigated membrane arm mutants were remarkably consistent. In particular, the membrane arm mutants nuo11.5 (Fig. 6D), nuo14 (Fig. 6E), nuo20.8 (Fig. 6F) and nuo9.8 (not shown) revealed essentially the same pattern for the 30.4 kDa subunit, as part of complexes with apparent masses from ~50 kDa to at least 1,500 kDa. The 450 kDa subcomplex detected by in-gel NADH-DH staining (Fig. 4A, see above) represented the peripheral arm, whose constituents like the 78 kDa, 49 kDa and 40 kDa subunits were discernible as weakly stained spots in the 2D BN/SDS gels (Fig. 6D-F). The peripheral arm could also be separated by BN-PAGE of membrane arm mutants after solubilization with DDM (53). Due to tiny amounts of assembled peripheral arm in the mutant nuo11.5, no peripheral arm could be detected in the first report in which we used less sensitive approaches (55). It is unlikely that the subcomplexes smaller than the peripheral arm originate from the breakdown of the assembled peripheral arm induced by the experimental conditions. In fact, their mobilities correspond to assembly intermediates of the 30 kDa subunit (the mammalian homologue of the 30.4 kDa subunit) and/or the free 30 kDa subunit found in human mitochondria, which appears to represent an early indispensable assembly step of the peripheral arm (86, 90).

Due to the fact that the 30.4 kDa subunit was separated to a large extent as part of high-mobility species, it is unlikely that the distinct low-mobility species of the rather hydrophilic 30.4 kDa subunit arose from any aberrant aggregation triggered by the experimental conditions or *in vivo*. Indeed, these immunosignals represent distinct high-molecular weight complexes which comigrated with the supercomplexes IV₂, III₂IV₁ and III₂IV₂ and the prohibitin complex with apparent masses of ~550 up to ~1,250 kDa as indicated by black bars in Fig. 6D–F. In nuo14 and nuo20.8 mutants even larger complexes up to about 2,000 kDa of the 30.4 kDa subunit were found comigrating with III-IV-supercomplexes and displaying COX in-gel activity (Fig. 6E and F). Taken together, these high-molecular-weight complexes seem to be assembly intermediates of the peripheral arm, which likely function in assisting the biogenesis of the peripheral arm and/or the whole complex I. Nonetheless, the comigration of proteins in the first dimension BN-PAGE with consistent band shapes in the second dimension SDS gel is a necessary precondition to propose an interaction between them (46, 72) and, thus, III-IV-supercomplexes and/or the prohibitin complex, respectively, are conceivable constituents of the various large 30.4 kDa species. If subcomplexes of complex I containing the 30.4 kDa subunit are actually bound to the supercomplexes IV₂, III₂IV₁,

III₂IV₂, it would follow that these subcomplexes are indeed rather small, well below the size of the peripheral arm, since no major migration shift of the supercomplexes III₂IV₁ and III₂IV₂ was recognized in the first dimension BN gel.

Discussion

Mammalian-like respiratory I-III-IV-supercomplexes and complex I dimers in *N. crassa* mitochondria.

Herein we report the first extensive survey of the OXPHOS system in the *N. crassa* wild-type and a representative set of complex-I-deletion mutants displaying a variety of complex I assembly defects. In particular, we detected complexes I, III, and IV associated in mammalian-like supercomplexes in both wild-type and nuo21, nuo29.9 and nuo51 mutants, as previously reported in wild-type mitochondria of the fungus *P. anserina* (45). In addition, we could detect the digitonin-stable supercomplex I₁IV₁ comigrating with ATP synthase dimers (Fig. 1A), which was not previously identified in *Podospora* mitochondria (45). This indicates a direct complex-I-IV-interaction demonstrated to occur in bovine heart mitochondria by native electrophoresis (48, 67) and in particular by a 2D (66) and 3D projection map of a single particle structure of the bovine heart supercomplex I₁III₂IV₁ (Schäfer, E., N. A. Dencher, J. Vonck, and D. Parcej, submitted). Furthermore, the I-III-IV-supercomplexes (Fig. 1A) with apparent masses higher than 2,300 kDa and up to $\geq 3,000$ kDa seem to be larger fragments of a respirasome network of complexes I, III, and IV as proposed for bovine heart (67, 68, 70, 95) possibly composed of I₁III₂IV₅₋₆. Such stoichiometries could arise from the breakdown of two copies of supercomplex I₁III₂IV₄ or else from one copy of each supercomplexes I₁III₂IV₄ and III₂IV₄, which could be connected by a direct interaction between adjacent complex IV dimers (70, 95). Indeed, similar supercomplexes with masses up to about 3,300 kDa could be observed in 2D BN/SDS gels of digitonin-solubilized mitochondria isolated from fresh bovine heart (40, 46, 48). Taken together, these results indicate a very similar respirasome organization in fungi and mammals. Moreover, when disrupting all I-III-IV-supercomplexes upon solubilization with either Triton X-100 or DDM, we found evidences for the existence of complex I dimers (I₂) in the respiratory chain of *N. crassa* wild-type (Fig. 3) as well as in the nuo21, nuo29.9 and nuo51 mutants (not shown). It is likely that small amounts of complex I dimers are also stable upon digitonin solubilization, although their identification may be hampered due to comigration with I-III-IV-supercomplexes during BN-PAGE. In fact, complex I dimers as well as the larger supercomplex I₂III₂ were found in BN gels of digitonin-solubilized mitochondria of COX-deficient *Podospora* strains (45). It is possible that the presence of complex I dimers occurs only when alternative respiratory enzymes are present in the organism, hence the

reason why they have not been detected so far in mammalian mitochondria. Whereas the AOX as well as the alternative NADH dehydrogenases were found to be constitutively expressed and active in wild-type *P. anserina* (34), there are no evidences for any AOX respiration in the wild-type of *N. crassa* under normal growth conditions (e.g., 20, 80). Therefore, we propose that the occurrence of dimeric complex I is a general feature of the respiratory chain in *N. crassa*.

III-IV-supercomplexes are formed without the presence of assembled complex I.

Our results demonstrate that complex I in *N. crassa* is neither essential for assembly/stabilization of the other OXPHOS complexes nor for assembly/stabilization of III-IV-supercomplexes and ATP synthase dimers (Fig. 6). On the other hand, analysis of the three mutants that assemble complex I indicates that the biogenesis of complex I is obligatorily linked with its assembly into supercomplexes. Furthermore, we demonstrated that neither of the 21 kDa, 29.9 kDa and 51 kDa peripheral arm subunits play a pivotal role in the physical interaction of complex I with complexes III and IV, suggesting that the binding interfaces are predominantly provided by the membrane arm, in accordance with structural data of the bovine heart supercomplex $I_1III_2IV_1$ (66, Schäfer, E., N. A. Dencher, J. Vonck and D. Parcej, submitted), and/or by other peripheral arm subunits. Mitochondria of the nuo21 and nuo51 mutants have wild-type-like amounts of assembled complex I as part of supercomplexes, which results in a similar distribution of individual complex I and its supercomplexes (I-III-IV and I_2) after solubilization with digitonin (Fig. 5) or either Triton X-100 or DDM, respectively. Strikingly, while complex I in nuo21 is enzymatically active, the complex I of nuo51 lacks the highly conserved 51 kDa subunit hosting the NADH-binding site and has therefore no respiratory activity. This is the first direct demonstration that the incorporation of complex I into supercomplexes as part of a proposed supramolecular network has major non-respiratory significance and cannot be solely related to conceivable enzymatic advantages like substrate channeling. Furthermore, the nuo29.9 mutant can assemble only low amounts of complex I, about 20% of that of the wild-type (23, 87), which is nonetheless incorporated into supercomplexes as in wild-type, nuo21 and nuo51 strains (Fig. 6B). Altogether, the results from nuo21, nuo29.9 and nuo51 imply that III-IV-supercomplexes “trap” essentially all complex I, even when complex I is inactive. This may occur either after initial formation or at the early stages of complex I biogenesis, suggesting that III-IV-supercomplexes could be involved in assembly/stabilization of complex I. The situation in COX-deficient mutants of *P. anserina* possessing I-III-supercomplexes is different to that of the nuo51 mutant (45). Indeed, this suggests the assembly/stabilization of complex I as a key function of complex III in *Podospira*, but complex III is active although it does not contribute to respiration (45). Evidence for a role of either complex III or IV in the assembly of complex I has also been described in human patients and mammals (1,

7, 18, 21, 56, 71) as well as in bacteria (79).

III-IV-supercomplexes and/or the prohibitin complex might serve as assembly factors for complex I.

