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Minireview

Attempts to Define Distinct Parts of NADH:Ubiquinone Oxidoreductase (Complex I)

Thorsten Friedrich,[1] Uwe Weidner,[1] Uwe Nehls,[1] Wolfgang Fecke,[1] Regina Schneider,[1] and Hanns Weiss[1]

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The NADH:ubiquinone oxidoreductase (complex I) is made up of a peripheral part and a membrane part. The two parts are arranged perpendicular to each other and give the complex an unusual L-shaped structure. The peripheral part protrudes into the matrix space and constitutes the proximal segment of the electron pathway with the NADH-binding site, the FMN and at least three iron-sulfur clusters. The membrane part constitutes the distal segment of the electron pathway with at least one iron-sulfur cluster and the ubiquinone-binding site. Both parts are assembled separately and relationships of the major structural modules of the two parts with different bacterial enzymes suggest, that both parts also emerged independently in evolution. This assumption is further supported by the conserved order of bacterial complex I genes, which correlates with the topological arrangement of the corresponding subunits in the two parts of complex I.

KEY WORDS: NADH:ubiquinone oxidoreductase; complex I; iron-sulfur cluster; assembly; gene disruption; *Neurospora crassa, Escherichia coli.*

INTRODUCTION

NADH:ubiquinone oxidoreductase (EC 1.6.99.3.), also known as respiratory complex I of the mitochondria, transfers electrons from NADH to ubiquinone and links this process with the translocation of protons. One flavin mononucleotide (FMN) and a still not exactly defined number of iron-sulfur (FeS) clusters serve as prosthetic groups in this reaction. The sequence of operation of these groups and their linkage to proton-translocation are not yet understood (see also Weiss and Friedrich, 1991; Walker, 1992). This is due to the extraordinary complexity of the enzyme. In mammals complex I contains 41 different subunits giving a total molecular mass of 907 kDa (Arizmendi *et al.*, 1992a; Walker, 1992). The enzyme of the fungus *Neurospora* (*N.*) *crassa* seems to be somewhat smaller but still consists of at least 30 different subunits (Weiss *et al.*, 1991). Furthermore, the mitochondrial enzyme is under dual genetic control. The majority of subunits is nuclear-encoded and made in the cytoplasm, whereas seven more hydrophobic subunits are encoded and synthesized within the mitochondrion (for reviews, see Weiss *et al.*, 1991; Walker, 1992).

The related bacterial counterparts of mitochondrial complex I are also highly complicated enzymes. The proton-translocating NADH:ubiquinone oxidoreductase of *Paracoccus* (*P.*) *denitrificans* might turn out to contain up to 17 different subunits (Xu *et al.*, 1992, 1993) and the *Escherichia* (*E.*) *coli* enzyme 14 subunits. These 14 subunits appear to be the minimal structural framework for the enzyme, because homologues to them are found in all the proton-translocating NADH:ubiquinone oxidoreductases studied so far (Walker, 1992; Weidner *et al.*, 1993; Xu *et al.*, 1993).

The primary structures of all the subunits of complex I from bovine (Arizmendi *et al.*, 1992a; Walker, 1992), *P. denitrificans* (Xu *et al.*, 1993), and *E. coli* (Weidner *et al.*, 1992) and many subunits of the *N. crassa* complex I are now known (Weiss *et al.*,

[1] Heinrich-Heine-Universität Düsseldorf, Institut für Biochemie, Universitätsstraße 1, 4000 Düsseldorf 1, Germany,

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1991). Conserved sequence motifs provide information about the binding sites of the substrates and the internal redox groups and reveal interesting relationships with different bacterial electron transfer enzymes. Most remarkably, all subunits predicted to harbor the redox groups appear to be peripheral proteins with either none or only one hydrophobic segment (Table I). These are all nuclear-encoded mitochondrial complex I subunits. With the exception of the binding site for ubiquinone (Friedrich *et al.*, 1990; Walker, 1992), nothing is known about the function of the membrane intrinsic subunits which in the mitochondrial complex I are mitochondrially encoded proteins.

In this review an attempt is made toward the definition of distinct parts of the NADH:ubiquinone oxidoreductase. Two parts of the mitochondrial complex I can easily be distinguished by their different topography and separate pathways of assembly. The conserved clustering of genes in bacteria leads to an outline of similar parts. This subdivision of complex I is supported by relationships with different bacterial electron transfer enzymes.

