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Toward a Characterization of the Connecting Module of Complex I

Alain Dupuis,[1,2] Isabelle Prieur,[1] and Joël Lunardi[1]

Complex I [NADH–ubiquinone oxidoreductase (complex I, EC 1.6.5.3)] couples electron transfer between NADH and ubiquinone to proton transport across the bacterial cytoplasmic membrane and the mitochondrial inner membrane. This sophisticated enzyme consists of three specialized modules: (1) a hydrophilic NADH-oxidizing module that constitutes the input machinery of the enzyme; (2) a hydrophobic module that anchors the enzyme in the membrane and must take part in proton transport; and (3) a connecting domain that links the two previous modules. Using the complex I of *Rhodobacter capsulatus*, we developed a genetic study of the structure and function of the connecting module. In the present review, we put together the salient results of these studies, with recent reports of the literature, to try and elucidate the structure of the connecting module and its potential role in the coupling process between electron and proton flux within complex I. From this overview, we conclude that the NUOB–NUOD dimer of the connecting module and a hydrophobic subunit such as NUOH must share a quinone-reduction site. The function of this site in the mechanism of complex I is discussed.

KEY WORDS: Complex I; NADH–ubiquinone oxidoreductase; *nuo* operon; quinone; piericidin; rotenone; *Rhodobacter capsulatus*; Fe–S; NiFe; hydrogenase.

INTRODUCTION

The complex I (type I NADH–ubiquinone oxidoreductase, EC 1.6.5.3) is a ubiquitous enzyme present both in the inner membrane of mitochondria and in the cytoplasmic membrane of numerous bacteria. In mitochondria, this enzyme couples the transfer of two electrons from NADH to ubiquinone with the active transport of four protons across the membrane. The oligomeric composition of complex I ranges from 13 to 14 subunits for the bacterial enzyme to up to 43 different subunits for the bovine mitochondrial complex I (Fig. 1). However, this great variability contrasts with the remarkable conservation of the characteristic "L"-shaped quaternary structure of this enzyme (Friedrich, 1998). The membrane embedded arm of the "L" is a module constituted mainly of seven hydrophobic proteins called subunits NUO-H, -N, -A, -M, -K, -L, and -J in bacteria, such as *Rhodobacter capsulatus* and, respectively, subunits ND1, ND2, ND3, ND4, ND4L, ND5, and ND6 in mitochondria (Fig. 1). The protruding arm, pointing out of the membrane, is considered to contain all the identified prosthetic groups of complex I except quinones. It is composed of two modules: (1) the first one, the NADH-oxidizing unit, is composed of subunits NUO-E, -F, -G in the bacterial enzyme (subunits 24, 51, and 75 kDa in the bovine enzyme). It contains at least one FMN and four [Fe–S] clusters and is able to catalyze the oxidation of NADH by artificial electron acceptors like ferricyanide or hexammine ruthenium (III). (2) The second module is constituted of four proteins, namely, NUO-B, -C, -D, and -I in bacteria (equivalent subunits are PSST, 30 kDa, 49 kDa, and TYKY in mitochondria). It connects the NADH-oxidizing unit to the membrane arm (Fig. 1).

Complex I contains both hydrogen carriers (FMN and ubiquinone) and electron carriers ([Fe–S] clusters). According to a classical loop mechanism, the association of these two types of cofactors allows a net transport of protons resulting schematically from the transfer of

[1] Laboratoire de Bioénergétique Cellulaire et Pathologique (EA 2943-UJF), Département de Biologie Moléculaire et Structurale, CEA Grenoble, 17 Rue des Martyrs, 38054 Grenoble Cedex 9, France.

