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The 49-kDa subunit of NADH-ubiquinone oxidoreductase (Complex I) is involved in the binding of piericidin and rotenone, two quinone-related inhibitors

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Abstract Piericidin is a potent inhibitor of the mitochondrial and bacterial type I NADH-ubiquinone oxidoreductases (Complex I) and is considered to bind at or close to the ubiquinone binding site(s) of the enzyme. Piericidin-resistant mutants of the bacterium *Rhodobacter capsulatus* have been isolated and the present work demonstrates that a single missense mutation at the level of the gene encoding the peripheral 49-kDa/NUOD subunit of Complex I is definitely associated with this resistance. Based on this original observation, we propose a model locating the binding site for piericidin (and quinone) at the interface between the hydrophilic and hydrophobic domains of Complex I.

Key words: Complex I; Mitochondrion; Piericidin; Rotenone; Ubiquinone; *Rhodobacter capsulatus* mutant

1. Introduction

The NADH-ubiquinone oxidoreductase (Complex I, EC 1.6.5.3) catalyzes the first step of oxidative phosphorylation, i.e. the oxidation of NADH by ubiquinone. Complex I couples this oxidoreduction reaction with an active proton transport across the mitochondrial inner membrane or the bacterial cytoplasmic membrane. In the case of bovine Complex I, this oligomeric enzyme is composed of up to 43 different subunits [1] assembled in two major domains. The peripheral domain is nuclearly encoded and bears the catalytic NADH binding site. The membranous domain is mainly constituted of the 7 so-called ND subunits (ND1, ND2, ND3, ND4, ND4L, ND5 and ND6) encoded by the mitochondrial genome or the equivalent NUOH-NUON plus NUOB subunits in the bacteria. The most recent enzymatic models attribute a key role to bound ubiquinones distinct from the substrate quinone in proton transport processes carried out by Complex I [2–4]. Many inhibitors targeting the Complex I are considered to bind at, or close to, the quinone binding sites (for review see [5]). Photoreactive analogues of the classical Complex I inhibitor rotenone were found to label ND1 [6,7] but the definite location of the quinone binding site remains to be ascertained. Genetic studies of inhibitor-resistant mutants have proved an efficient and reliable way to map the quinone binding sites of other quinone-dependent enzymes, such as the photosynthetic reaction center and the cytochrome bc_1 complex of photosynthetic purple bacteria [8,9], as has been confirmed by recent crystallographic studies [10,11]. In the specific case of Complex I, pathogenic mutations of the human ND1 or ND4 genes have been reported to slightly alter both the interaction of Complex I with quinones and its sensitivity to rotenone [12,13]. This pointed to the subunits ND1 and ND4 as potential candidates for the binding of quinone and rotenone. In the eukaryotic system, these subunits are mitochondrially encoded, which severely limits the possibility for genetic studies of inhibitor and quinone binding sites. Human and *Drosophila* cell lines resistant to rotenone have been isolated in vitro [14,15] but their resistance did not appear to be related to Complex I. On the other hand, taking advantage of the efficiency of bacterial genetics, we isolated *Rhodobacter capsulatus* mutants which synthesize a piericidin- and rotenone-resistant Complex I [16]. In the present report, we demonstrate that this resistance results from a mutation in the bacterial NUOD subunit equivalent to the hydrophilic 49-kDa subunit of mitochondrial Complex I. This unexpected result appears to contradict the data presented above that suggested a localization of the rotenone and piericidin binding sites at the level of the ND subunits. However, this apparent discrepancy can be rationalized in a model locating the piericidin binding site at the interface between the peripheral and membranous domains of Complex I.

2. Materials and methods

The mutants of *R. capsulatus* resistant to piericidin are named Pi^RA, Pi^RB, Pi^RC and Pi^RD [16] and were derived from strain W1 [17]. This strain was obtained from the wild-type strain B10 by silent insertion of the KIXX kanamycin resistance cartridge in the intergenic region between *nuoI* and *nuoJ* [17]. In the present work, the *nuoA-D* region from strain B10 was cloned and sequenced using manual techniques previously described [18]. The *nuo* operon of the Pi^RC mutant strain was sequenced after amplification by PCR, using the automated sequencing facility of Genome Express SA (France). Automated re-sequencing of the previously characterized *nuoH-nuoN* region of wild-type *R. capsulatus* (B10) *nuo* operon revealed a single discrepancy with the manually determined sequence [18] (an additional G should be added to a track of 3 Gs within the *nuoL* gene). This correction has been entered in the sequence of the entire *R. capsulatus nuo* operon we deposited in GenBank (GenBank accession no. AF029365).