VISUALIZATION OF A PERIPHERAL AND A MEMBRANE PART OF MITOCHONDRIAL COMPLEX I BY ELECTRON MICROSCOPY

Complex I from *N. crassa* can be isolated as a monodisperse protein–phospholipid–detergent preparation. When the preparation is incubated at high concentrations of NaBr 14 peripheral subunits fall apart, whereas the membrane part of the preparation, as judged from phospholipid and detergent binding, resists this treatment (Fig. 1A; Tuschen *et al.*, 1990).

Preparations of the whole complex I, the membrane part obtained as described above and a complementary peripheral part isolated from chloramphenicol-treated *N. crassa* as will be described below, were analyzed by electron microscopy (Hofhaus *et al.*, 1991). Single particles of the whole complex revealed an unusual L-shaped structure with two distinguishable arms arranged perpendicularly. The longer arm of the "L" resembled in size and shape the single particle of the membrane part, whereas the more bulky, shorter arm roughly resembled that of the peripheral part (Fig. 1A).

Membrane crystals were prepared of the whole complex I and of the peripheral and membrane parts. From tilted electron microscopic views the low-resolution structures were calculated. The reconstructions

Table I. Predicted Properties and Relationship to Other Bacterial Enzymes of the "Minimal" Set of Complex I Subunits[a]

Name of related subunits *E.c./B.t.*	Molecular mass *E.c./B.t.* (kDa)	Membrane helices *E.c./B.t.*	Predicted function or cofactor-binding	Different bacterial enzyme with related subunit
NUO1/ND3[a]	16.3/13.1	3/3		
NUO2/PSST[b]	25.1/20.1	1/0	1 × [4Fe-4S]	Formate hydrogenlyase of *E.c.*
NUO3/30(IP)[c]	21.5/26.4	0		
NUO4/49(IP)[d]	45.9/48.9	0		Formate hydrogenlyase of *E.c.*
NUO5/24(FP)[e]	18.6/23.7	0	1 × [2Fe-2S]	NAD^+-reducing hydrogenase of *A.e.*
NUO6/51(FP)[f]	49.6/48.4	0	NADH-binding; FMN; 1 × [4Fe-4S]	NAD^+-reducing hydrogenase of *A.e.*
NUO7/75(IP)[g]	91.2/77.1	0	2 × [4Fe-4S]; 1 × [2Fe-2S]	NAD^+-reducing hydrogenase of *A.e.*
NUO8/ND1[a]	36.3/35.7	8/8	Ubiquinone-binding	Formate hydrogenlyase of *E.c.*
NUO9/TYKY[h]	20.4/20.2	1/0	2 × [4Fe-4S]	Formate hydrogenlyase of *E.c.*
NUO10/ND6[a]	19.9/19.1	5/5		
NUO11/ND4L[a]	11.2/10.8	3/3		
NUO12/ND5[a]	66.3/68.3	13/11		Formate hydrogenlyase of *E.c.*
NUO13/ND4[a]	50.8/52.1	10/12		
NUO14/ND2[a]	51.6/39.7	12/8		

[a] The subunits are listed according to the order of their genes in the *E. coli nuo* locus. *E.c.* refers to *E. coli*, *b.t.* to *Bos taurus*, and *A.e.* to *A. eutrophus*. The molecular masses are calculated from sequence. The data for the *E. coli* proteins are from Weidner *et al.* (1993), and the data and nomenclature for the bovine proteins are from Walker (1992). The references for the bovine sequences are (a) Anderson *et al.*, 1982; (b) Arizmendi *et al.*, 1992b; (c) Pilkington *et al.*, 1991b; (d) Fearnley *et al.*, 1989; (e) Pilkington and Walker, 1989; (f) Pilkington *et al.*, 1991a; (g) Runswick *et al.*, 1989; (h) Dupuis *et al.*, 1991. The numbers of transmembranous alpha-helices are calculated by the method of Klein *et al.*, 1985; see also Fearnley and Walker (1992).

