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Complex I from the fungus *Neurospora crassa*

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Abstract

Respiratory chain complex I is a complicated enzyme of mitochondria, that couples electron transfer from NADH to ubiquinone to the proton translocation across the inner membrane of the organelle. The fungus *Neurospora crassa* has been used as one of the main model organisms to study this enzyme. Complex I is composed of multiple polypeptide subunits of dual genetic origin and contains several prosthetic groups involved in its activity. Most subunits have been cloned and those binding redox centres have been identified. Yet, the functional role of certain complex I proteins remains unknown. Insight into the possible origin and the mechanisms of complex I assembly has been gained. Several mutant strains of *N. crassa*, in which specific subunits of complex I were disrupted, have been isolated and characterised. This review concerns many aspects of the structure, function and biogenesis of complex I that are being elucidated. © 1998 Elsevier Science B.V.

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

1. Introduction

Respiratory chain NADH dehydrogenase or complex I is an amazing mitochondrial enzyme, located in the inner membrane of the organelle. It is well known that it catalyses the transfer of electrons from NADH to ubiquinone, through a number of protein-bound prosthetic groups, and that it also couples this reaction to proton translocation across the inner mitochondrial membrane [1]. As in the case of mitochondria, complex I has a dual genetic origin. The majority of the multiple protein subunits of the enzyme are coded by the nucleus, synthesised in the cytoplasm and imported into mitochondria, where they join a few mtDNA-encoded polypeptides [2,3]. Research efforts on complex I have been conducted for a long time as evident from the first description of isolation of the enzyme from bovine heart mitochondria that appeared in 1962 [4]. Two main points raised considerably the interest in complex I: (i) the discovery that several unidentified reading frames of mammalian mtDNA (previously called URFs, now renamed NDs) specify complex I subunits, implying that a great portion of this genome is devoted to encode the enzyme [5,6], and (ii) the more recent findings that complex I deficiencies are associated with different human diseases [7] and might also contribute to the development of cellular processes such as apoptosis and ageing.

Along with bovine, *Neurospora crassa* has been used as one of the main model organisms to investigate complex I. The fungal and mammalian enzymes

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are very similar [2,3,8]. The fungus can be easily manipulated in the laboratory and grows quite rapidly, yet it is an eukaryote. Besides this, its genetics and biochemistry are quite well known [9] allowing many different kind of experiments to be performed. For example, mutant strains lacking particular subunits of complex I or expressing specifically mutated proteins can be constructed and this is certainly a powerful approach for the study of the enzyme. Several organisms are now being used to study many aspects related to the biogenesis, structure and function of complex I, as well as, e.g., the inhibitory action of drugs and their potential use, or the involvement of complex I in disease. This review focus on results that were obtained from research with the fungus *N. crassa*. Nevertheless, bovine names for specific subunits of complex I will be widely used because they are more generally known.

2. Subunit composition, genetics and relationship with other proteins

The complex I of *N. crassa* contains around 35 polypeptide subunits [2]. At least seven of these genes are encoded by mtDNA. They are the homologues of the human ND1–ND6 and ND4L mitochondrial genes [10–12]. It is not known if additional unidentified reading frames, that are present in the fungal mtDNA genome (e.g., Refs. [13,14]), also encode proteins related to complex I. The primary structure of 41 proteins, believed to represent almost all subunits of bovine complex I, are known [15]. In *N. crassa*, 20 of the nuclear-coded subunits have been cloned so far (Table 1). Some of these genes, called *nuo* for *N*ADH:*u*biquinone *o*xidoreductase, have been assigned to specific chromosomes of the fungal genome by classical genetic crosses or the

Table 1
Characteristics of nuclear-coded subunits of complex I from *N. crassa*

N. crassa protein (kDa)	Bovine homologue	Relationship with FHL and NH	Prosthetic group	Location	Mutant available
78	75 IP	NH	N-4, N-1?	p	+
51	51 FP	NH	FMN, N-3	p	+
49	49 IP	FHL		p	+
40	39			p	+
30.4	30 IP	FHL		p	+
29.9	B13			p	+
24	24 FP	NH	N-1?	p	+
21.3a	?			p	+
21.3b	?			m	+
21.3c	TYKY	FHL	N-2?	p	+
21	18 IP			p	
20.9	?			m	+
20.8	PGIV			m	+
19.3	PSST	FHL	N-2?	?	
17.8	?			m	
14.8	B14			p	
12.3	PDSW			m	+
10.5	B8			p	
9.3	B9		Q?	m	
ACP	SDAP			p	+

The references for the *N. crassa* polypeptides are given in Ref. [8], except for the 24-kDa [16], 21.3c-kDa [17], 21-kDa [16], 19.3-kDa (unpublished) and 14.8-kDa proteins [18].
All (and only) the proteins related to bacterial formate hydrogenlyase (FHL) and NAD^+-reducing hydrogenase (NH) have also homologues in the prokaryotic complex I.
p: Peripheral arm.
m: Membrane arm.
FP, flavoprotein fragment.
IP, iron–sulphur protein fragment.