To check for the causality of the mutation NUODV407M with respect to piericidin-resistant phenotype, the mutated *nuoD* gene from Pi^RC mutant was reintroduced into an independent wild-type background by homologous recombination. For this, a *Bam*HI-*Xho*I fragment (5868 bp long) containing the wild-type *R. capsulatus nuoA-E* genes (Fig. 1) was cloned into suicide plasmid pPHU281 [19]. The resulting construction (pJP7DB) presents two *Bgl*II restriction sites

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Abbreviations: bp, nucleotide base pair; IC_{50}, 50% inhibitory concentration; Myx^R, myxothiazol resistant; ND, mitochondrially encoded subunit of the mitochondrial Complex I; *nuo*/NUO, gene/subunit of the bacterial Complex I; Pi^R, piericidin resistant

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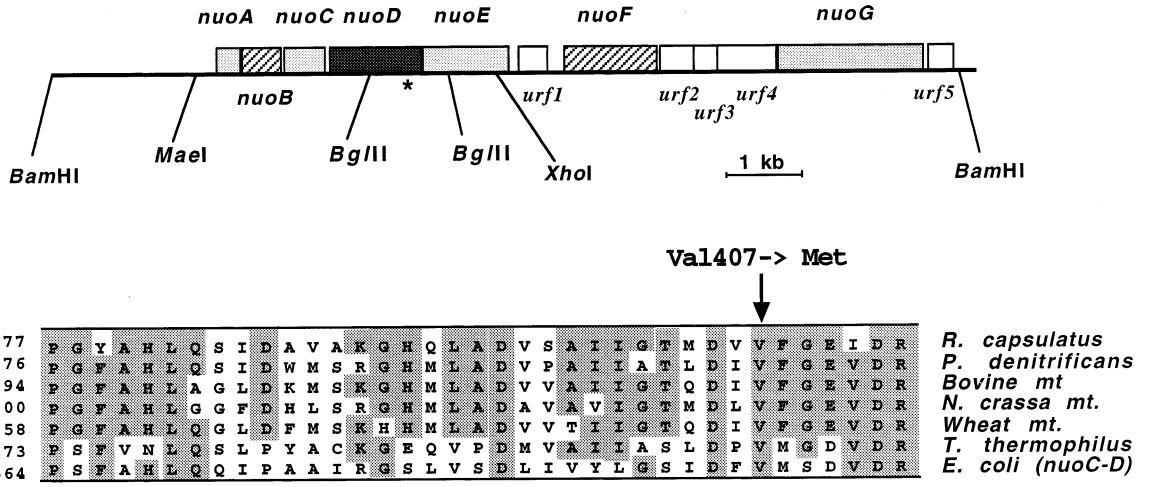

Fig. 1. Structure of the *nuoA-nuoG* part of the *R. capsulatus* operon and localization of the NUODV407M mutation. Top: Map of the 5′ region of *R. capsulatus nuo* operon. This region (from the *Mae*I restriction site to the previously identified *Bam*HI site) has now been completely sequenced (this work, GenBank accession no. AF029365). The *nuoH-nuoN* region of the *R. capsulatus nuo* operon [18] is not represented and starts at the *Bam*HI site indicated at the right of the present map. The mutation NUODV407M is located at the extreme 3′ end of *nuoD* gene (position indicated by a star). The *Bam*HI (left site) and *Xho*I restriction sites delimiting the insert of plasmid pJP7DB are indicated, as well as the two *Bgl*II restriction sites present in this insert. Bottom: Alignments of the peptidic sequences of the carboxy-terminus of NUOD/49-kDa subunits from different origins: *R. capsulatus* NUOD (this work); *P. denitrificans* NQO4 (accession number: P29916); bovine 49 kDa (accession number: P17694); *N. crassa* 49 kDa (accession number: P22142); wheat mitochondrial 49 kDa (accession number: S53862); *Thermus thermophilus* NQO4 (accession number: Q56220); *E. coli* NUOCD (accession number: AE000317). Shaded blocks correspond to residues conserved in at least 5 out of the 7 aligned sequences. $Val^{407} \rightarrow$ Met mutation observed in Pi^R mutants is indicated by an arrow.