Herein we provide direct evidence that high-molecular-weight complexes bind subcomplexes of the peripheral arm containing at least the 30.4 kDa subunit (Fig. 6), as shown in western blots of 2D BN/SDS gels of digitonin-solubilized mitochondria from the four membrane arm mutants nuo9.8, nuo11.5, nuo14 and nuo20.8. These subcomplexes were found to be comigrating with III-IV-supercomplexes and/or the prohibitin complex, thus pointing them as putative candidates for interaction partners. This is in line with the above discussion on mutants forming complex I. In fact, an interaction of the prohibitin complex with a subcomplex of complex I, containing at least the 23, 30 and 49 kDa subunits, has been demonstrated by co-immunoprecipitation in human cells missing mitochondrial-encoded membrane subunits, similarly to what we observed in the four membrane arm mutants (9). The prohibitin complex (Fig. 2, Table 1) can interact with subunits of respiratory complexes, thus probably having a chaperone-like function (9, 57). In addition, the interaction of both the human 30 kDa (homologous to the 30.4 kDa subunit) and 49 kDa subunits has been proposed as the first assembly step towards the peripheral arm (86). These results were corroborated in a very recent study which traced complex I assembly in human cell culture by using a GFP-tagged 30 kDa subunit (90). The poorly understood biogenesis of complex I, the largest and most complicated respiratory enzyme, is probably assisted by many proteins and/or protein complexes of which only a few are known to date (25, 50, 58, 91). In particular, there are two proteins transiently interacting with two membrane arm intermediates, which are essential for assembly of the membrane arm in *N. crassa* (50). Of these, a human homologue appears to have a corresponding role in assembly/stabilization of complex I (25, 91).

In summary, the present survey demonstrates the importance of III-IV-supercomplexes in assembly/stabilization of complex I, highlighting the unique potential of the comprehensive set of *Neurospora* complex-I-deletion mutants to decipher the biogenesis of complex I.

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Figure Legends

Figure 1. Respiratory supercomplexes and ATP synthase dimers in *N. crassa* wild-type mitochondria.

Digitonin-solubilized crude mitochondria analyzed by (A) BN-PAGE and (B) CN-PAGE in the first dimension. (A) and (B) In-gel activity of cytochrome *c* oxidase (COX, upper panels) and subsequent 2D SDS-PAGE (silver-stained) to resolve the subunits of all OXPHOS complexes and their supercomplexes (middle panels) and 2D BN-PAGE (Coomassie-stained) with 0.02 % DDM in the cathode buffer to dissociate the OXPHOS supercomplexes into their individual complexes (lower panels) are shown. For mass calibration, digitonin-solubilized bovine heart mitochondria were used: individual complexes I-V (130-1,000 kDa) and supercomplexes a-e ($I_1III_2IV_{0-4}$, 1,500-2,300 kDa). Additionally, 31 subunits of all five OXPHOS complexes separated in 2D BN/SDS-PAGE (A) were verified by MALDI-MS, which are marked in detail in Figure 2 for sake of clarity. The OXPHOS supercomplexes were assigned according to their subunit compositions and apparent molecular masses. Besides ATP synthase monomers and dimers (V_1 and V_2) the individual respiratory complexes I–IV as well as the respiratory supercomplexes $I_xIII_yIV_z$, I_1III_2 , I_1IV_1 , III_2IV_2 , III_2IV_1 , and IV_2 are indicated. Additionally, a subunit of complex IV (spot 43, Table 1) in the 2D SDS-PAGE (A) as well as the separated complex IV monomers, complex III dimers and complex I monomers, which constitute the supercomplexes IV_2 , III_2IV_1 , III_2IV_2 , I_1IV_1 and $I_xIII_yIV_z$, in the 2D BN-PAGE (A, B) are marked by arrowheads. Note that supercomplexes I_1IV_1 and III_2IV_2 have very similar apparent masses. Similarly, the mobility of dimeric complex IV (IV_2) is only slightly higher than that of complex III.

Figure 2: Identification of proteins in a representative 2D BN/SDS gel of digitonin-solubilized *N. crassa* wild-type crude mitochondria.

The same gel as in Figure 1A is shown. The proteins identified by MALDI-TOF-MS peptide mass fingerprint are labeled by circles and numbers in different colors indicating their function: OXPHOS, complex I (light blue); complex II (red); complex III (pink); complex IV (yellow); complex V (green); tricarboxylic acid cycle and glycolysis (white); lipid metabolism (light green); amino acid and urea cycle (orange); chaperones (violet); transport and carrier proteins (dark blue); and other proteins (brown). The identified proteins are listed by their numbers in Table 1. All proteins described in the text are highlighted by arrows. The other markings correspond to Figure 1(a) and are according to the legend of Figure 1.

Figure 3: Putative complex I dimers in wild-type mitochondria after solubilization with Triton X-100 or DDM.

2D BN/SDS-PAGE of crude mitochondria solubilized with (A) Triton X-100 or (B) DDM at a detergent/protein ratio of 1.5 g/g. The subunits of monomeric complex I (I_1) and putative complex I dimers (I_2) are marked by boxes. One subunit of the pyruvate dehydrogenase complex (PDC, spot 4, Table 1) and two subunits of the NAD^+ -dependent isocitrate dehydrogenase (IDH, spots 41 and 42, Table 1) are indicated as in Figure 2. (C) 2D BN/BN-PAGE of crude mitochondria solubilized with Triton X-100 at a detergent/protein ratio of 1.5 g/g. The separated complex I monomers of individual complex I (I_1) and dimeric complex I (I_2) are marked by arrowheads.

Figure 4: In-gel activity assays of NADH dehydrogenase, cytochrome *c* oxidase, ATP hydrolase and succinate dehydrogenase.

BN-PAGE of digitonin-solubilized mitochondria from bovine heart (BHM) as control, *N. crassa* wild-type (wt) and eight complex I deletion mutants (nuo9.8, nuo11.5, nuo14, nuo20.8, nuo21, nuo29.9, nuo30.4, nuo51). (A) NADH dehydrogenase (purple bands), reactive bands of wt and nuo21 corresponding to complex I are marked by red bars, the red box indicates the active band of the peripheral arm of complex I from the four membrane arm mutants. (B) cytochrome *c* oxidase (brown-yellowish bands), (C) ATP hydrolase (black-white bands), (D) succinate dehydrogenase (purple band). (A-D) Some OXPHOS complexes and supercomplexes like a–e ($I_1III_2IV_{0-4}$) of bovine heart mitochondria are marked on the left side.

Figure 5: Wild-type-like respiratory supercomplexes in peripheral arm mutants nuo21 and nuo51. The nuo51 mutant lacking the 51 kDa subunit forms stable respirasomes containing complexes I, III, and IV without NADH oxidase activity.

2D BN/SDS-PAGE of digitonin-solubilized crude mitochondria of (A) nuo21 and (B) nuo51). The markings are according to the legend of Figure 1. Additionally, each one subunit of complex I (spot 12, Table 1), complex III (spot 24, Table 1), complex IV (spot 43, Table 1), and complex V (spot 22, Table 1) are indicated as in Figure 2.

Figure 6: High-molecular-weight complexes of the 30.4 kDa subunit in complex-I-deficient mutants lacking each a membrane arm subunit.

(A–F) Alignment between silver-stained 2D BN/SDS gels and corresponding immunoblots probed with antibodies against complex I subunits as well as (D–F) corresponding 1D BN gel strips tested for COX in-gel activity from digitonin-solubilized crude mitochondria of (A) wt, (B) nuo29.9, (C) nuo30.4, (D) nuo11.5, (E) nuo14 and (F) nuo20.8. The employed antibodies against the 30.4 kDa subunit of the peripheral arm and the 9.8, 11.5, 14 and 20.8 kDa subunits of the membrane arm are indicated on the left side of the immunoblots. The subunits of ATP synthase monomers (V_1) and dimers (V_2), complex IV dimers, supercomplexes III_2IV_1 and III_2IV_2 as well as of complex I and its peripheral arm are indicated by red, black, green or white continuous vertical lines. (A) and (B) The subunits of supercomplexes of complex I ($I_xIII_yIV_z$, inclusively I_1III_2 and I_1IV_1) are marked with a black box. (D–F) The membrane arm mutants display distinct high-molecular weight-species of the 30.4 kDa subunit which contain none of the tested membrane subunits and comigrate with supercomplexes IV_2 , III_2IV_1 and III_2IV_2 as well as the prohibitin complex whose subunits (spots 7 and 8, Table 1) are encircled. Furthermore, the 30.4 kDa subunit of the peripheral arm and at least three distinct subcomplexes containing the 30.4 kDa subunit marked by arrows are immunodetected.