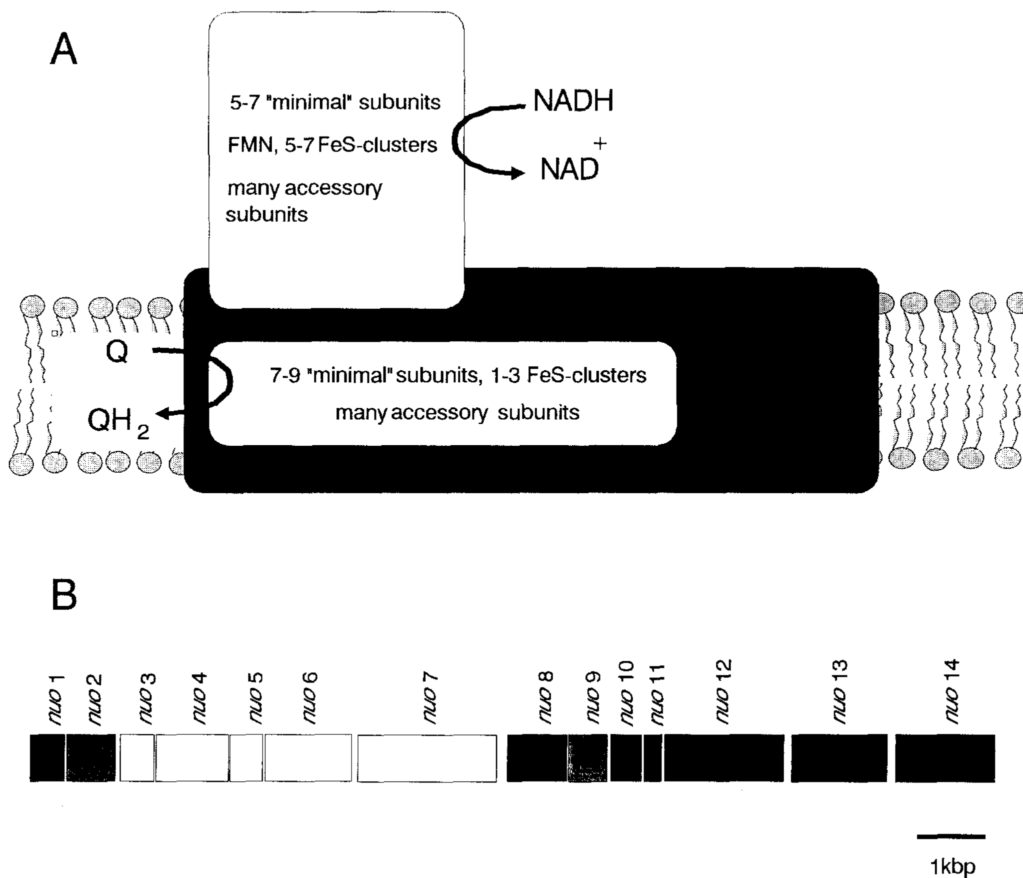

Fig. 1. (A) Schematic representation of the L-shaped mitochondrial complex I with the peripheral part shown in white and the membrane part shown in black. "Minimal" subunits refer to subunits of which homologues are found in the "minimal complex I" of *E. coli.* "Accessory" subunits refer to subunits that are not found in the bacterial complex I. Their number amounts to some 15 in the *N. crassa* complex I and 27 in the bovine complex I. (B) Schematic representation of the nuo-locus of *E. coli.* The genes are shown in white or black according to the location of their proteins in the peripheral or membrane parts of complex I. The genes of the two proteins that have not yet been allocated to one of the two parts are shown in gray.

confirmed the overall L-shaped structure of the complex and showed that the shorter arm of the "L" extends into the aqueous phase while the longer arm is buried in the membrane (Fig. 1A; Hofhaus *et al.*, 1991).

SEPARATE ASSEMBLY OF THE PERIPHERAL AND MEMBRANE PARTS

The existence of separate assembly pathways for the peripheral and membrane parts of mitochondrial complex I could be demonstrated by several approaches performed with *N. crassa.*

The fungus was grown in the presence of chloramphenicol, leading to an inhibition of mitochondrial protein synthesis. Total mitochondrial protein from such cells was then fractionated by sucrose gradient centrifugation in detergent solution. The distribution in the gradient of the NADH/ferricyanide reductase activity, a marker activity of the peripheral part of complex I, was determined. This activity sedimented like a 350,000 M_r protein through the gradient and not like the 700,000 M_r complex I as in a control experiment performed with normal hyphae. Only the peripheral part of complex I could be isolated from these cells (Friedrich *et al.*, 1989). This experiment showed that in *N. crassa* if the membrane part of complex I cannot be formed, the fungus still assembles the peripheral part.