delimiting a 1013-bp restriction fragment that contains the 3′ part of the *nuoD* gene and the 5′ part of *nuoE* (Fig. 1). The wild-type 1013-bp *Bgl*II fragment was replaced by the equivalent fragment amplified by PCR from Pi^RC genomic DNA giving plasmid pED16. The plasmid pED16, or the control plasmid pJP7DB, were transferred into B10 by biparental conjugation as previously described [17]. After conjugational transfer, the cells were spread onto a selective medium to screen for piericidin-resistant clones. As direct selection on piericidin containing medium is not stringent enough to select for piericidin-resistant mutants, we used a two-step screening strategy [16]. Conjugated cells were first spread on a solid medium containing 2.5 μM piericidin-A and 10 μM myxothiazol and incubated under aerobic conditions. These conditions allow the growth of *R. capsulatus* colonies resistant either to piericidin or to myxothiazol. Myxothiazol totally inhibits photosynthetic growth of *R. capsulatus* by blockade of cyclic photosynthesis at the level of the bc_1 complex, so we could then easily discriminate myxothiazol-resistant mutants from piericidin-resistant mutants [16].

All other biochemical and microbiological procedures used in the present publication were as previously described [16,17].

3. Results

We previously isolated piericidin-resistant mutants (Pi^R) of *R. capsulatus* displaying a decrease in piericidin sensitivity of at least 30 times in comparison to strains W1 or B10 [16]. The maximal NADH oxidase activities of the mutants were slightly decreased (about 30%) whereas their deamino-NADH-ferricyanide oxidoreductase activities were identical to those of the wild-type enzyme [16]. This suggested that piericidin resistance was associated with a rather limited alteration in the quinone binding region of the bacterial Complex I. Piericidin-resistant mutants also appeared to be resistant to rotenone. In the literature, the mitochondrial subunits ND1 and ND4 are considered as the best candidates for the location of the quinone and rotenone binding sites. However, no mutation was identified either in *nuoH* and *nuoM* equivalent genes, or in the remaining 3′ region of the *nuo* operon from the mutants Pi^RA, Pi^RB and Pi^RC. This prompted us to analyze the 5′ region of *R. capsulatus nuo* operon.

This region of the *R. capsulatus nuo* operon (*nuoA-nuoG*) has only been partially characterized so far [20,21]. To locate the genetic alteration associated with piericidin resistance, we undertook the complete sequencing of the 5′ regions of the Pi^RC and wild-type *nuo* operons, corresponding to *nuoA-G* genes (Fig. 1) (10 376 bp). Except for an extension of the *nuoE* gene and some divergences in the supernumerary open reading frames previously noted [20,21], this 5′ region of the *nuo* operon is highly homologous to the *P. denitrificans nqo* operon [22]. In contrast to *E. coli*, in which *nuoC* and *nuoD* are fused [23], the *R. capsulatus* and *P. denitrificans nuoC* and *nuoD* genes encode two separate proteins, which are related to subunits 30 kDa and 49 kDa of mitochondrial Complex I. The comparative sequencing of the wild-type (B10) and Pi^RC *nuo* operons allowed the identification of a unique $G \rightarrow A$ transition at position 1219 of the *nuoD* gene of Pi^RC (Fig. 1). This missense mutation changes the valine residue at position 407 of *R. capsulatus* NUOD subunit into a methionine (mutation NUODV407M).

The NUODV407M mutation introduces an additional *Nla*III restriction site within the *nuoD* gene. This allowed us to design a direct restriction site-PCR test for the presence of the mutation. A 111 bp long fragment encompassing position 1219 of the *nuoD* gene was generated by PCR using primers IJPR2 and IJPR9. When digested with *Nla*III PCR products presenting the NUODV407M mutation are cleaved into two fragments 87 bp and 24 bp long, respectively, whereas the wild-type PCR product remains uncleaved. Restriction analysis of the PCR products thus demonstrated the presence of the mutation in mutants Pi^RA, Pi^RB and Pi^RD and its absence in B10, W1, and myxothiazol resistant mutants (Myx^R) isolated in the same experiment as the Pi^R mutants (Fig. 2).