Table 1: Summary of Proteins from 2D BN/SDS Gels Identified by MALDI-TOF-MS-PMF

The proteins are listed according to their functions and the numbers (colors) marked in Figure 2. The theoretical molecular masses (M_r), pI , and GRAVY were calculated from the amino acid sequences found in the NCBI database without including possible processing, post-translational modifications and cofactors. The number of transmembrane helices (TM) was predicted by the TMHMM algorithm (<http://www.cbs.dtu.dk/services/TMHMM>).

Figure 1

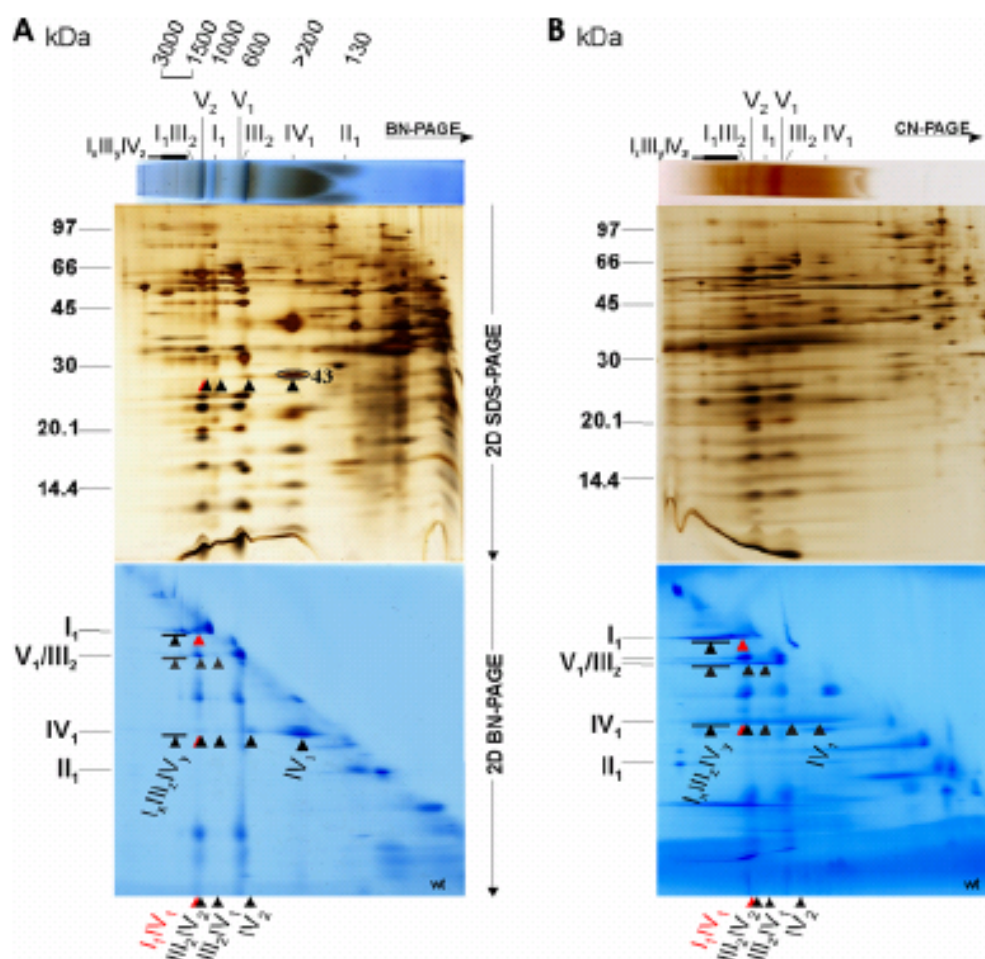


Figure 2

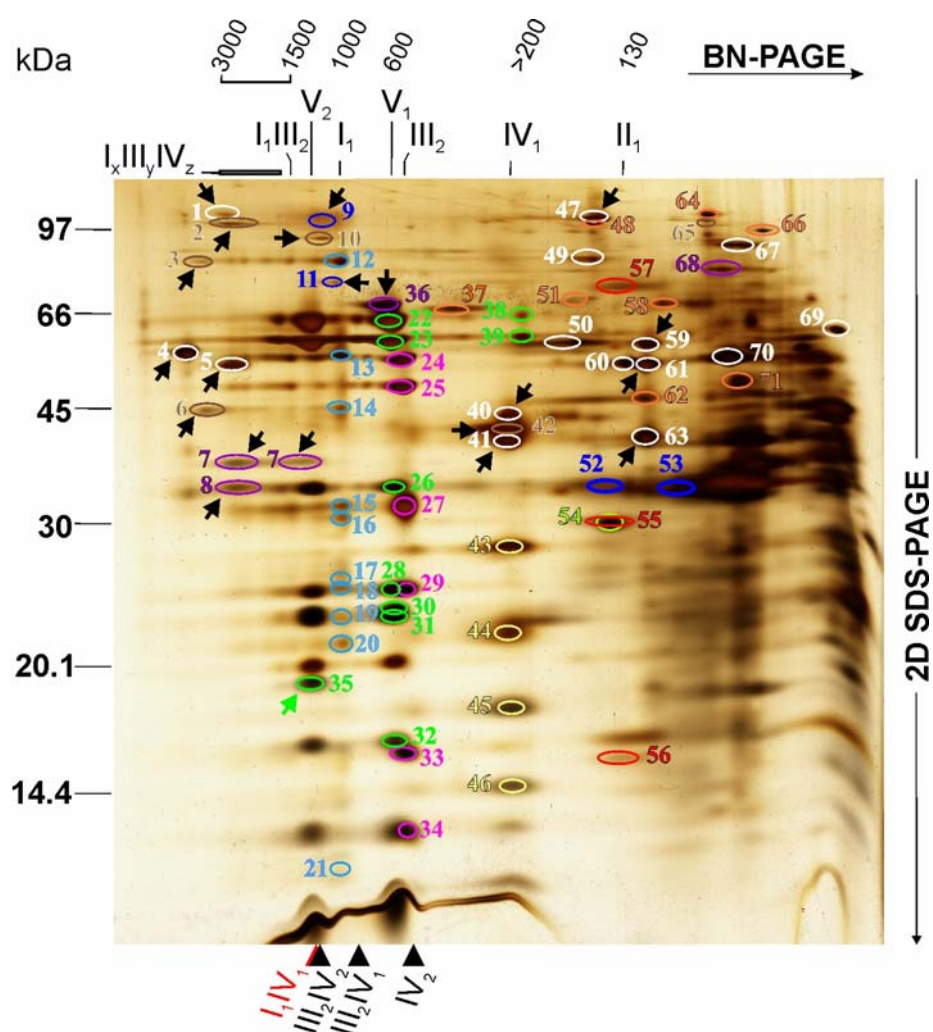


Figure 3

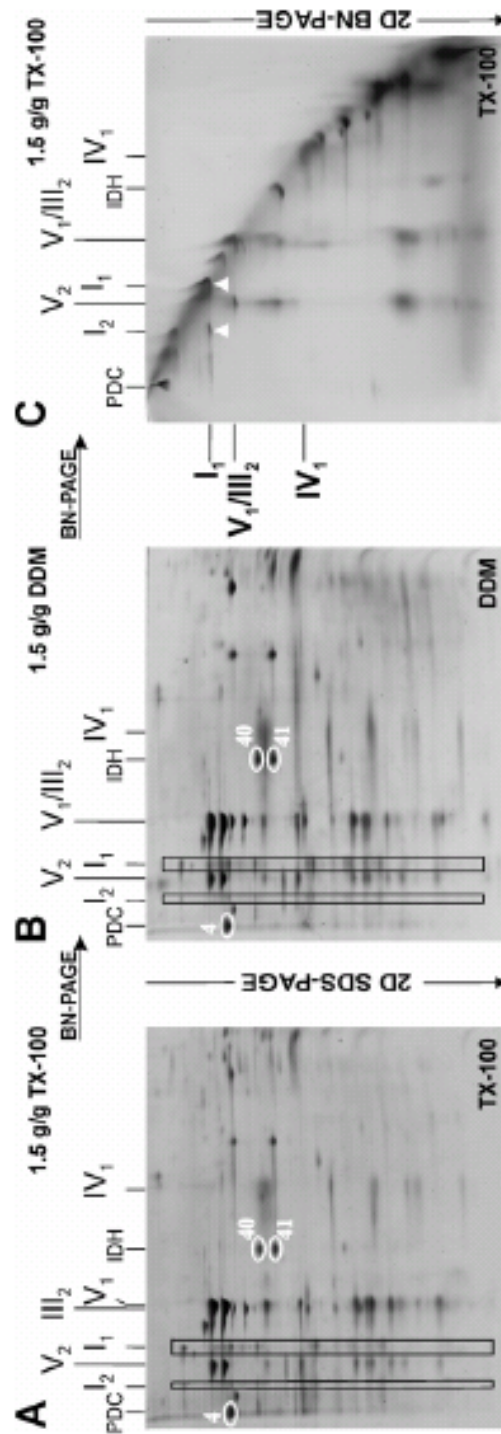


Figure 4

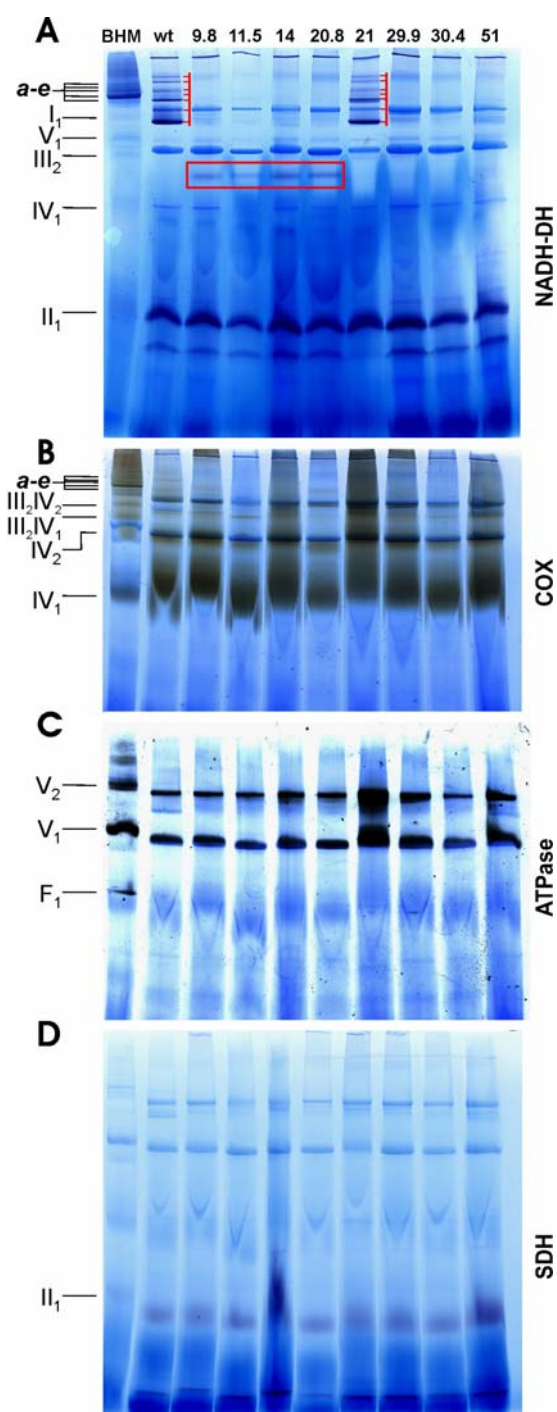


Figure 5

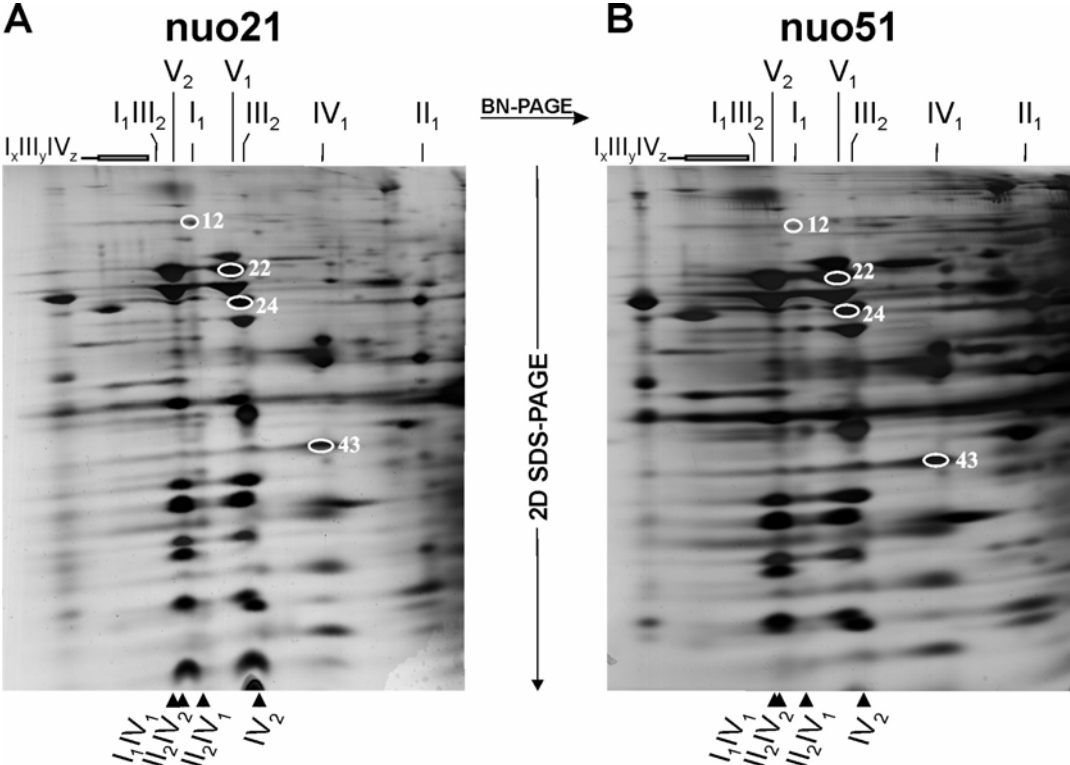


Figure 6

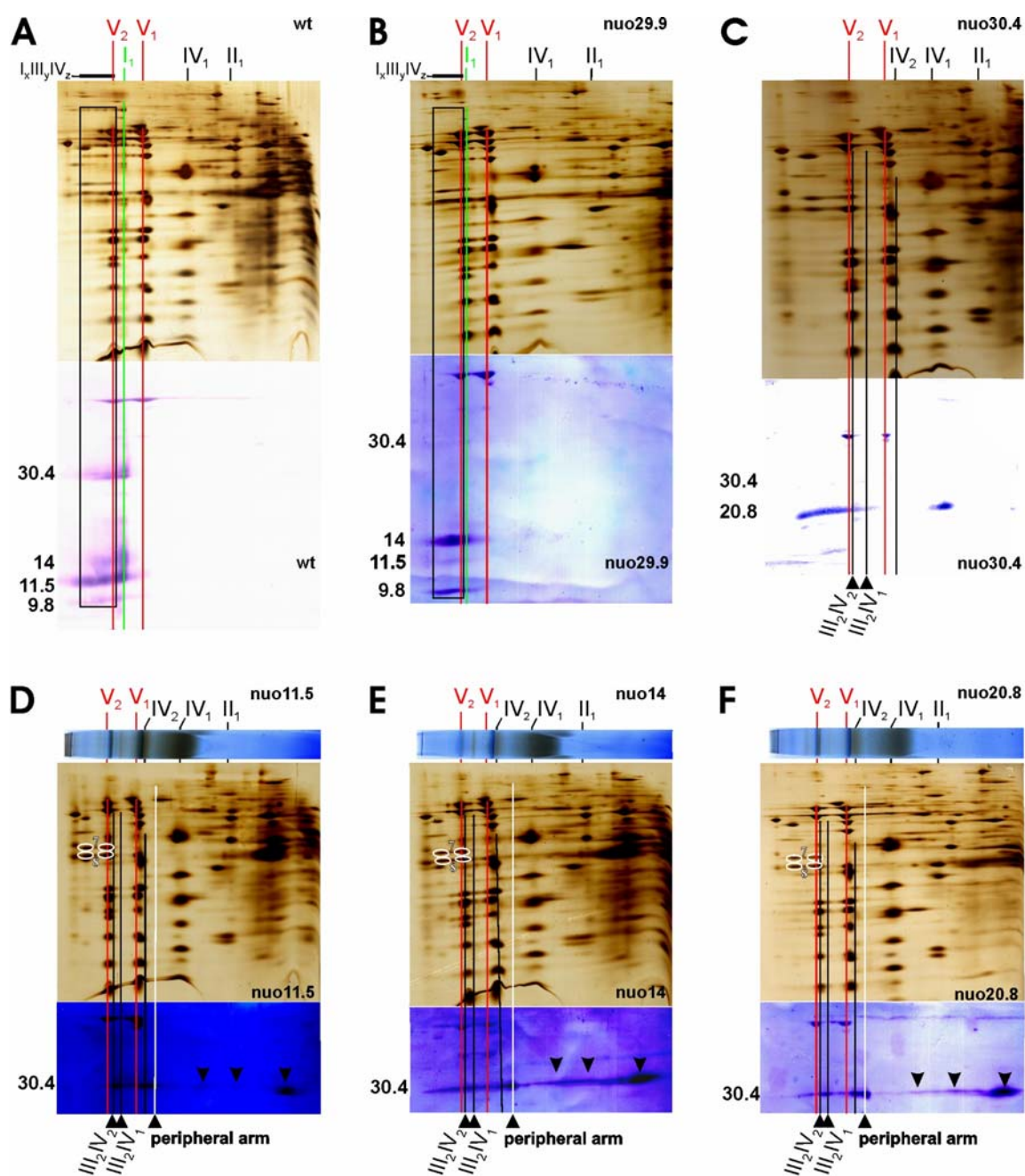


Table 1

	Protein name/ Blast homology	MW theor. (kDa)	pI theor.	TM pred.	GRAVY	gI Protein	Accession nr	Gene Locus ^(a)	Score	ppm
<u>Oxidative Phosphorylation</u>										
Complex I	(Light blue)									
12	NADH-ubiquinone oxidoreductase 78kDa subunit precursor	81.6	6.05	0	-0.330	85083792	NCBI # XP_957188	NCU01765.2	198	30
13	NADH-ubiquinone oxidoreductase 49kDa subunit precursor	54.0	6.26	0	-0.265	85119577	NCBI # XP_965665	NCU02534.2	128	30
14	NADH-ubiquinone oxidoreductase 40kDa subunit precursor	42.9	6.62	0	-0.445	85093948	NCBI # XP_959794	NCU02373.2	202	30
15	NADH-ubiquinone oxidoreductase 29.9kDa subunit precursor	30.9	5.12	0	-0.558	85105954	NCBI # XP_962070	NCU05299.2	45	60
16	NADH-ubiquinone oxidoreductase 30.4kDa subunit precursor	32.2	8.78	0	-0.534	85086366	NCBI # XP_957689	NCU04074.2	111	30
17	NADH-ubiquinone oxidoreductase 21.3a kDa subunit	21.3	7.77	0	-0.322	85099504	NCBI # XP_960797	NCU08930.2	90	60
18	hypothetical protein Blast search: NADH dehydrogenase (ubiquinone) 21.3b	21.3	9.69	3	-0.213	85094628	NCBI # XP_959924	NCU02280.2	86	30
19	19.3 kDa iron-sulfur subunit	25.0	10.02	0	-0.182	2764632	embl # CAA04802	NCU03953.2	42	60
