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In vivo dissection of the mitochondrial respiratory NADH:ubiquinone oxidoreductase (complex I)

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1. Introduction

The proton pumping NADH:ubiquinone oxidoreductase (EC 1.6.99.3), called complex I, is part of the mitochondrial as well as bacterial respiratory chains [1,2]. The minimal form of the enzyme found in prokaryotes is composed of 14 different subunits [3,4], while the mitochondrial enzyme contains up to 28 additional subunits [5,6]. The bacterial genes for complex I subunits are clustered or organized in an operon [3,4] whereas eukaryotic genes are distributed among the nuclear and the mitochondrial genome. Primary structures have been determined for the complex I subunits of *Escherichia coli* [4], *Paracoccus denitrificans* [3], bovine and *Neurospora crassa* [7]. Conserved sequence motifs have provided some clues about the binding sites of substrates and internal redox centres including 1 FMN and up to 7 iron-sulphur clusters [5].

The subunits of the mitochondrial complex I are arranged in an L-shaped structure [8]. One arm of the structure is embedded in the mitochondrial inner membrane, while the other arm protrudes into the matrix. The bipartite structure is reflected in the functional organization of the complex as well as in the organization of the genes and the mechanism of assembly. The peripheral arm including the FMN and at least 4 iron-sulphur clusters forms the NADH dehydrogenase part. The membrane arm contains all seven mitochondrially encoded subunits of complex I, 1 or 2 iron-sulphur clusters and constitutes the ubiquinone hydrogenase part. The two arms are assembled independently before joining to form the mature complex [9]. The particular clustering of genes of the peripheral NADH dehydrogenase part and the membrane intrinsic ubiquinone hydrogenase part, respectively, indicates a likewise arrangement in bacteria. On the basis of these findings a modular evolution of complex I from pre-existing electron transfer enzymes and proton-pumping devices was suggested [4,5].

Very little is known about the function of the many accessory subunits of the mitochondrial complex I. One of the more remarkable of these subunits is a 'prokaryotic' type of acyl carrier protein carrying a phosphopantetheine group [10,11]. This subunit appears to participate in a bacterial type of fatty acid synthesis in mitochondria [12].

To advance our insight into the structure and function of complex I, we followed an approach to dissect the complex in vivo using *N. crassa* mutants in which genes coding for complex I subunits were disrupted. Characterization of the mutants provides access to functional studies of distinct parts of the complex as well as information about the assembly pathway.

2. Accumulation of assembly intermediates

Assembly of complex I involves formation of fairly stable intermediates. In wild type *N. crassa* growing under standard conditions the steady state concentrations of these intermediates are small. However accumulation is achieved by applying growth conditions disturbing distinct steps in the assembly process. Fol-

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lowing pulse-chase labelling, subcomplexes could be identified as assembly intermediates by their transient accumulation of radioactivity [9,13]. In cells growing under manganese starvation, assembly of the peripheral arm is retarded and these cells accumulate the membrane arm [13]. On the other hand, cells growing in the presence of chloramphenicol show a deficiency in mitochondrially translated subunits present only in the membrane arm. This leads to accumulation of the peripheral arm [14].

A more definite disturbance of the assembly process can be achieved by disruption of single nuclear genes of complex I subunits. This involves transformation of *N. crassa* with a genomic fragment containing the gene for the subunit interrupted by a gene providing resistance to hygromycin B [15]. Some 5–40% of the transfomants integrate the fragment by homologous recombination, leaving the transformant with an inactive copy of the subunit gene. So far, five mutants have been characterized, designated *nuoX*, with *X* being the molecular mass (in kDa) of the missing subunit. Two of the mutants lack a subunit of the membrane arm and three are deficient for synthesis of a subunit of the peripheral arm.

3. Membrane arm mutants

*Nuo*21 and *nuo*20.9 are mutants affected in two different subunits of the membrane arm. Assembly of this arm is blocked at distinct stages in these mutants, whereas the pre-assembled peripheral arm is unaffected and accumulates. The remaining subunits of the membrane arm are found in two subcomplexes designated large and small assembly intermediates, respectively. They have complementary subunit compositions (Table 1) and are assembled independently. The large intermediate found in the mutant *nuo*21 consists of 5 mitochondrially encoded subunits, 6 nuclear-encoded subunits and an 80 kDa extra-protein not part of mature complex I (Table 1) [15]. The small intermediate, present in the mutant *nuo*20.9, is composed of the 2 other mitochondrially and 5 nuclear-encoded subunits. While small amounts of the large intermediate are also evident in chloramphenicol-treated and standard wild type cells, the small intermediate has not yet been identified in wild type and its designation as a bona fide intermediate is speculative.

EPR studies of mitochondrial membranes of the mutants revealed three iron-sulphur clusters (N1, N3, N4) assigned to the peripheral arm, but absence of cluster N2 assumed to be located in the membrane arm. The peripheral arm accumulating in the mutants shows NADH/ubiquinone-2 oxidoreductase activity; however, the K_m for ubiquinone-2 is 10-times higher than that for complex I [14]. No enzymatic activity has been related to the membrane arm or its intermediates so far.