Table 2
Chromosomal location of complex I genes of *N. crassa*

Gene name	Linkage group
nuo-12.3	I R; close to *lys-4* [19]
nuo-19.3	VI L; close to *Bml* (F. Ferreirinha, unpublished)
nuo-20.8	I L; linked to markers R58.2 and AP36a.2 [20]
nuo-21	IV R; close to *Fsr-4* (F. Ferreirinha, unpublished)
nuo-21.3a	V R; left of *inl* (18% recombination) [21]
nuo-21.3c	VI R; close to Tel VI R (M. Duarte, unpublished)
nuo-24	V R; close to *υma-1* (T. Almeida, unpublished)
nuo-29.9	IV R; left of *Fsr-4* [22]
nuo-30.4	VI L; linked to *Bml* [23]
nuo-78	II R; close to *Fsr-55* [24]

analysis of restriction fragment length polymorphisms (Table 2).

In addition to non-proton-pumping NADH dehydrogenases (like NDH2 of *Escherichia coli*), prokaryotic organisms, e.g., *Paracoccus denitrificans* and *E. coli*, contain complex I-like enzymes named NDH1 [25,26]. These enzymes are made up of at least 14 polypeptide subunits and contain redox groups similar to those found in mitochondria. Seven of the bacterial polypeptides represent the homologues of the hydrophobic mtDNA-encoded subunits of mitochondrial complex I, whereas the other seven are equivalent to the 75, 51 49, 30, 24, 23 (TYKY) and 20-kDa (PSST) proteins (bovine nomenclature). These proteins are considered as the 'minimal' subunits required to build a complex I with competent electron transfer and proton translocation activities [3,26,27]. With the recent cloning of the *N. crassa* homologue of the bovine PSST protein (R. Sousa and B. Barquera, unpublished results), all subunits that are homologous to the subunits of prokaryotic complex I have been identified in the fungus.

The cloning of complex I subunits lead to the identification of proteins that share homology with chloroplast proteins [28,29]. These organelles contain at least 11 genes that encode homologues of the bacterial complex I. They appear to be part of a ferredoxin:plastoquinone or NADPH:plastoquinone oxidoreductase transferring electrons from photosystem I to plastoquinone, thus participating in a cyclic electron pathway [27,30]. The 75-kDa, 51-kDa and 24-kDa subunits of complex I are related to the $\alpha\gamma$ dimer (NADH oxidoreductase part) of a bacterial NAD^+-reducing hydrogenase. Subunit α is similar to a fusion of the 24-kDa and 51-kDa proteins and subunit γ is similar to the N-terminal region of the 75-kDa protein [31]. The bacterial enzyme also contains the $\beta\delta$ dimer responsible for the hydrogenase activity. Homologues of these complex I subunits are not present in the chloroplast enzyme, reflecting a different electron input device between the mitochondrial and plastidal complexes [27,30]. However, homologues of these genes have now been found within the operon encoding the NAD(P)-reducing nickel hydrogenase of a cyanobacterium [32]. Four out of the seven nuclear-coded subunits of complex I that have prokaryotic homologues in NDH1 (the 49, 30, TYKY and PSST proteins) together with two mtDNA encoded subunits (the ND1 and ND5 gene products) are related to a bacterial formate hydrogenlyase [26,33,34], an enzyme that catalyses the oxidation of formate coupled to the reduction of protons. The 49-kDa and PSST subunits were found to have a broad relationship with nickel hydrogenases [35]. It is worth noting that all nuclear-coded subunits of complex I from fungi and mammals that represent homologues of prokaryotic complex I are related to either the NAD^+-reducing hydrogenase of *Alcaligenes eutrophus* or to the formate hydrogenlyase of *E. coli*.

An interesting finding was that an acyl-carrier protein of mitochondria [36] is part of complex I, indicating that it performs other functions besides its participation in oxidative phosphorylation [37,38]. This small protein might be involved in lipid biosynthesis and/or lipid repair [39]. One of its roles is to carry lipoic acid that is then transferred to the pyruvate dehydrogenase complex by lipoate transferases [40]. Analysis of a *N. crassa* mutant lacking the protein suggested that ACP has a specific role in the biosynthesis of complex I [39]. Another complex I subunit believed to participate in some kind of synthetic pathway is the 40-kDa (bovine 39 kDa). It contains a putative NADH binding site and shows similarity to β-hydroxysteroid dehydrogenases [41] and to several other enzymes involved in NAD(P)H dependent reactions of biosynthetic pathways (U. Schulte, personal communication). It has been suggested that the protein possesses a NADH-NAD^+ transhydrogenase activity or a second NADH dehydrogenase activity or that it helps in binding other dehydrogenases in order to channel NADH into complex I [41]. It has also been suggested that the 40-kDa

protein is similar to mitochondrial processing peptidases [42], enzymes that remove the N-terminal signal sequences of imported mitochondrial proteins [43], although the similarity was questioned [41]. Analysis of a *N. crassa* mutant lacking this protein revealed that it assembles an inactive complex I which also lacks the signal of a putative chromophore of the enzyme showing a spectroscopic absorption maximum at 295 nm. The 40-kDa protein could be involved in the mechanism of synthesis of this hypothetical chromophore [44] (U. Schulte, personal communication). Without more conclusive experimental