20	NADH dehydrogenase, 20.9 kDa subunit	21.0	9.40	1	-0.280	3030	embl # CAA43221	NCU01859.2	57	60
34	NADH-ubiquinone oxidoreductase 10.5 kDa subunit	10.5	9.46	0	-0.509	2833211	Swiss-prot # Q07842	NCU03156.2	51	90
Complex II	(Red)									
55	hypothetical protein Blast search: succinate dehydrogenase iron-sulphur protein (<i>Aspergillus fumigatus</i> Af293)	31.7	9.12	0	-0.450	85116983	NCBI # XP_965152	NCU00959.2	42	30

56	hypothetical protein Blast search: succinate dehydrogenase cytochrome b560 subunit (<i>A. fumigatus</i> Af293)	19.3	10.38	3	0.464	85090713	NCBI # XP_958549	NCU07756.2	58	60
57	hypothetical protein Blast search: succinate dehydrogenase flavoprotein subunit (<i>A. fumigatus</i> Af293)	65.3 ^(b)	5.72	0	-0.397	85117365	NCBI # XP_965239	NCU08336.2	77	30
Complex III (Pink)										
24	ubiquinol-cytochrome c reductase complex core protein I	52.5	5.63	0	-0.309	127289	Swiss-prot # P11913	NCU02549.2	70	30
25	ubiquinol-cytochrome c reductase complex core protein 2 precursor	47.0	8.95	0	0.053	18376040	embl # CAD21046	NCU03559.2	63	30
27	hypothetical protein Blast search: Cytochrome c1, heme protein, mitochondrial precursor	35.4	7.66	0	-0.305	85111062	NCBI # XP_963756	NCU09816.2	106	30
29	hypothetical protein Blast search: ubiquinol-cytochrome c reductase iron-sulfur subunit precursor (<i>A. fumigatus</i> Af293)	24.8	8.84	0	-0.065	85107305	NCBI # XP_962348	NCU06606.2	64	60
33	hypothetical protein Blast search: probable ubiquinol-cytochrome c reductase complex 14 kDa protein (subunit VII)	14.0	6.59	0	-0.472	85099543	NCBI # XP_960807	NCU08940.2	54	60
34	hypothetical protein Blast search: ubiquinol-cytochrome c reductase chain VIII	11.8	9.89	0	-0.466	85099572	NCBI # XP_960814	NCU08947.2	50	30
Complex IV (Yellow)										
43	cytochrome oxidase subunit 2 prepeptide	28.7	4.44	2	0.317	7145096	NCBI # AAA31959		23	30
44	hypothetical protein Blast search: cytochrome c subunit Vb (<i>A. fumigatus</i> Af293)	20.6	5.90	0	-0.551	85109713	NCBI # XP_963051	NCU05689.2	59	30