The separate assembly of the membrane part was first demonstrated by pulse-labeling of *N. crassa* with

[^{35}S]-methionine and following the flux of radioactivity into the fully assembled complex I. By immunoprecipitation, labeled subunits were transiently found in an intermediate complex that in terms of size and subunit composition resembled the membrane part (Tuschen *et al.*, 1990). A similar conclusion was drawn from growing *N. crassa* under manganese-limited conditions. For reasons not yet understood, the assembly of the peripheral part of complex I was disturbed, leading to the pre-assembly of the membrane part alone (Schmidt *et al.*, 1992).

Gene disruption is now the most elegant way to accumulate either of the two pre-assembled parts of complex I in *N. crassa* (Nehls *et al.*, 1992). The fungus is transformed with a genomic DNA fragment, including the gene to be deleted, interrupted by a resistance marker gene. Heterokaryotic transformants were selected and crossed with the parental strain to obtain homokaryonts. Two mutants have so far been characterized in more detail. In one mutant, called *nuo*21, the nuclear gene of the 21 kDa subunit of the membrane part was disrupted. The mutant pre-assembles the entire peripheral part, but the assembly of the membrane part is blocked at a distinct step, leading to the accumulation of smaller assembly intermediates of the membrane part (Nehls *et al.*, 1992). In another mutant, called *nuo*51, the gene of the 51 kDa NADH-binding subunit of the peripheral part was disrupted. This mutant cannot fully assemble the peripheral part but still makes the entire membrane part (W. Fecke, unpublished results).

These experiments also demonstrated that the obligate aerobic fungus *N. crassa* can grow without a fully assembled complex I. An alternative nonproton-translocating NADH:ubiquinone oxidoreductase which is found in mitochondria of fungi and plants and in many bacteria (Weiss *et al.* 1991; Yagi, 1991) can take charge of the electron transfer function of complex I. This occurs at the expense of one energy-coupling site. Mitochondrial cytochrome contents and respiratory activities of the mutants were normal. Respiration with pyruvate/malate was inhibited by antimycin A or KCN but was insensitive to piericidin A, the well-known inhibitor of complex I (Nehls *et al.*, 1992).

ISOLATION AND CHARACTERIZATION OF THE PERIPHERAL PART

The peripheral part of the *N. crassa* complex I was first isolated from chloramphenicol-treated hyphae (Friedrich *et al.*, 1989) and, subsequently, from the mutant *nuo*21 (Nehls *et al.*, 1992). SDS/polyacrylamide gel electrophoresis of the preparation clearly showed the bands of 14 subunits. Biochemical analysis and EPR spectroscopy showed the presence of FMN and three FeS-clusters N-1, N-3, and N-4 (Wang *et al.*, 1991). The putative binding sites for NADH, FMN, and up to five FeS-clusters in the peripheral part were identified by conserved sequence motifs in this part (Fearnley and Walker, 1992; Weidner *et al.*, 1993). Since only three FeS-clusters have been detected so far by EPR spectroscopy, there still may be hidden or EPR-silent FeS-clusters in the peripheral part.

Most remarkably the isolated peripheral part alone has NADH/ubiquinone-2 reductase activity which is insensitive to piericidin A. The K_M^{app} for NADH was the same as with the whole complex, namely 2 μM, but the K_M^{app} for ubiquinone-2 was higher, namely 20 μM instead of 5 μM. This putative ubiquinone-reduction site of the peripheral part could be a cryptic one in the whole complex I. This observation was used to propose a (still hypothetical) internal quinone cycle as the electronic link between the peripheral part and the membrane part. Such an internalized quinone (or quinoid group) could also serve as a device for redox-linked proton translocation (Weiss and Friedrich, 1991; Wang *et al.*, 1991).

Taken together, these data clearly show that the peripheral part of complex I comprises the electron input site and the proximal section of the electron pathway (see Fig. 1).