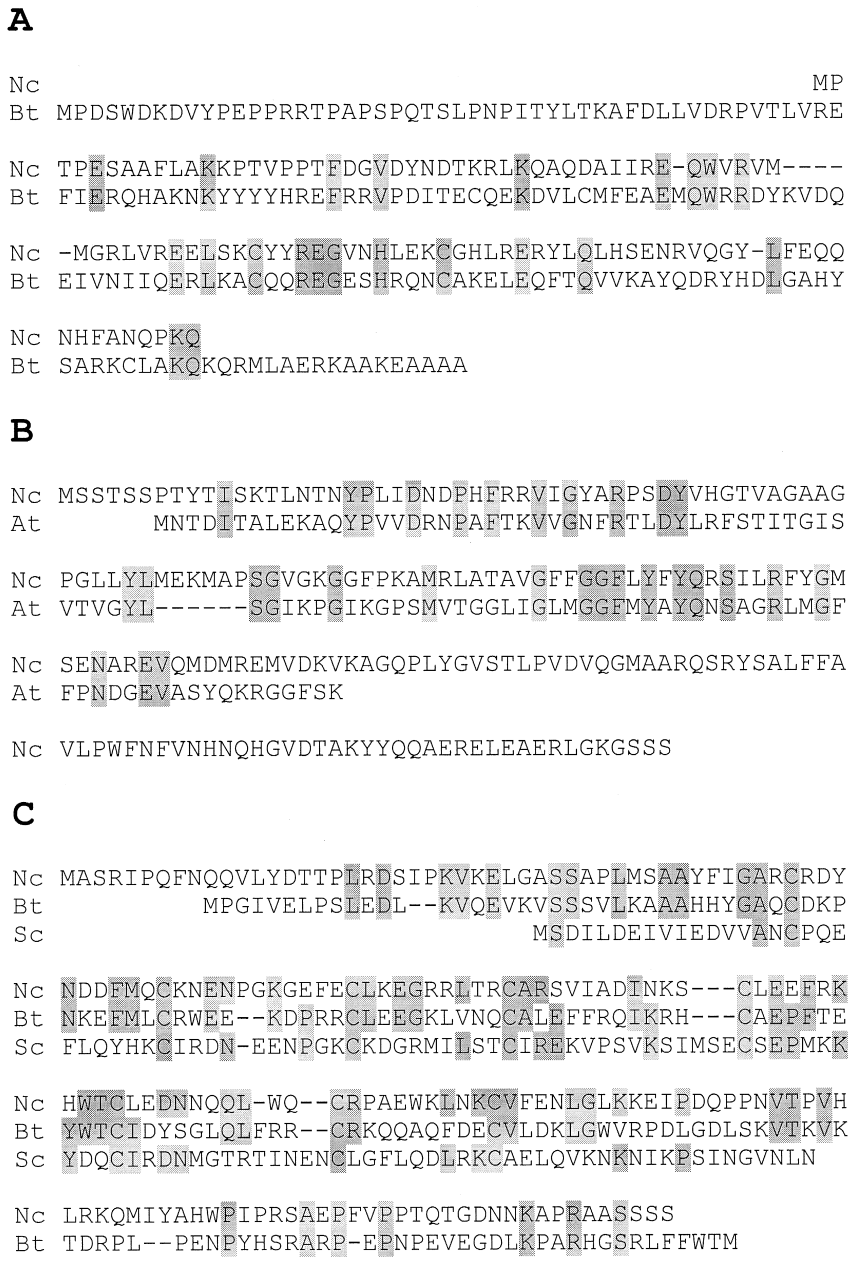

Fig. 1. Alignment of protein sequences. Identical residues are shadowed. (A) *N. crassa* 12.3-kDa protein (Nc) and bovine PDSW (Bt); (B) *N. crassa* 20.9-kDa protein (Nc) and a protein of *A. thaliana* (At); (C) *N. crassa* 20.8-kDa protein (Nc), bovine PGIV (Bt) and the yeast hypothetical protein YDR031w (Sc).

data, the role of the 40-kDa protein remains speculative.

The primary structure of the mtDNA encoded subunits of complex I is neither indicative that they could bind prosthetic groups of complex I nor suggestive of any special function [41]. Recently, a relationship between ND subunits of complex I and bacterial cation/H^+ antiporters was found [45], lending some support to the view that the more hydrophobic membrane arm of complex I is responsible for the proton translocation activity of the enzyme [2].