45	cytochrome c oxidase polypeptide V precursor	18.8	9.88	1	-0.308	85110415	NCBI # XP_963448	NCU05457.2	32	30
46	hypothetical protein Blast search: cytochrome c oxidase subunit Va (<i>A. fumigatus</i> Af293)	16.8	5.79	0	-0.404	85100681	NCBI # XP_961010	NCU06695.2	86	60

Complex V (Green)

22,38	ATP synthase alpha chain, mitochondrial precursor	59.5	9.07	0	-0.110	85119497	NCBI # XP_965645	NCU02514.2	191	30
23, 39	ATP synthase beta chain, mitochondrial precursor	55.6	5.10	0	-0.045	85074641	NCBI # XP_963253	NCU05430.2	145	30
26	hypothetical protein Blast search: ATP synthase gamma chain, mitochondrial precursor, putative (<i>A. fumigatus</i> Af293)	32.3	8.27	0	-0.116	85091417	NCBI # XP_958891	NCU09119.2	111	30
28	hypothetical protein Blast search: ATP synthase subunit 4, mitochondrial precursor (<i>Saccharomyces cerevisiae</i>)	26.3	9.35	0	-0.162	85112239	NCBI # XP_964306	NCU00502.2	66	30
30	probable oligomycin sensitivity conferring protein (OSCP)	23.0	9.72	0	0.136	85080302	NCBI # XP_956517	NCU01606.2	59	60
31	hypothetical protein Blast search: ATP synthase D chain, mitochondrial (<i>A. fumigatus</i> Af293)	19.4	9.02	0	-0.503	85074817	NCBI # XP_965776	NCU00636.2	97	60
32	hypothetical protein Blast search: Subunit h of the F _o sector (<i>S. cerevisiae</i>)	13.6	4.79	0	-0.456	85117672	NCBI # XP_965299	NCU03199.2	40	30
35	hypothetical protein Blast search: F ₁ F _o ATP synthase g subunit, putative (<i>A. fumigatus</i> Af293)	22.8	10.58	0	0.037	85074833	NCBI # XP_965784	NCU00644.2	80	30

Glycolysis and Tricarboxylic Acid Cycle (White)

1, 47	oxoglutarate dehydrogenase precursor	134.1	8.19	0	-0.517	85074631	NCBI # XP_963248	NCU05425.2	136	30
4	dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial precursor (PDC-E2) (MRP3)	48.6	6.23	0	-0.196	85109166	NCBI # XP_962786	NCU07659.2	78	60
5	hypothetical protein Blast search: 2-oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succinyltransferase (<i>A. fumigatus</i> Af293)	46.0	8.24	0	-0.332	85092528	NCBI # XP_959443	NCU02438.2	91	60