To confirm the effect of the identified NUODV407M muta-

tion, this mutation was reintroduced into an independent genetic background, i.e. the parental B10 strain. For this purpose, we first constructed a suicide plasmid (pJP7DB) containing the 5′ region of the B10 *nuo* operon from *nuoA* to *nuoE* (see Section 2). In a second step, this construction was modified by replacement of the 1013-bp *Bgl*II restriction fragment encompassing the 3′ end of *nuoD* (see Fig. 1) by the mutated *Bgl*II fragment issued from the $Pi^{R}C$ genome. This construction was called pED16. Following conjugational transfers of pJP7DB and pED16 plasmids into B10 strain, the *R. capsulatus* conjugates were plated on solid medium containing both myxothiazol and piericidin under aerobic dark growth conditions as previously described [16]. This screening allows for the isolation of mutants resistant to either myxothiazol or piericidin. Applying this screening procedure to bacteria issued from the conjugation with the pJP7DB construction led to the appearance of few clones, and all these clones proved myxothiazol resistant. On the contrary, more than 80% of the clones issued from pED16 conjugation proved

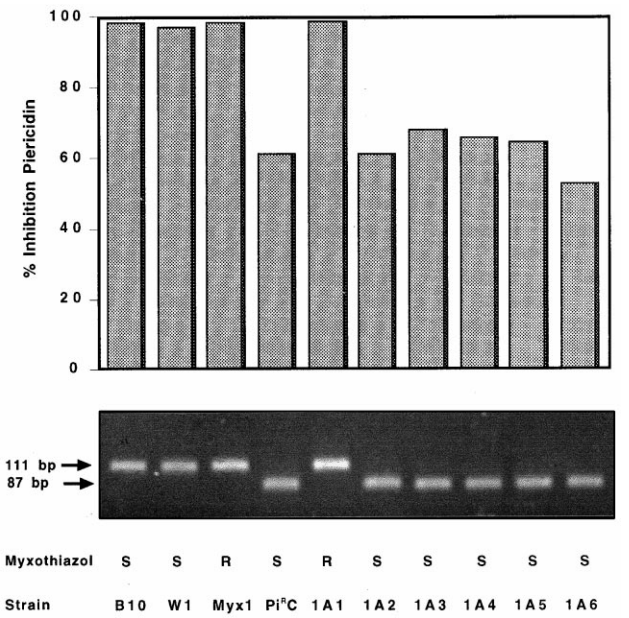

Fig. 2. Reconstruction of NUODV407M mutation in the parental B10 strain. Mutation NUODV407M was reintroduced into the B10 background by homologous recombination. Genotypes and phenotypes of recombinant clones 1A1–1A6 were analyzed in comparison to the different reference and parental strains (B10, W1, Myx1, $Pi^{R}C$) used in this study. From bottom to top: line 1: Names of the different strains. Line 2: Myxothiazol resistance phenotype; the letters R/S stand for myxothiazol resistant/sensitive phenotype when grown under anaerobic photosynthetic conditions in the presence of this inhibitor. Lower panel: Restriction site-PCR characterization of *nuoD* gene. A 111-bp long fragment encompassing NUODV407M mutation was generated by PCR using primers IJPR2 (ATCATCGGCACGATG) and IJPR9 (CCCAGGCGCGGTTGGC). When digested with the restriction enzyme *Nla*III, PCR products presenting the NUODV407M mutation are cleaved into 2 fragments of 87 bp and 24 bp (the 24-bp fragment is not observable in the present picture) whereas the wild-type product remains uncleaved (111 bp). Upper panel: Piericidin sensitivity of Complex I in the different strains. Membranes of mutants and parental *R. capsulatus* were characterized by oxygraphy. The NADH dependent respiration was measured in the absence or presence of 12 nM piericidin. This piericidin concentration was chosen as it resulted in a total inhibition of respiration for membranes of B10, W1 and Myx1 (50% inhibitory concentration (IC_{50}) = 0.2 nM) but induced only about 60% inhibition of the respiration of $Pi^{R}C$ mutant (IC_{50} = 6 nM).