CHARACTERIZATION OF THE MEMBRANE PART

The last step of the electron transfer through complex I is believed to occur from the FeS-cluster N-2, which has the most positive redox potential (Ohnishi, 1979), to ubiquinone (for reviews, see Weiss *et al.*, 1991; Weiss and Friedrich, 1991; Walker, 1992). The membrane part obtained by NaBr treatment of the *N. crassa* complex I contained no EPR-detectable FeS cluster. The cluster N-2 must have been destroyed in this preparation because it is found in the pre-assembled membrane part *in situ*, i.e., in mitochondria from *N. crassa* in which the assembly of the peripheral part was blocked. Mitochondria from cells grown under manganese-limited condi-

tions significantly exhibit only the signal of cluster N-2 at the same *g*-values and with the same power saturation dependence as the signal from cluster N-2 in mitochondria from normally grown *N. crassa*. Small signals arising from the clusters N-1, N-3, and N-4 from some residual whole complex I, which were still made under the manganese-limited condition, were also found. In the mitochondria from the mutant *nuo*51, cluster N-2 was the only EPR-detectable FeS cluster of complex I (unpublished for *N. crassa*; published for *Aspergillus (A.) niger* in Weidner *et al.*, 1992). Due to the complete absence of the whole complex I, EPR spectra of this mutant show no signals of the clusters N-1, N-3, and N-4.

The ubiquinone-binding site was identified indirectly. The mitochondrially encoded ND1 subunit in the membrane part was photolabeled by a rotenone analogue, and because rotenone (like piericidin A) inhibits complex I at the ubiquinone-catalytic site, the subunit was suggested to contain the ubiquinone-binding site (Earley *et al.*, 1987). This suggestion was supported by sequence comparison with a bacterial glucose dehydrogenase. This single polypeptide enzyme oxidizes glucose to gluconolactone in the periplasmic space and delivers the electrons via pyrroloquinoline-quinone to ubiquinone. The glucose dehydrogenase is inhibited by piericidin A like complex I. Only the N-terminal region of the glucose dehydrogenase is hydrophobic enough to span the membrane. Comparisons of this N-terminal sequence with a consensus sequence derived from the ND1 subunit of many different species revealed considerable similarity (Friedrich *et al.*, 1990).

In summary, the membrane part, as defined by its separate assembly, comprises all seven mitochondrially encoded subunits and some eight nuclear-encoded subunits. So far, the FeS cluster N-2 has been found as the only redox group in this part. The subunit that ligates this cluster has not yet been identified. The only function known so far for a mitochondrially encoded subunit is that of ubiquinone binding.

CLUSTERED GENES OF THE BACTERIAL COMPLEX I

The *nuo* locus encoding the proton-translocating NADH:ubiquinone oxidoreductase in *E. coli* was sequenced. The locus comprises 14 genes designated *nuo*1 to *nuo*14 (Fig. 1B and Table I). Seven proteins derived from these genes are homologous to nuclear-encoded and seven to mitochondrially encoded subunits of mitochondrial complex I (Weidner *et al.*, 1993). Summing up the calculated molecular masses of the 14 derived proteins, a protein molecular mass of 525,000 for the *E. coli* complex I is obtained. The *E. coli* complex I comprises homologues to all subunits of the mitochondrial complex I considered to be essential for binding of the substrates and the internal redox groups. The 14 *E. coli* subunits can therefore be considered as the minimal set of proteins required for the proton-translocating NADH:ubiquinone oxidoreductase (see Table I). Secondary structure predictions classify five *E. coli* subunits as peripheral proteins, in agreement with their mitochondrial counterparts which are all found in the peripheral part of complex I. The *E. coli* counterparts of the seven mitochondrially encoded subunits are likewise predicted to be buried in the membrane (Table I). The location of the NUO2 and NUO9 subunits is not known. Depending on the method used for folding prediction, they contain either none or one membrane-spanning helix (Table I). The *E. coli nuo* locus is bordered by regions resembling bacterial promoter and terminator sequences. It was therefore suggested that the 14 *nuo* genes are organized in one transcriptional unit (Weidner *et al.*, 1993).

Yagi and coworkers (Xu *et al.*, 1992, 1993) have sequenced the gene cluster encoding the *P. denitrificans* complex I. Besides the 14 genes which correspond to the *E. coli nuo* genes, six additional reading frames were found in the cluster coding for unknown proteins. Whether some of them are accessory subunits, specific for the *P. denitrificans* complex, could not be determined yet because the intact complex has not yet been isolated from this bacterium.