40	hypothetical protein Blast search: probable isocitrate dehydrogenase [NAD] subunit 1, mitochondrial precursor	43.8	8.76	0	-0.076	85115775	NCBI # XP_964931	NCU00775.2	152	30
41	hypothetical protein Blast search: isocitrate dehydrogenase, NAD-dependent (<i>A. fumigatus</i> Af293)	41.0	6.97	0	-0.046	85106968	NCBI # XP_962283	NCU07697.2	117	30
49	glycerol-3-phosphate dehydrogenase precursor	76.9	7.60	0	-0.309	85110409	NCBI # XP_963445	NCU05454.2	79	60
50	hypothetical protein Blast search: fumarate hydratase, putative (<i>A. fumigatus</i> Af293)	57.2	8.83	0	-0.153	85092553	NCBI # XP_959454	NCU10008.2	111	30
59	hypothetical protein Blast search: dihydrolipoamide dehydrogenase (<i>A. fumigatus</i> Af293)	56.5	7.64	0	-0.078	85092766	NCBI # XP_959535	NCU02407.2	107	30
60, 70	citrate synthase, mitochondrial	52.0	8.10	0	-0.244	85082342	NCBI # XP_956898	NCU01692.2	147	30
61	hypothetical protein Blast search: pyruvate dehydrogenase complex alpha subunit (E1) putative (<i>A. fumigatus</i> Af293)	46.1	8.49	0	-0.362	85083464	NCBI # XP_957122	NCU06482.2	132	30
63	probable pyruvate dehydrogenase beta chain precursor (E1) (PDB1)	40.9	6.04	0	0.018	85118132	NCBI # XP_965390	NCU03004.2	64	30
67	hypothetical protein Blast search: aconitate hydratase, mitochondrial (<i>A. fumigatus</i> Af293)	85.0	6.22	0	-0.335	85093919	NCBI # XP_959787	NCU02366.2	152	30
69	hypothetical protein Blast search: D-lactate dehydrogenase, Dld2p (<i>S. cerevisiae</i>)	60.8	6.12	0	-0.333	85084552	NCBI # XP_957332	NCU06441.2	79	60
<u>Lipid Metabolism</u> (Light green)										
54	hypothetical protein Blast search: enoyl-CoA hydratase/ isomerase family protein (<i>A. fumigatus</i> Af293)	32.2	9.24	0	-0.134	85084582	NCBI # XP_957339	NCU06448.2	96	30

Amino Acid Metabolism and Urea Cycle (Orange)

37	hypothetical protein Blast search: delta-1-pyrroline-5-carboxylate dehydrogenase (<i>A. fumigatus</i> Af293)	66.0	8.91	0	-0.232	85111922	NCBI # XP_964169	NCU03076.2	152	60
48	hypothetical protein Blast search: glycine dehydrogenase (<i>A. fumigatus</i> Af293)	120.5	6.77	0	-0.228	85116528	NCBI # XP_965069	NCU02475.2	66	30
51	hypothetical protein Blast search: serine hydroxymethyltransferase (<i>A. fumigatus</i> Af293)	57.5	9.08	0	-0.375	85095341	NCBI # XP_960065	NCU05805.2	86	30
58	hypothetical protein Blast search: mitochondrial dihydroxy acid dehydratase (<i>A. fumigatus</i> Af293)	64.0	6.97	0	-0.208	85090149	NCBI # XP_958280	NCU04579.2	60	30
62	hypothetical protein Blast search: aspartate aminotransferase, putative (<i>A. fumigatus</i> Af293)	47.2	9.01	0	-0.201	85110141	NCBI # XP_963283	NCU08411.2	66	30
64	hypothetical protein Blast search: carbamoyl-phosphate synthase large subunit (<i>A. fumigatus</i> Af293)	128.3	5.65	0	-0.168	85118469	NCBI # XP_965450	NCU02677.2	125	30
66	hypothetical protein Blast search: aminopeptidase (peptide metabolism) (<i>A. fumigatus</i> Af293)	101.4	5.16	0	-0.311	85091989	NCBI # XP_959172	NCU09228.2	49	60
71	hypothetical protein Blast search: glycine cleavage system T protein (<i>A. fumigatus</i> Af293)	48.4	9.25	0	-0.289	85116758	NCBI # XP_965112	NCU02727.2	69	30

Chaperones (Violet)

7	hypothetical protein Blast search: Subunit Phb2p of the prohibitin complex (<i>S. cerevisiae</i>)	34.1	9.73	0	-0.309	85113233	NCBI # XP_964487	NCU03310.2	60	30
8	hypothetical protein Blast search: probable prohibitin PHB1 (<i>A. fumigatus</i> Af293)	30.3	9.07	0	-0.107	85099568	NCBI # XP_960813	NCU08946.2	149	30
36	probable heat-shock protein hsp60	60.5	5.60	0	-0.062	85080225	NCBI # XP_956500	NCU01589.2	130	30
68	hypothetical protein (AF401236) heat shock protein 70kDa (<i>Coccidioides immitis</i>)	56.2	5.09	0	-0.389	85104522	NCBI # XP_961753	NCU08693.1	49	30

Transport and Carrier Proteins (Dark blue)

9	plasma membrane ATPase	99.9	5.03	9	0.148	85082294	NCBI # XP_956886	NCU01680.2	143	30
11	H ⁺ -transporting ATPase, vacuolar, 67K chain	67.1	5.32	0	-0.282	18376302	embl # CAD21414	NCU01207.2	97	30
52	outer mitochondrial membrane protein porin (VDAC)	30.0	9.02	0	-0.166	85100389	NCBI # XP_960950	NCU04304.2	80	30
53	ADP, ATP carrier protein (Adenine Nucleotide Translocator - ANT)	33.9	9.84	3	0.049	85110027	NCBI # XP_963201	NCU09477.2	94	30

Other Proteins (Brown)

2	hypothetical protein (AF323912) Matrix AAA protease MAP-1	103.0	7.21	1	-0.538	85081618	NCBI # XP_956756	NCU01479.2	77	30
3	hypothetical protein (AF323913) intermembrane space AAA protease IAP-1	80.1	8.98	1	-0.242	85080016	NCBI # XP_956468	NCU03359.2	60	90
6	hypothetical protein Blast search: <i>Homo sapiens</i> stomatin (EPB72)-like 2	45.8	9.20	0	-0.171	85095578	NCBI # XP_960112	NCU05633.2	107	30
10	hypothetical protein Blast search: RNA12 protein (<i>A. fumigatus</i> Af293]	99.9	9.17	0	-0.292	85109974	NCBI # XP_963176	NCU09598.2	80	60
42	mitochondrial import receptor subunit TOM40	38.1	5.65	0	-0.201	85103565	NCBI # XP_961545	NCU01179.2	95	30
65	hypothetical protein Blast search: pitrilysin family metalloprotease Cym1 (<i>A. fumigatus</i> Af293)	112.8	5.84	0	-0.398	85099301	NCBI # XP_960750	NCU01272.2	108	30

^(a) <http://www.broad.mit.edu/annotation/genome/neurospora/>

^(b) Mass calculated according to the *N. crassa* genome annotation

CHAPTER 4 – RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

Mitochondrial complex I transfers electrons from NADH to quinone and links this process with translocation of protons across the inner membrane, thereby contributing to the establishment of a transmembrane proton gradient needed for the synthesis of ATP. It is composed of multiple polypeptide subunits of dual genetic origin and contains one non-covalently bound FMN molecule and eight iron-sulfur clusters (Ohnishi *et al.*, 1998; Hinchliffe and Sazanov, 2005). The *Neurospora* L-shaped enzyme spans the mitochondrial inner membrane and consists of two distinct domains formed independently of each other before joining together, denominated membrane and peripheral arms. The first is composed by the more hydrophobic subunits, whereas the second is made of hydrophilic proteins and contains all known prosthetic groups (Videira, 1998).

Despite the growing interest to understand the structure, composition and function of mitochondrial complex I, since many neurodegenerative disorders have been associated with complex I deficiency, information about this large multiprotein complex is still limited. Thus, in order to have a better definition of the subunit composition of *N. crassa* complex I, the fungal genome available from the *Neurospora* Sequencing Project of the Whitehead Institute/MIT Center for Genome Research was screened with homologues from other organisms and a total of 39 dissimilar subunits were identified (**Chapter III, Study 2**). In addition to the fourteen core subunits that constitute the minimal functional structure, seven nuclear- and seven mitochondrial-encoded subunits (Chapter I, Table 1), at least 25 accessory subunits compose the mitochondrial complex I of *Neurospora* (Chapter I, Table 2). Furthermore, 35 of the 39 fungal complex I subunits are conserved in mammals, which demonstrate the high similarity between the enzyme from these two types of organisms. Three out of the remaining four proteins (21.3a kDa, 20.9 kDa, 17.8 kDa and 10.4 kDa) can be found in non-mammalian species. The 21.3a kDa subunit seems to be *N. crassa* specific, because it has no apparent homologue in any other organism. The 20.9 kDa has an apparent homologue in plants, whereas the 17.8 kDa and 10.4 kDa subunits have homologues in other fungi species. Of the 39 fungal subunits, 17 have predicted mitochondrial targeting presequences similar to the bovine homologues, except the bovine B22 (11.7 kDa) protein. With a few exceptions, most of the peripheral arm subunits possess and most of the membrane arm polypeptides lack presequences. Ten fungal nuclear encoded subunits contain one predicted transmembrane helice, and the 21.3b kDa (B14.7) subunit contains three transmembrane domains. The majority of the subunits were located either in the peripheral or membrane arms of complex I, in particular when *Neurospora* mutant strains are available. According to their localisation, none of the subunits of the peripheral arm of complex I exhibit predictable transmembrane helices (**Chapter III, Study 2**).