A Blast search [46,47] of existing protein databases revealed additional similarities between complex I subunits of *N. crassa* and other proteins (Fig. 1). All the comparisons were found to be statistically significant with the Dayhoff MDM-78 matrix. The 12.3-kDa subunit [19] seems to be related to bovine PDSW [48]. Despite the fact that the homology is not very impressive (Fig. 1A), both proteins belong to membrane subcomplexes of the respective complex I, namely the fungal membrane arm and bovine fragment Iβ, although none of them contains potential membrane spanning domains. Moreover, both proteins lack a mitochondrial cleavable signal sequence. The 20.9-kDa protein [49] was found similar to a 106 amino acid protein of *Arabidopsis thaliana* (EMBL Z97341) (Fig. 1B). Thus, it is possible that a homologue of this protein is also present in bovine complex I. Only a few of the *N. crassa* subunits of complex I have no known bovine homologues (Table 1), stressing the similarity between the two enzymes. The 20.8-kDa subunit of *N. crassa* complex I [50], homologue of bovine PGIV [51], seems related to a hypothetical protein YDR031w encoded by chromosome IV of the yeast *Saccharomyces cerevisiae* [52]. The overall similarity is not high but this is also true between the fungal and bovine homologues (Fig. 1C). The most striking resemblance between these proteins is the conservation of eight regularly-spaced cysteine residues. It can be noticed further that there are always nine amino acids between each pair of cysteine residues (cysteines 1 and 2, 3 and 4, 5 and 6, and 7 and 8). Due to the presence of several cysteines, despite the fact that some are involved in intramolecular disulphide bridges [11], it is possible that the 20.8-kDa protein participates in Fe–S cluster binding [50,51]. It is known that *S. cerevisiae* does not possess complex I. It is tempting to speculate that special (unknown) functions are associated with mitochondrial complex I and performed independently by the latter organism. An example of this is represented by the acyl-carrier protein, which belongs to *N. crassa* complex I and is located in the mitochondrial matrix in *S. cerevisiae* [39]. Several hypothetical proteins of the yeast protein database are referred as related to complex I subunits encoded by mtDNA, but the similarity is not readily apparent.

3. Structure and activity

The appearance of *N. crassa* complex I under the electron microscope is that of two arms arranged perpendicularly to each other in an L-shaped structure [53,54]. The membrane arm is embedded in the mitochondrial inner membrane, while the peripheral arm is mainly protruding into the mitochondrial matrix. Roughly, these two arms are the equivalents of two subcomplexes that can be obtained by resolution of bovine complex I, fragments Iβ and Iα respectively [55,56]. Besides minor differences in the location of a few homologous polypeptides [8], one of the differences between the structures is that bovine fragment Iα contains all detectable Fe–S clusters of complex I [55] while cluster N-2 was not observed in the fungal peripheral arm [57,58].

Several aspects of the complex I main function, such as the exact route of the electron-flow from NADH to ubiquinone through the prosthetic groups of the enzyme, or the mechanisms of proton-pumping and its interrelationship with electron transfer, are far from being understood. However, several advances have been made and are discussed in accompanying articles. An important contribution to understand those questions is the identification of proteins that bind the prosthetic groups. In *N. crassa*, FMN and only four Fe–S clusters (binuclear N-1 and tetranuclear N-2, N-3 and N-4) have been observed [57]. Additional centres detected in other organisms might also be present, like bovine clusters N-1a and N-5 [3] (*E. coli* also contains cluster N-1c [59]). The assignment of both FMN and cluster N-3 to the 51-kDa protein, N-1b to the 24-kDa protein, and the possibility that either the 75-kDa or the TYKY or PSST proteins provide ligands for clusters N-2 or N-4 was reviewed [3]. The isolation of a *N. crassa* mutant that (only)

lacks the 51-kDa protein, FMN and cluster N-3 further confirmed the assignment of both prosthetic groups to this protein [60]. Expression of individual proteins of *P. denitrificans* in *E. coli* and EPR analysis of the isolated proteins tentatively assigned clusters N-3 and N-4 to the 51-kDa and 75-kDa polypeptides, respectively [61,62]. Similar studies suggested that clusters N-1a and N-1b are bound by the 24-kDa and 75-kDa proteins, respectively [61–63], as advanced before [56]. Previous work suggested that the 24-kDa binds N-1b, because both co-segregated in the flavoprotein fragment of bovine complex I, which does not contain the 75-kDa protein [64]. Since the EPR signals of Fe–S clusters bound to individual proteins are somewhat different from those in intact complex I, these assignments may need further verification. Another matter of controversy regards the binding of cluster N-2, which is thought to be the direct reducer of ubiquinone and contribute to the proton translocation activity of complex I [65,66]. ND5 was suggested to bind cluster N-2 [2], but this is unlikely since the protein and the redox group were found in different domains of bovine complex I, fragments Iβ and Iα, respectively [55]. Furthermore, the cysteine residues of the protein are not conserved in other organisms [41]. Two proteins, TYKY and PSST are currently the best candidates to bind cluster N-2. This is in agreement with work in *E. coli* NDH1, where characterisation of a 'connecting fragment' indicated that it contains the homologues of both proteins, a tetranuclear Fe–S cluster resembling N-2 and the homologues of two other (non-Fe–S) subunits of complex I [59]. PSST would be an interesting candidate, since the expected unusual properties of a cluster bound to it is in agreement with the pH-dependent midpoint-potential of cluster N-2 [65–67]. We recently obtained some indirect evidence in support of this assumption by clearly demonstrating that the TYKY homologue of *N. crassa* [17] belongs to the peripheral arm of complex I [67], which lacks cluster N-2 [57]. Other authors sustain that TYKY instead binds two N-2 clusters [68,69]. A small 9.3-kDa subunit of *N. crassa* was identified as a ubiquinone binding protein by photo-