[2] To whom all correspondence should be addressed. e-mail: adupuis@cea.fr

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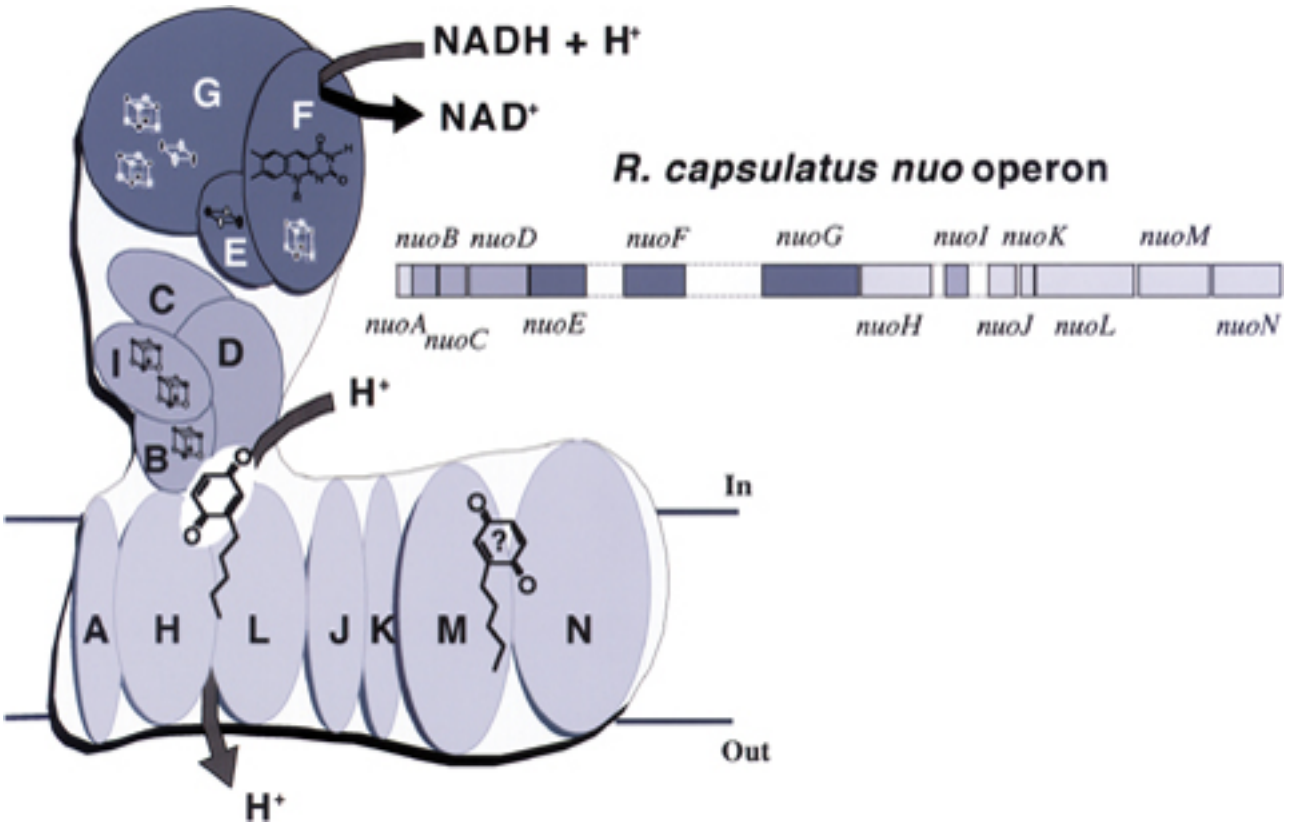


Fig. 1. The complex I of *R. capsulatus*. Both in the operon and in the schematic structure of the enzyme, the subunits of the NADH-oxidizing domain (NUOE, -F, -G) are drawn in dark gray. The subunits comprised in the connecting fragment (NUOB, -C, -D, -I) are in medium gray and the subunits located in the membrane-embedded arm (NUOA, -H, -J, -K, -L, -M, and -N) are in light gray. The *nuo* operon of *R. capsulatus* is 18.3 kbp long. Location of the first quinone-binding site at the interface between NUOB and NUOD subunits is discussed in this review. No data shall be presented about the location of a putative second quinone-binding site.

two hydrogens across the membrane and the consecutive return of two electrons. However, this mechanism would account for a stoichiometry of only two protons transported per molecule of NADH oxidized. Thus, complex I must function according to a more sophisticated mechanism (Vinogradov, 1993; Degli Esposti and Ghelli, 1994; Dutton *et al.*, 1998; Brandt, 1999). The NADH-oxidizing unit, which is the best understood part of the enzyme, appears to be a kind of interchangeable input module (see below) and is not directly involved in the coupling mechanism of complex I (see Friedrich and Scheide, 2000). At the other edge, the membrane module has to be involved in vectorial proton transfer, but its hydrophobic nature and the absence of any associated enzymic activity have restrained its characterization. Finally, although the connecting module has no measurable catalytic activity, the presence of two to three [Fe–S] clusters and its clear phylogenetic relation with multisubunits [NiFe] hydrogenases (Böhm *et al.*, 1990) point to this module as a strategic crossroad between the electron and proton avenues.

Using the bacterium *R. capsulatus*, we developed a genetic study of the connecting module. This review surveys the salient points of this genetic study. It especially develops original evidence for the direct role of subunit NUOD in the binding of quinone. We propose that a quinone-reduction site must be located at the interface between subunits NUOD and NUOB of complex I. The putative contribution of this reduction site to the mechanism of complex I is discussed.

GENETIC STUDIES OF *Rhodobacter capsulatus* COMPLEX I

Identification of the Complex I Encoding Genes in *Rhodobacter capsulatus*

The presence of a NADH-dehydrogenase related to mitochondrial complex I in the bacterium *R. capsulatus* was reported in the early 1970s (Baccarini Melandri *et al.*,

1973). However, due to its instability, the enzyme proved very difficult to isolate (Duborjal *et al.*, 1997; Herter *et al.*, 1997). In fact, most of our knowledge on this enzyme came from the characterization of the *nuo* operon (Dupuis, 1992; Dupuis *et al.*, 1995; Duborjal *et al.*, 1997; Herter *et al.*, 1997). This operon is 18345 bp long and is composed of fourteen *nuo* genes encoding the fourteen subunits of *R. capsulatus* complex I. Its use for different genetic studies has been extensively presented in a recent review (Dupuis *et al.*, 1998b) and, thus, shall be surveyed only briefly in the present publication.