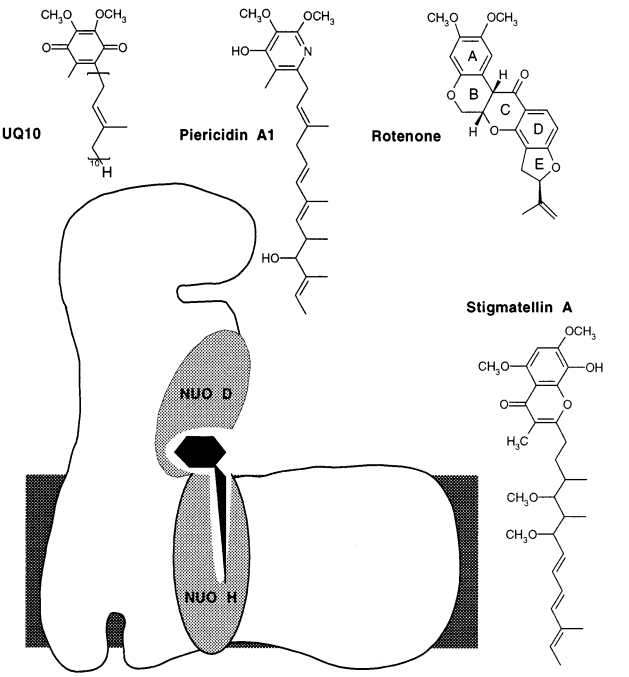

Fig. 3. Model for the organization of the piericidin/rotenone binding site at the interface between membranous and peripheral domains of Complex I. This model represents the possible contribution of NUOD (49 kDa) and NUOH (ND1) subunits in the organization of the piericidin binding site. The NUOD subunit has been represented on the internal side of the L-shaped Complex I to comply with the localization from electron-microscopic studies [5]. Bound piericidin or ubiquinone is schematized as a black molecule.

piericidin resistant as illustrated in Fig. 2 for six randomly picked clones (clones 1A1 to 1A6). Among these clones only clone 1A1 remained sensitive to piericidin and displayed myxothiazol resistance. Using the restriction site-PCR test described above, we demonstrated that all the clones except clone 1A1 had actually integrated the NUODV407M mutation (Fig. 2). Restriction site-PCR further demonstrated that piericidin resistance was associated with a true gene replacement as only the mutated copy of *nuoD* gene (i.e. the copy presenting the *Nla*III site) was observed in piericidin-resistant mutants (Fig. 2). Thus, we observed a perfect correlation between the appearance of piericidin resistance and the transfer of NUODV407M mutation.

4. Discussion

The NUOD/49-kDa subunit is associated with the peripheral domain of Complex I [24]. An electron microscopy study of *N. crassa* Complex I using an Fab fragment directed against the 49-kDa subunit for immunodecoration indicated that this protein is located in the vicinity of the membrane domain [25]. Quinones and inhibitors are usually considered to bind to two binding sites located within the membrane domain of Complex I [5–7,12,13]. However, quinones are amphiphatic molecules presenting a polar cyclic head and a hydrophobic isoprenyl tail. Identification of a mutation in the *nuoD* gene associated with piericidin resistance demonstrates the necessity for a critical reappraisal of the location of Complex I quinone and inhibitor binding sites in the light of the structural data available for other quinone dependent en-