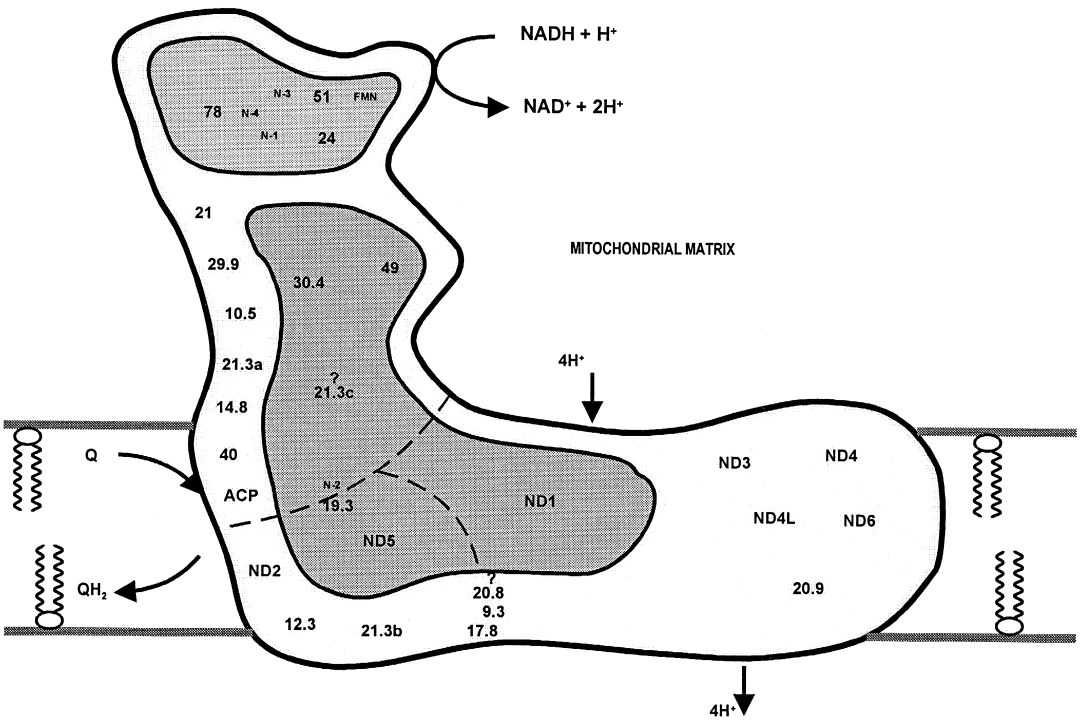

Fig. 2. A structural model of *N. crassa* complex I. The peripheral and membrane arms of the enzyme, as well as the large and small subcomplexes of the latter, are tentatively separated by broken lines. Two domains of complex I related to bacterial NAD^+-reducing hydrogenase (upper) and formate hydrogenlyase (lower) are shadowed. See text for more details.

affinity labelling with ubiquinone derivatives [70]. This has been questioned due to the fact that no homologue of this protein was found in prokaryotic complex I [26]. The 9.3-kDa protein may be part of a ubiquinone binding 'pocket' or could be related to a second ubiquinone binding site suggested to be present in complex I [71,72]. The product of the mitochondrial ND1 gene was also suggested to bind ubiquinone, based on (rather low [3]) similarity to bacterial glucose dehydrogenase [73].

Apart from the 'minimal' subunits required for oxidative phosphorylation, mitochondrial complex I contains a large number of additional polypeptides, considered as 'accessory' proteins [3,26]. With a few exceptions, like the acyl carrier protein, many subunits of complex I seem to have no relation with other proteins that might be indicative of their function. A few of them might even be specific of each organism. We could speculate as to their role. Some could participate in the binding of NADH generating enzymes, which are known to channel NADH to complex I [74]. It was also suggested that these proteins could have a role merely in providing a shield to prevent electrons from escaping the enzyme and making electron transfer in mitochondrial complex I less leaky than in bacteria [45]. Some proteins may carry out other (unknown) functions, however, and some observations can be taken as a support of this view. For instance, the identification of a subunit of complex I that is phosphorylated by a cAMP-dependent kinase [75] or the finding that a chloroplast protein, related to TYKY, binds the origin of replication of the plastid DNA [76].