The order of the 14 *nuo* genes in *E. coli* is exactly the same as that of the 14 related genes in *P. denitrificans* (Weidner *et al.*, 1993; Xu *et al.*, 1993). This order is also found in clustered genes encoding the putative NAD(P)H:quinone oxidoreductase in cyanobacteria and chloroplasts as well as in the related genes of the NAD-reducing hydrogenase operon of *A. eutrophus* (Weiss *et al.*, 1991; Walker, 1992). To some extent, this conserved gene order reflects the topological arrangement of the subunits in the two parts of the mitochondrial complex I. If we take the L-shaped model of the *N. crassa* complex I to approximate the *E. coli* enzyme, the peripheral part would then contain the subunits encoded by the *nuo* genes

3–7, and the membrane part would contain the subunits encoded by *nuo*1, 8, and 10–14. The allocation of the subunits encoded by *nuo*2 and 9 in the two parts is not yet clear (Fig. 1; Table I).

RELATIONSHIP OF COMPLEX I WITH DIFFERENT BACTERIAL ENZYMES

A major functional module of the peripheral part of complex I is made up by three subunits called 24(FP), 51(FP), and 75(IP) in the bovine complex I (Walker, 1992) and called NUO5, NUO6, and NUO7 in the *E. coli* complex I (Weidner *et al.*, 1993). They contain the binding sites for NADH, FMN, and up to five FeS clusters (see Table I). These three peripheral complex I-subunits are closely related to the diaphorase part of the soluble NAD^+-reducing hydrogenase of *A. eutrophus* (Tran-Betcke *et al.*, 1990; Pilkington *et al.*, 1991a; Preis *et al.*, 1991; Xu *et al.*, 1993; Weidner *et al.*, 1993). These three subunits constitute the electron input site and at least part of the proximal section of the electron pathway in complex I.

There is another most remarkable relationship between a part of complex I and a completely different bacterial enzyme. We believe that the peripheral subunit 49(IP)/NUO4 (see Table I), the two subunits of unclear topographic allocation PSST/NUO2 and TYKY/NUO9, and the membrane-intrinsic subunits ND1/NUO8 and ND5/NUO12 are related to the proteins Hyc E, G, F, D, and C of the hydrogenase 3 part of the *E. coli* formate hydrogenlyase system (Sauter *et al.*, 1992; Böhm *et al.*, 1990; Weiss *et al.*, 1991; Fearnley and Walker, 1992). The TYKY/NUO9/Hyc E subunits are ferredoxin-type FeS proteins, the PSST/NUO2/Hyc G subunits are possible candidates of being iron-sulfur proteins, and the subunits ND1/NUO8/Hyc F are suggested to contain the ubiquinone-binding site. It might turn out that these five subunits constitute another functional module, namely the distal section of the electron pathway and the electron output site. In terms of function, one could then define three parts of complex I. One part would be located exclusively peripherally and comprise the three subunits which contribute the electron input site and the proximal section of the electron pathway. This part is related with the diaphorase part of the NAD^+-reducing hydrogenase of *A. eutrophus*. The second part would be located partly peripherally and partly membrane intrinsic and constitutes the distal section of the electron pathway and the electron output to ubiquinone. This part would comprise the five subunits related to the hydrogenase 3 of the *E. coli* formate hydrogenlyase. The third and most hypothetical part of the remaining "minimal" subunits (see Table I) would be purely membrane intrinsic. This part could be involved in proton translocation. Since this third part probably does not contain redox groups, proton translocation could then be driven by conformational changes.

This subdivision of complex I would be different from the subdivision discussed above. It would not follow the order of the genes in the *E. coli* or *P. denitrificans* gene cluster. Compared to the model discussed above, the peripheral part of the complex would contain the electron input section and the membrane part the proton-translocation machinery. Both functional parts are linked by the electron output section which is found partly in the peripheral and partly in the membrane part.

EVOLUTION OF COMPLEX I FROM PREEXISTING ENZYMES

The subdivision of complex I discussed in this review suggests that complex I has arisen not by the accretion of the many subunits one by one, but by the association of preexisting subunit-complexes *en bloc*. Complete sections of the electron pathway and the device(s) of proton translocation would thus have evolved independently as separate structural modules and then come together to form the complex (Weiss *et al.*, 1991; Walker, 1992). This is further underlined by the fact that the pre-assembled membrane and peripheral part of complex I are completely equipped with their known redox groups before they unify to the fully assembled complex I. Such a modular evolution has also been discussed for the proton-translocating ATPase (Walker and Cozens, 1986). It has long been known that this enzyme consists of two structurally and functionally distinct parts, the peripheral F1 concerned with ATP synthesis and the membrane intrinsic F_0 concerned with proton translocation.

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