zymes. The 2.45 Å resolution structure of Q_A and Q_B quinone binding sites of the reaction center of photosynthetic bacteria suggests that quinone binding sites are located at the interface between the membranous domain and the cytosolic domain of quinone binding enzymes [10]. The 'quinone ring pocket' of the Q_B site is located at the interface between the transmembranous L subunit and the cytosolic H subunit of the reaction center whereas the isoprenyl motif is diving into the membranous part of subunit L [10]. In this regard, it is interesting to note that the reaction center inhibitor stigmatellin essentially occupies the 'quinone ring pocket'. It is also worth noting that mutations associated with stigmatellin resistance precisely target essential residues of this pocket [8,10]. On the other hand, one molecule of lauryl dimethyl aminoxide (LDAO) binds to the Q_B 'isoprenyl subsite' of the reaction center crystallized in the absence of added quinone [10]. In the case of bc_1 complex, crystallographic data also revealed that the inhibitors myxothiazol and antimycin, known to target, respectively, the Q_P and Q_N ubiquinone binding sites of the bc_1 complex, bind close to the interface between the membrane and the peripheral parts of the enzyme [11]. Here again, the efficiency and reliability of genetic studies of inhibitor resistant mutants for the mapping of quinone/inhibitor binding sites was validated by crystallographic data [9,11]. By analogy with the structure of the Q_B site of the reaction center, it can be proposed that each quinone binding site of Complex I would be constituted of a polar 'quinone ring' subsite and a membranous 'isoprenyl tail' subsite. Along this line, the different inhibitors of Complex I should be classified as inhibitors targeting preferentially the polar subsite(s) and inhibitors targeting preferentially the hydrophobic part of the quinone sites. This rationale would explain the great structural diversity and the observed partial competition between inhibitors of Complex I. The Complex I situation would parallel the observations made on the Q_P site inhibitors stigmatellin and methoxyacrylates in the case of the bc_1 complex. The different behavior of stigmatellin and methoxyacrylate derivatives appears to result from differential interaction with, respectively, the 'quinone ring'- and 'isoprenyl tail'-binding regions of the Q_P site [27].

Among the numerous inhibitors of Complex I, piericidin is one of the most potent. It displays a very high affinity for mitochondrial and bacterial Complexes I and is considered to be a direct antagonist of quinones [5,28]. As noted previously [29], the overall chemical structure of piericidin is clearly related to that of ubiquinones: it presents a polar heterocycle similar to the ubiquinone ring with a branched aliphatic chain reminiscent of the isoprenyl motif of ubiquinones (Fig. 3). Semiquinones have long been observed in association with Complex I and are postulated to take part in the energetic coupling between proton and electron fluxes [3,30–32]. In this regard, it is interesting to note that, like stigmatellin [10], piericidin can be viewed as an analogue of semi-ubiquinone. Similarly to stigmatellin in the case of the reaction center and the bc_1 complex, piericidin would thus essentially target the 'quinone ring'-binding subsite(s) of Complex I. Accordingly, the identification of the NUODV407M mutation characterized here constitutes the first direct evidence for the participation of the C-terminal part of the NUOD subunit in the structure of a 'quinone ring' subsite of Complex I. As our mutants are resistant both to rotenone and piericidin, this subsite would be shared by rotenone, piericidin and quinone. The carboxy-terminus of NUOD is remarkably conserved, both in similarity and length, between higher eukaryotes and bacteria (Fig. 1). Interestingly, Complex I of *E. coli* is relatively insensitive to piericidin and rotenone [28] and the enzymes of both *E. coli* and *T. thermophilus* are reported to preferentially use menaquinone instead of ubiquinone as substrate [33,34]. This may be tentatively associated with the fact that, although Val^{407} is conserved in *E. coli* NUOCD and *T. thermophilus* NQO4, the carboxy-termini of these proteins are only weakly homologous with those of mitochondria, *R. capsulatus* and *P. denitrificans* (Fig. 1).

The dimethoxy derivatized ring A of rotenone appears structurally related to the quinone head of ubiquinone (Fig. 3). Structure-function relationship studies of rotenone analogues suggest that the A-B cycles of rotenone mimic the quinone ring of ubiquinone [35]. Along this line, the C-D-E cycles must functionally correspond to the hydrophobic 'isoprenyl tail' of ubiquinone. Both arylazidomorphigenin and dihydrorotenone photochemically reactive groups are located at the level of the E cycle and must therefore react essentially with the peptides lining the 'isoprenyl-tail' binding subsite. Thus, photolabelling with these rotenones actually points to the ND1 subunit as a candidate subunit for the 'isoprenyl-tail' binding subsite [6,7]. It is well known that an isolated peripheral fragment of Complex I devoid of all the ND subunits is still able to catalyze NADH-quinone oxidoreduction, especially with short-tailed quinones [24,26]. This last observation is consistent with the idea that ubiquinone binding sites are actually located at the interface between membranous and peripheral domains of Complex I. The overall organization of the piericidin/quinone binding site can therefore be proposed to be constituted of a hydrophobic pocket borne by the ND1/NUOH subunit and a polar head borne by the 49-kDa/NUOD subunit as illustrated in Fig. 3.

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