A structural model of complex I is depicted in Fig. 2 to illustrate this review. The shape of the enzyme is a rough drawing of a three-dimensional model [54]. The 49-kDa subunit was located in this structure [54]. It is close to the 30.4-kDa subunit since a similarity was found between a fusion of these proteins and subunit HycE of bacterial formate hydrogenlyase, suggesting that they are in close contact [34]. The same is true for the 51/24-kDa pair, since a fusion of both is related to the α subunit of the NAD^+-reducing hydrogenase of *A. eutrophus*. They probably are in contact with the 78-kDa protein, since it is related to the γ subunit of the NAD^+-reducing hydrogenase and the α and γ subunits form a dimer responsible for NAD^+ reduction [31]. The composition of the peripheral arm and the small and large intermediates of the membrane arm of complex I has been described [12]. All ND subunits were located [12]. The 12.3-kDa may be part of the small intermediate [20] and the 20.9-kDa belongs to the large intermediate of the membrane arm [12]. Other proteins were placed more arbitrarily in the model. The ND2, 20.8-kDa and 9.3-kDa subunits of the membrane arm are probably close to the peripheral arm [8], because their homologues were found in fragment Iα of bovine complex I [55]. Since ND2 belongs to the small intermediate of the membrane arm in *N. crassa*, this subcomplex is also probably close to the peripheral arm of complex I. The ND1 and ND5 subunits could contact each other and subunits of the peripheral arm, based on their common relationship to bacterial formate hydrogenlyase.

4. Origin and assembly

Experiments in which *N. crassa* cells were pulse-labelled with radioactive amino acids and the flux of radioactivity in complex I polypeptides was followed demonstrated that the peripheral and membrane arms of complex I undergo independent assembly [77]. Independent formation of these two domains of the enzyme can be achieved by cultivating *N. crassa* cells under special growth conditions. In the presence of chloramphenicol, an inhibitor of mitochondrial translation, a small isoform containing only nuclear-coded proteins is formed [71], while the membrane arm can be accumulated in cells grown in manganese-depleted medium [78]. Gene disruption experiments further confirmed independent assembly of the peripheral and membrane arms of complex I (see below). In addition, it appears that membrane arm assembly is also preceded by the formation of two subcomplexes called the small and large intermediates [12]. The latter can be found associated with two complex I-specific proteins, called CIA proteins, that are not present in the final enzyme (chaperones) [12]. Disruption of the CIA genes prevents formation of the large intermediate and further confirms that the large and small intermediates are true assembly intermediates (U. Schulte, personal communication). These kind of observations support the idea that complex I is assembled by different modules. As

mentioned above, four nuclear-coded subunits (of the peripheral arm of complex I) as well as two mtDNA-encoded subunits (the ND1 and ND5 constituents of the large and small intermediates of the membrane arm, respectively [12]) are related to a bacterial formate hydrogenlyase. It is tempting to speculate that association between the peripheral and membranes arms, or even between the two intermediates of the latter arm, relies on interactions between the aforementioned proteins, reminiscent of their association in an 'ancestor' complex.

An interesting prospect will be to investigate whether the mechanism of complex I assembly in *N. crassa* is analogous to that in other organisms. Whereas in the fungus the mtDNA encoded polypeptides are exclusively located in the membrane arm of the enzyme, several homologues of nuclear-coded components of the peripheral arm are encoded by the mitochondrial genome in plants and other organisms. An extreme case is represented by mitochondria of the flagellate *Reclinomonas americana*, which codes for 12 complex I-homologous genes [79]. In this organism, all complex I homologues of the bacterial formate hydrogenlyase, together with one homologue of the NAD^+-reducing hydrogenase of *A. eutrophus* (the 75-kDa), are coded by mtDNA.

The observations that some portions of mitochondrial complex I are related to other enzyme complexes of chloroplasts and bacteria suggests that they represent functional modules and had a common origin. Thus, the structure of complex I would be the result of the association of pre-existing enzymes, occurring during the course of evolution. The conservation of the gene order in different organisms also concurred to this theory. This issue has been reviewed recently [45].