Table 1
Subunit composition of *N. crassa* complex I and its assembly intermediates

Complex I	Peripheral arm	Membrane arm	Large intermediate	Small intermediate
			80	
78.2	78.2			
ND5		ND5		ND5
51.4	51.4			
ND4		ND4	ND4	
49.2	49.2			
ND2		ND2		ND2
40.1	40.1			
ND1		ND1	ND1	
29.9	29.9			
28.6	28.6			
28	*28*			
27	*27*			
ND6		ND6	ND6	
21.3a	21.3a			
21.3b		21.3b		21.3b
21		*21*	*21*	
20.9		20.9	20.9	
20.8	20.8			
20		*20*		*20*
ND3		ND3	ND3	
16		*16*	*16*	
15	*15*			
14a		*14a*	*14a*	
14b		*14b*		*14b*
13a		*13a*	*13a*	
13b		*13b*		*13b*
13c				
ND4L		ND4L	ND4L	
11		*11*	*11*	
10a		*10a*	*10a*	
10b				
9.6	9.6			

Mitochondrially encoded subunits are listed according to the respective genes (ND1–6). Nuclear-encoded subunits are characterized by their molecular mass derived from sequence data if available or as found in SDS-PAGE (shown italicized).

4. The intermediate associated protein

An interesting feature of the large assembly intermediate of the membrane arm is its association with an 80 kDa protein. The protein is co-precipitated by various antibodies against single subunits present in the intermediate. It remains tightly bound to the detergent-solubilized intermediate purified chromatographically. Antibodies against the protein do not cross-react with any subunit of mature complex I. Furthermore in pulse-chase experiments with wild type, labelling of the 80 kDa protein bound to the intermediate is permanent, while the other subunits of the intermediate are only transiently labelled. This indicates that the 80 kDa

protein is continuously recycled in the assembly process. In *N. crassa* wild type and the mutant *nuo*21, the 80 kDa protein is found bound to the 250 kDa intermediate and also in the free state. On the other hand, in the mutant nuo20.9, which lacks the large intermediate, the 80 kDa protein is detectable only in the free state. This suggests that it is a complex-I-specific protein and it has been termed *CIA*-protein, standing for *c*omplex I *i*ntermediate *a*ssociated protein. Functional analysis is still preliminary, but the specific association with an assembly intermediate indicates a role of the *CIA*-protein in the biogenesis of the membrane arm. One suggestion is a chaperone type function, keeping the intermediate competent for further assembly.

5. Peripheral arm mutants

The mutant *nuo*51 does not show any complex I related NADH dehydrogenase activity [16]. The alternative, non-proton-pumping NADH:ubiquinone oxidoreductase is used for respiratory oxidation of NADH in the mutant. The mutant assembles an enzymatically inactive complex I lacking probably only the 51 kDa subunit. EPR spectroscopy shows the iron-sulphur clusters N1, N2 and N4. Cluster N3 and the FMN are missing, indicating their association with the 51 kDa subunit. The phenotype of the mutant is in full agreement with the primary structure of the 51 kDa subunit, suggesting it to provide the binding sites for NADH, the FMN and a tetranuclear iron-sulphur cluster [17,18]. This subunit was also labelled by photoaffinity analogues of NADH [19].

A different phenotype is shown by mutant *nuo*49 lacking the 49 kDa subunit of the peripheral arm. No stable assembly of subunits of the peripheral arm is found and the NADH/ferricyanide reductase activity characteristic for this arm is not detectable. Assembly of the membrane arm seems to be unaffected, demonstrating again the independent assembly of the two arms.

A most striking phenotype is shown by mutant *nuo*9.6 lacking the acyl carrier protein, also a subunit of the peripheral arm. Neither subunits of the peripheral arm nor subunits of the membrane arm were found as stable assemblies. None of the complex I specific iron-sulphur clusters could be detected by EPR spectroscopy. All the other respiratory complexes are, however, made in normal amounts in the mutant. It is hardly imaginable that the complete complex I deficiency of the mutant is brought about by structural properties of the acyl carrier protein, since this small peripheral subunit is not expected to have a major impact on the structural integrity of particularly the membrane arm of complex I. Therefore, further studies will focus on the functional importance of the acyl carrier protein for complex I assembly.

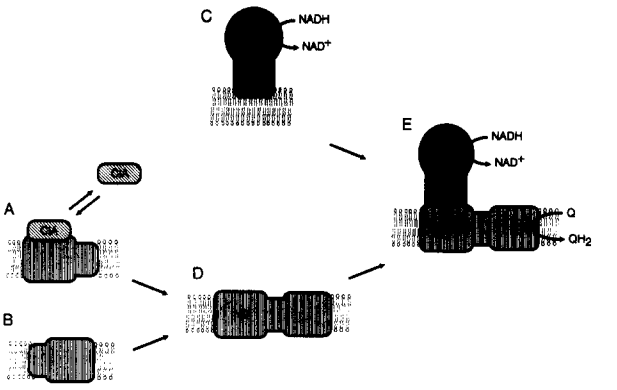

Fig. 1. Assembly pathway of complex I in *N. crassa*. Shown are the characterized assembly intermediates and the proposed stepwise association. A, large assembly intermediate of the membrane arm; B, small assembly intermediate of the membrane arm; C, peripheral arm; D, membrane arm; E, complex I. CIA is the complex I intermediate associated protein, N1–4 are the EPR-visible iron-sulpher clusters. For subunit compositions of A, B, C, D, E, see Table 1.

6. The assembly pathway of complex I

Considering the subunit composition of the assembly intermediates of complex I (Table 1) and their independent formation, we propose that assembly of the complex is advanced by stepwise association of pre-assembled parts. Following this scheme, the membrane arm is formed by the large and small intermediate and in a second step the membrane arm and the peripheral arm join to form complex I (Fig. 1). The *CIA*-protein bound to the large intermediate is released as assembly of the membrane arm is completed and will be reused in the next assembly process. The pre-assembled peripheral arm and the membrane arm are already equipped with the respective prosthetic groups, while no group seems to be present in the intermediates of the membrane arm. This indicates that at least iron-sulphur cluster N2 is formed late in the assembly process.

Assembly intermediates as well as mutant forms of complex I are readily accessible for biochemical studies and provide valuable tools for further analysis of complex I.

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