The role of the accessory subunits has long intrigued researchers and several questions can be raised about the functions of these subunits in mitochondrial complex I: Are they essential for complex I biogenesis?; Are they required for assembly/stability of complex I?; Do they regulate complex I activity?; Do they confer other cellular functions to complex I? Some progress has been made in determining the functions of some of the accessory proteins and their requirement for complex I assembly and/or activity. In order to contribute to a better understanding of these accessory subunits, we have identified and studied two of the *N. crassa* accessory subunits, the 9.8 kDa (**Chapter III, Study 1**) and the 11.5 kDa polypeptides (**Chapter III, Study 2**), homologues of the correspondingly mammalian MWFE and 15 kDa, in the assembly and function of *N. crassa* complex I. These two small conserved accessory subunits, 9.8 kDa (MWFE) and 11.5 kDa (15 kDa), are constituted by correspondingly 86 and 105 amino acid residues, without predictable presequences. Curiously, it was found a similarity between the MWFE homologues and the nqrA subunit of bacterial Na⁺-NQR, which suggests a relation between H⁺- and Na⁺-pumping NADH dehydrogenases that might be indicative of a common origin (**Chapter III, Study 1**).

In order to determine the localisation of the 9.8 kDa (MWFE) and 11.5 kDa (15 kDa) proteins within complex I we checked their presence in Western blots of *Neurospora* complex I mutants using specific antibodies against these proteins. Both proteins are visible in wild-type mitochondria, as well as in mitochondria from peripheral arm mutants, and in contrast they are faintly or not detected in mitochondria from the membrane arm mutants. Thus, we concluded that both proteins are localised in the membrane domain of the fungal enzyme (**Chapter III, Studies 1 and 2**). Indeed, the N-terminal of the mammalian MWFE protein as well as of its fungal homologue possess one transmembrane predicted domain that function as membrane anchor, while the C-terminal must be exposed to the intermembrane space (Yadava and Scheffler, 2004). The 11.5 kDa subunit must be located in the boundary between the peripheral and membrane arms of complex I, accordingly with the bovine homologue that is likely in subcomplex I_α and was also associated with the iron-sulfur fragment of complex I (Carroll *et al.*, 2006).

To further characterise the role of these two nuclear-encoded accessory subunits the corresponding *Neurospora* genes were separately inactivated by RIPing, and the corresponding null-mutant strains were isolated and characterised. The state of assembly and activity of complex I in the nuo9.8 and nuo11.5 mutants, lacking respectively the 9.8 kDa (MWFE) and 11.5 kDa (15 kDa) subunits, was evaluated by an assortment of techniques.

Analyses of the mitochondrial proteins from the nuo9.8 mutant indicate that complex I fail to assemble. Like for mammalian complex I, the 9.8 kDa (MWFE) subunit was found essential for the assembly and activity of fungal complex I, accumulating intermediates of the membrane and peripheral arms. The 9.8 kDa polypeptide must be required at an intermediate step of complex I assembly. Furthermore, respiration of *Neurospora* mutant mitochondria on matrix NADH is

rotenone-insensitive, demonstrating that all the activity is due to the alternative enzyme NDI1 and not to complex I (**Chapter III, Study 1**). Interestingly, in mitochondria of a Chinese hamster mutant cell line, lacking all the ND subunits, the MWFE protein insertion and stabilisation was not observed and it was found that the MWFE incorporation requires at least the ND4 and ND6 subunits, demonstrating that its stabilisation is a coordinated and inter-dependent process likely involving more subunits (Scheffler *et al.*, 2004).

The nuo11.5 mutant strain, lacking the 11.5 kDa (15 kDa) subunit, is also not able to assemble complex I as was demonstrated by BN-PAGE, and accumulates intermediates of the membrane arm. We were unable to detect the formation of an active peripheral arm of the enzyme, as was evidenced by the absence of NADH:hexaamineruthenium (III) and NADH:ferricyanide reductase activities analysed in the sucrose gradient fractions. Furthermore, the peripheral arm subunits identified by Western blotting analysis of the same gradient fractions were predominantly detected as free subunits or smaller subcomplexes, which support the nuo11.5 mutant disability to assemble complex I (**Chapter III, Study 2**). Overall, the results obtained with a combination of different techniques have demonstrated an absolute need for these two accessory subunits for complex I biogenesis and function (**Chapter III, Studies 1 and 2**).

Mammalian complex I exists as a mixture of two interconvertible active (A) and de-activated (D) forms, the latter being sensitive to SH-modifying compounds. The A/D transition has also been described in *N. crassa* (Grivennikova *et al.*, 2003; Maklashina *et al.*, 2003). The 11.5 kDa (15 kDa) subunit was suggested to participate in complex I A/D transitions, since its bovine homologue was among a few candidate proteins that might have been labelled by a fluorescence analogue of NEM, in the de-activated form of the enzyme (Gavrikova and Vinogradov, 1999). In fact, the 11.5 kDa polypeptide as well as homologues from different organisms contains four highly conserved cysteine residues that can possibly react with these SH-reagents.

As previously described, the lack of the 11.5 kDa subunit prevents complex I assembly, and consequently measurements of any kinetic activity using nuo11.5 mitochondria was impracticable. To circumvent this result, we performed independently site-directed mutagenesis of the four conserved cysteines, substituting them with serine residues and the resulting proteins were independently expressed in the null-mutant nuo11.5. All of the obtained site-directed mutant strains were able to assemble a complex I with similar kinetic properties to those observed in the wild-type enzyme, indicating that none of the cysteine residues of the 11.5 kDa protein is individually relevant for the A/D transition process. Diminished amounts of assembled complex I seem to be the major effect of these specific mutations. Thus, the cysteine residues were found essential to the acquisition and stabilization of the correct 11.5 kDa (15 kDa) protein conformation and this is reflected in the assembly/stability of complex I (**Chapter III, Study 3**).

To characterise the mitochondrial respiratory chain of *N. crassa* we investigate the supramolecular organization of the OXPHOS complexes. The existence of these supramolecular assemblies is considered of functional importance. Hence, in order to assess whether a respirasome organization exists in *N. crassa* mitochondria, the mitochondrial protein complexes were gently solubilised with the mild detergent digitonin and subsequently separated by three types of gel systems: 1D gradient BN(CN)-PAGE, 2D BN(CN)/SDS-PAGE and 2D BN(CN)/BN-PAGE (**Chapter III, Study 4**).

To fully characterise the respirasome organization of wild-type *N. crassa* mitochondria, the subunits of native protein complexes were resolved according to their denatured molecular mass. The 2D BN/SDS-PAGE technique has advantages for the study of multiprotein complexes since it can give information about the size, number, protein composition, stoichiometry or relative abundance of complexes (Krause, 2006). Overall, the pattern of OXPHOS complexes in *Neurospora* wild-type mitochondria is similar to mammalian, plant and *P. anserina* mitochondria with high amounts of large respiratory supercomplexes comprising complexes I, III, and IV (I_1IV_1 , I_1III_2 , $I_xIII_yIV_z$), as well as smaller ones (III_2IV_{1-2}). The apparent masses up to 3,000 kDa of the largest supercomplexes, obtained by comparison with the ketoglutarate dehydrogenase complex, suggest that they may have compositions like $I_1III_2IV_{5-6}$.

Interestingly, the individual dimeric complex III (III_2) migrates nearly at the same position as monomeric complex V with an apparent molecular weight of about 600 kDa, which is probably due to boundary lipids that are known to be important to the respiratory function of complex III (Krause *et al*, 2004b; Lange *et al*, 2001). Indeed, using high detergent/protein ratios of DDM or TX-100 to solubilized *N. crassa* mitochondria, we could barely identify substantial amounts of assembled complex III_2 suggesting that complex III dissociates into its subunits, which might be due to the loss of bound lipids. In *Neurospora*, the amount of dimeric complex V (V_2) is about 50% of the total ATP synthase observed. Moreover, there are evidences for the occurrence of even larger complex V assemblies in yeast and mammalian mitochondria (Krause *et al.*, 2005; Wittig and Schagger, 2005), which were not identified in *Neurospora* possibly due to a lower stability of these assemblies.