5. Gene disruption

Two approaches have been followed to construct mutant strains of *N. crassa* lacking particular subunits of complex I. One is the replacement of the endogenous genes with defective copies by homologous recombination [44]. Another method relies on the ability of *N. crassa* to recognise and mutate duplicated DNA sequences when they are passed through a genetic cross, a unique phenomenon called repeat-induced point-mutation (RIP) [80]. In either technique, a bacterial gene encoding hygromycin B resistance, driven by an *Aspergillus nidulans* promoter, has been a major genetic marker used to select for *N. crassa* transformants [81]. In the RIP technique, a cloned gene is introduced by transformation into a strain in order to create a duplication. The strain is mated with another strain, which may result in the induction of DNA methylations and GC to AT transitions in both copies of the duplication and lead to gene inactivation [82]. Mutant strains can be recovered from the progeny of the cross [21]. This method has been refined in order to achieve the disruption of essential genes [83]. In this case, briefly, the end result is an heterokaryotic strain in which a wild type nucleus complements the effects of disrupting the essential gene that is present in the other nucleus type. Additional genetic markers can be used to force the mutated nucleus to predominate in the heterokaryon, thus mimicking the situation of a 'pure' mutant.

Both methods have been successfully employed and 14 mutants in specific nuclear-coded subunits of complex I are now available, 10 in subunits of the peripheral arm and four in subunits of the membrane arm of the enzyme (Table 1). All mutants are viable in the vegetative state although their growth rate may be somewhat slower than in the wild type *N. crassa*. Disruption of specific subunits disturbs the assembly of complex I more or less drastically, leading to the accumulation of subcomplexes of the enzyme. Even though the exact polypeptide composition has not been determined for most mutant strains, some conclusions can be anticipated from the analysis of the sedimentation behaviour of the subcomplexes in sucrose gradients. In some cases, mutations in peripheral arm subunits do not prevent the assembly of the others, like in mutants *nuo51* [60], *nuo40* [44], *nuo24* (T. Almeida, unpublished results) and *nuo21.3a* [21]. The presumed peripheral location of the 51-kDa and 24-kDa polypeptides is consistent with these results. In other cases, like mutants *nuo78* [24], *nuo49* [44], *nuo30.4* [23], *nuo29.9* [22] (but see also Ref. [23]), *nuo21.3c* (M. Duarte, unpublished results) and the ACP mutant [39], the peripheral arm seems to be totally disrupted. This indicates that these proteins have a role in the assembly and/or stability of complex I. With the exception of the ACP mutant

[39], mutations in peripheral arm subunits do not interfere with formation of the membrane arm of the enzyme, in agreement with the discovery that these two domains of complex I are assembled independently of each other.

The peripheral arm of complex I can be formed in all mutants disrupted for subunits of the membrane arm that were analysed up to now. These mutations lead to the accumulation of a so-called small intermediate of the membrane arm (*nuo20.9* [44]), or both a small and a large intermediate (*nuo21.3b* [58] and *nuo20.8* [20]), or even an almost complete membrane arm (*nuo12.3* [22]). An interesting observation is that, while the prosthetic groups of complex I are mostly (or even all) associated with the peripheral arm and none was convincingly detected in the membrane arm, several strains seem to be completely devoid of the former but no strain was found that completely lacked the latter. Presumably, a complete disruption of the membrane arm of complex I would affect strongly the structure of the inner mitochondrial membrane. Recently, we have analysed complex I assembly in *N. crassa* stopper strains, that display a severe phenotype of irregular stop-start growth due to mutations in mtDNA [84]. In an analysis of mutant E35, that lacks the ND2 and ND3 proteins [85], we could observe formation of the peripheral arm of complex I but no assembly of membrane arm subunits (P.C. Alves, unpublished results). It is not clear whether the complex I phenotype is responsible for the growth characteristics of the strain.

An exciting observation is that the membrane arm of complex I cannot assemble properly in the ACP mutant, presumably due to the absence of some specific function of this peripheral protein [39], while it is formed in the other mutants disrupted in subunits of the peripheral arm. This suggests that at least the ACP, and perhaps even other isolated proteins or subcomplexes of complex I, can function independently of their association with the complete enzyme. It was indeed shown that mitochondria of mutant *nuo49*, which does not assemble the peripheral arm of complex I, contains the ACP protein. The protein can be precipitated with an antiserum against complex I [39], but it is not clear whether this arises because ACP is associated with other proteins or because it has antigenic determinants recognised by the serum.

Mutant *nuo24* appears quite interesting and might give some insights into complex I function. It lacks the characteristic NADH:ferricyanide reductase activity of the enzyme (T. Almeida, unpublished results). This suggests that the protein may be essential for this activity, although there is other possible explanations. For instance, electrons may not be accessible for the reduction of ferricyanide or the 51-kDa may not be properly assembled and becomes unable to oxidise NADH. A clue to this possibility comes from experiments in which *P. denitrificans NDH1* subunits are expressed in *E. coli*. The 51-kDa (homologous) protein was expressed in an insoluble form unless it was co-expressed with the 24-kDa protein, in which case both proteins formed a soluble complex [61]. It is not yet clear whether *N. crassa* mutant *nuo24* is able to perform the 'normal' rotenone-sensitive NADH:ubiquinone reductase activity of complex I. In the cases where the absence of a complex I protein has a pleiotropic effect on the assembly of the other subunits, it is more difficult to study their role in the enzyme. A way to circumvent this problem will be probably to express specifically mutated proteins, hoping that they lose their function and yet are able to promote a stable assembly of complex I.

6. The importance of complex I

From bacteria to mammals, complex I is an enzyme that can be regulated, in most cases probably reflecting the need to fulfil different energy demands. For example, regulation of complex I expression was studied in the *nuo* operon of *E. coli* [86] and *Salmonella typhimurium* [87], or in rat brain mitochondria during postnatal development [88]. In addition, the expression of complex I might vary in different stages of the life cycle of an organism, like in *Trypanosoma brucei brucei* [89], or between different tissues, as in plants where an increased expression of complex I subunits in flowers was observed [90–92]. Thus, the significance of complex I is expected to vary depending on the organism, stage of development or the specific tissue concerned.

In the case of *N. crassa*, the isolation of mutants in so many different subunits of complex I is leading to the idea that virtually any subunit of the enzyme can be inactivated. It also shows that complex I itself

is dispensable during the vegetative growth of the fungus, albeit at some cost in terms of growth rate. This contrasts to the importance that complex I appears to have in humans where mutations in mtDNA-coded genes may be responsible for severe phenotypes [7]. One explanation for this is that the fungus possesses alternative NADH dehydrogenases [93]. However, the life cycle of *N. crassa* includes an asexual (vegetative) phase, that results in the production of (macro- and micro-) conidia, and a sexual phase. The sexual phase occurs when two strains of opposite mating types are crossed. Briefly, one of the strains forms protoperithecia, which develop into perithecia after fecundated by cells of the other strain. Inside perithecia, following cellular and nuclear fusion, a meiosis event in asci cells results in the production of ascospores that can germinate and reinitiate the vegetative growth [94]. The germination of conidia seems to depend upon respiratory chain activity [95], and an increase in NADH and NADPH content [96] suggests a specific (obviously not crucial) role for complex I during this process. Recently, we obtained evidence that complex I is essential for *N. crassa* to complete the sexual phase of its life cycle [23,97]. While heterozygous crosses between complex I mutants work well and double mutants can be easily obtained, homozygous crosses between several complex I mutants fail to produce ascospores. The block in the sexual sporulation seems to occur at early stages, probably before fusion of the two nuclei of opposite mating types (N.B. Raju, personal communication). Thus, it seems that during sexual development of *N. crassa*, lack of complex I can not be compensated by other enzymes. We are currently sequencing a putative alternative NADH dehydrogenase of *N. crassa* (A. Melo, unpublished results) and it will be interesting to study its expression throughout the life cycle of the fungus.

Complex I seems to be important for bacteria under certain conditions. *E. coli* mutants lacking NDH1 activity have a competitive disadvantage in stationary phase [98]; the enzyme is required for electron transport from NADH to fumarate [99]; and *nuo* mutants of *S. typhimurium* cannot apparently activate ATP-dependent proteolysis under carbon starvation [100]. Inactivation of a subunit of *NDH1* from *Myxococcus xanthus* prevents development and sporulation presumably due to a decrease in the proton-motive force across the membrane [101]. Disruption of *NDH1* genes of *P. denitrificans* seems to be a lethal event. Deletion of the *nqo8* and *nqo9* genes was only achieved in strains expressing NDH2 from *E. coli* [102]. Likewise, it seems that disruption of NDH1 in the cyanobacterium *Anabaena* PCC 7120 is lethal because there are probably no other enzymes to duplicate its role [103]. It should be noticed that the screening for mutants was done under photosynthetic conditions [103] (*Anabaena* is an obligate photoautothroph), and disruption of complex I subunits in *Rhodobacter capsulatus* gave an unexpected photosynthesis-negative phenotype [104]. Anyway, it is not clear if NDH1 is connected with photosynthesis in *Anabaena*, because the enzyme was located in the plasma membrane and not in the thylakoid membrane [103]. In plants, a reduction of the expression of the NADH-binding subunit of complex I, by antisense repression in transgenic potato, disturbed pollen maturation resulting in reduced male fertility [105].

One hypothesis to explain why complex I might be essential for certain cellular processes, like sexual sporulation in *N. crassa*, is that it is a question of energy requirements. Defects in mitochondrial activities are well known as responsible for cytoplasmic male sterility in plants. It was suggested that a threshold level of cytochrome oxidase activity is required for proper anther and pollen development in maize [106]. Furthermore, the development of pathogenic human conditions have been attributed to a decrease of oxidative phosphorylation capacity to below organ-specific energetic thresholds [7]. This ‘energetic’ explanation has also been advanced for the impairment in development and sporulation of *M. xanthus* [101] or the reduced male fertility of transgenic potato [105]. Another possibility to explain the need for complex I in certain situations, particularly in what concerns the more elaborated mitochondrial complex I, is that some (yet unknown) function of the enzyme is involved. These issues deserve further investigation.

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