We have also analysed how different complex I mutations affect the stability of the other OXPHOS complexes and supercomplexes. Thus, in order to analyse the role of complex I in the (super)complexes formation, eight *N. crassa* deletion mutants harbouring nuclear genes mutations representative of various defects in complex I biogenesis were used (nuo9.8, nuo11.5, nuo14, nuo20.8, nuo21, nuo29.9, nuo30.4 and nuo51). The mutants nuo9.8, nuo11.5, nuo14 and nuo20.8, lacking each a membrane arm subunit of complex I, are only competent to assemble the peripheral arm and intermediates of the membrane arm. The mutant nuo30.4, lacking the peripheral arm 30.4

kDa subunit, can not assemble the peripheral arm and accumulates the membrane arm. Our results demonstrate that complex I in *N. crassa* is not essential for assembly/stability of the other OXPHOS complexes, nor for assembly/stability of III-IV-supercomplexes and ATP synthase dimers, since they are present in wild-type-comparable amounts in the five investigated mutants lacking an assembled complex I (**Chapter III, Study 4**).

The remaining peripheral arm mutants nuo21, nuo29.9 and nuo51, lacking respectively the 21 kDa, 29.9 kDa and 51 kDa subunits, are able to assemble the complete enzyme and have wild-type-like I-III-IV-supercomplexes. Although the nuo51 mutant strain is able to assemble complex I and I-III-IV-supercomplexes, these are devoid of NADH oxidase activity that is imposed by the missing subunit that hosts the NADH binding site. Thus, as revealed by these inactive supercomplexes, I-III-IV-supercomplexes are not only related to electron channeling and must have major non-respiratory importance. The formation of mitochondrial cristae was already suggested to be influenced by complex V dimerisation in yeast (Paumard *et al.*, 2002). This process might not only be a function of complex V but also of other OXPHOS complexes. Furthermore, it was demonstrated that complex III is essential for assembly and/or stability of complex I in humans (Acin-Perez *et al.*, 2004), which supports the existence of supramolecular structures according to the solid state model. The results obtained for nuo21, nuo29.9 and nuo51 demonstrate that preformed III-IV-supercomplexes capture essentially all complex I, even when it lacks activity. This occurs either after formation or at early stages of complex I biogenesis and hence supercomplexes are involved in assembly/stabilization of complex I.

We have extended the studies by using BN-PAGE in-gel activities of NADH dehydrogenase (complex I), cytochrome c oxidase (complex IV), ATP hydrolase (complex V), and succinate dehydrogenase (complex II) giving a qualitative indication of these four OXPHOS complexes. Overall, the pattern of active supercomplexes containing complexes III and IV found in wild-type, was also detected in all mutant strains, indicating that the lack of complex I does not impair the formation and stability of III-IV-supercomplexes (III₂IV₁₋₂). Furthermore, all mutant strains demonstrated identical levels of the IV₂ and V₂ dimers, as compared with wild-type. Interestingly, the four membrane arm mutant strains accumulating the assembled peripheral arm (nuo9.8, nuo11.5, nuo14 and nuo20.8), displayed a specific single band with both NADH-DH and deamino-NADH-DH activity at about 450 kDa that apparently corresponds to this hydrophilic domain. Complex II exists as a monomer with an apparent molecular mass of about 130 kDa and no higher species with succinate dehydrogenase activity were detected (**Chapter III, Study 4**).

To further characterise the complex I species in the *Neurospora* mutant strains, mitochondrial protein complexes were separated by 2D BN/SDS-PAGE system, blotted and probed with a set of antibodies against complex I subunits (30.4 kDa localised in the peripheral arm, and either the 9.8 kDa, 11.5 kDa and 14 kDa, or the 20.8 kDa subunits localized in the membrane arm).

In the nuo30.4 and nuo29.9 mutants, where the peripheral arm is the limiting step in complex I assembly, it seems that the surplus assembled membrane arm form low-mobility membrane aggregates. These low-mobility species of the membrane arm may represent aggregates of the partially assembled hydrophobic membrane subunits and/or association products of the membrane arm intermediates with “assembly factors”. The western blots of the membrane arm mutants nuo9.8, nuo11.5, nuo14 and nuo20.8 revealed that subcomplexes of the peripheral arm of complex I, containing at least the 30.4 kDa subunit, interact with III-IV-supercomplexes and/or the prohibitin complex forming high-molecular-weight complexes. In addition, the presence of a subcomplex of around 450 kDa in all membrane mutant strains, indicative of accumulation of the peripheral arm, is in accordance with the in-gel NADH dehydrogenase activity (**Chapter III, Study 4**).

In order to get more insights about the composition and organisation of *N. crassa* mitochondrial proteins, the technique of 2D BN/SDS-PAGE was combined with mass spectrometry analysis of separated polypeptides (**Chapter III, Study 4**). Overall, 67 different proteins were identified by MALDI-TOF-MS. Among them, 31 subunits belong to the OXPHOS respiratory complexes, including one complex V dimer-specific protein (subunit g). This subunit is also known to be dimer-specific in yeast (Arnold *et al.*, 1998), indicating a resemblance between yeast and *Neurospora* ATP synthase dimerisation. In fact, the yeast complex V dimer possesses three dimer-specific subunits (e, g and k) that are essentially to the stability of the dimers, strongly suggesting that supercomplexes are not aggregation products but true assemblies (Arnold *et al.*, 1998). Interestingly, deletion of the gene encoding subunit g not only disturbs the formation of complex V dimers, but also changes the mitochondrial ultrastructure in yeast. Mitochondria of the mutant yeast are enlarged and the mitochondrial inner membrane is devoid of the characteristic invaginations called cristae, which allow speculating that complex V dimerisation is essential for cristae formation (Giraud *et al.*, 2002; Paumard *et al.*, 2002).

The remaining 36 subunits identified are non-OXPHOS proteins such as enzymes of metabolic pathways, chaperones and carriers. Some examples of the non-OXPHOS proteins identified are the two large mitochondrial multienzyme assemblies, the pyruvate dehydrogenase complex (~8,000-10,000 kDa) and the ketoglutarate dehydrogenase complex (~2,800 kDa), the prohibitin complex with 1,250 kDa formed by two homologues (Phb1 and Phb2), the mitochondrial import receptor Tom40 of the TOM complex and the heat shock protein 60 (HSP60). Interestingly, it was detected an even heavier Phb complex with a molecular mass greater than 2,500 kDa, that we suggest to be a supercomplex of the prohibitin complex with the *m*-AAA protease (MAP-1). The *N. crassa* IAP-1, homologue of the *i*-AAA protease, was also identified as a putative supercomplex with an apparent mass of about 4,000-6,000 kDa much higher than that of the

individual protein. Deduced from their mobility in the first-dimension BN-PAGE we suggest that IAP-1 and stomatin-like 2 protein form a large supercomplex in the inner membrane of *N. crassa* mitochondria.

In summary, **study 4** demonstrates that the arrangement of the OXPHOS complexes into supercomplexes is according to the “respirasome” model, in which I:III:IV supercomplexes are associated in a 1:3:6 stoichiometry, and possibly have major non-enzymatic importance. Respiratory complexes do not always form part of supercomplexes and apparently individual complexes and supramolecular assemblies co-exist within the mitochondrial respiratory chain in a dynamic way. Moreover, the absence of an assembled complex I in *N. crassa* does not interfere with the assembly and association of the other respiratory chain complexes, as well as the other non-OXPHOS protein complexes. It is also suggested that III-IV-supercomplexes have a role in assembly/stabilization of complex I, probably at early stages of the peripheral arm assembly (**Chapter III, Study 4**).

CHAPTER 5 – FUTURE PERSPECTIVES

FUTURE PERSPECTIVES

The understanding of complex I remains as a major challenge for the future. This work presents several unanswered questions, which naturally lead to the following future aims:

- To clarify which complex I subunits are involved in the A/D transition.
- To study the role of III-IV supercomplexes in the assembly and stabilisation of complex I, through the characterisation of *N. crassa* mutants lacking specific subunits of complexes III and/or IV.
- To investigate which subunits of the individual complexes are in close contact within the supercomplexes and what drives their stability.
- To elucidate how the alternative respiratory enzymes function in the context of the respirasome organization in *Neurospora*. The analysis of *Neurospora* mutants, devoid of the alternative enzymes, will enable us to identify their role in the supramolecular organisation of the OXPHOS complexes.

CHAPTER 6 – REFERENCES

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