Genetic disruption of the *R. capsulatus nuoG, -H, -I, -J, -K, -L, -M, -N* individual genes, and of the *nuo*(*B-C-D*) cluster showed that any of the corresponding protein is essential for the assembly of an active complex I in *R. capsulatus* (Dupuis *et al.*, 1997, 1998a; Chevallet *et al.*, 1997 and unpublished data). As complex I is the first component of the respiratory chain, a complex I deficiency was expected to prevent the growth of *R. capsulatus* under dark aerobic conditions using a fermentable carbon source. Therefore, we intended to take advantage of the ability of *R. capsulatus* to grow photoheterotrophically under anaerobic conditions to bypass this drawback. However, to our surprise, none of the complex I-deficient mutants could grow under anaerobic photoheterotrophic growth conditions using either lactate, malate, or succinate as a carbon source. The single apparent exception to this phenotype reported so far was due to the fact that the B10A$^-$ mutant used by the authors was not a true disruption mutant. In fact, it resulted from a single crossover event that can restitute a normal copy of the corresponding *nuoF* gene (Herter *et al.*, 1998). This inability to grow photoheterotrophically under anaerobiosis could be explained by the requirement of the coupled complex I to ensure a reverse flow of electrons from quinol to NAD^+ under these metabolic conditions (Dupuis *et al.*, 1997, 1998b). To assess this hypothesis, we recently transcomplemented mutants disrupted in either *nuo*(BCD) or NUOH genes with a construct expressing *Escherichia coli* type II NADH dehydrogenase under the control of the conditional *R. capsulatus pFru* promotor. Under physiological conditions, this uncoupled enzyme can catalyze the exergonic NADH oxidation by ubiquinone but, contrary to complex I, it is unable to catalyze the reverse reaction. High levels of rotenone-insensitive type II NADH–dehydrogenase could be induced in the membranes of the complemented mutants. However, this uncoupled enzyme could hardly restore the ability of the mutants to grow under anaerobiosis (unpublished data). Thus the deficient phenotype of our mutants under photosynthetic anaerobic conditions can be used as a good physiological index to evaluate the energy coupling of altered complex I.

On the contrary, all our mutants can grow under dark aerobic conditions with a nonfermentable substrate like lactate. This second phenotypic feature was explained by the identification of a membrane-bound NAD-independent lactate dehydrogenase (iLDH) in *R. capsulatus* (Dupuis *et al.*, 1997; see also Dupuis *et al.*, 1998b). Unlike *E. coli*, *Paracoccus denitrificans* and *R. capsulatus* are naturally devoid of the type II NADH dehydrogenase. This potentially simplifies biochemical and enzymic studies. However, the absence of an alternative metabolic pathway is a serious limitation for genetic studies in *P. denitrificans* (Finel, 1996). In this regard, due to the presence of its iLDH, *R. capsulatus* is a privileged model for genetic studies of complex I.

As discussed in the introduction, the magic of complex I energy coupling must take place in the membrane and connecting modules of the enzyme. In an effort to investigate this coupling mechanism, we mainly followed two directions: (1) the mutagenesis study of the connecting [Fe–S] subunit NUOI and (2) the genetic study of the quinone-binding sites of complex I.

Mutagenesis Studies of the [Fe–S] Subunit NUOI

Among the six [Fe–S] clusters identified by electron paramagnetic resonance (EPR) studies of mitochondrial complex I, five centers (N1a, N1b, N3, N4, N5) were associated with the NADH-oxidizing input module and seemed to have little contribution in the coupling mechanism (for review, see Ohnishi, 1998). The remaining N2 [4Fe–4S] cluster is located in the connecting module of complex I. Its relatively high midpoint potential and the sensitivity of its EPR signature to piericidin, a specific inhibitor targeting quinone-binding sites of complex I, designate N2 as the direct reductant of ubiquinone. Furthermore, the N2 [Fe–S] cluster presents a pH-dependent midpoint potential. Thus the reduction/oxidation of N2 cluster is associated with protonation/deprotonation events that may be essential for proton transport across the membrane. Based on sequence data, two subunits of the connecting module, subunits NUOI and NUOB, can potentially harbor the N2 center.

NUOI subunit (TYKY in mitochondria) presents two canonical motifs [C64-$(X)_2$-C67-$(X)_2$-C70-$(X)_3$-C74-P and C103-$(X)_2$-C106-$(X)_2$-C109-$(X)_3$-C113-P (*R. capsulatus* numbering used as default numbering in the following, unless otherwise stated)] for the insertion of two [4Fe–4S] iron–sulfur clusters. From the crystallographic structure of the highly related bacterial two [4Fe–4S] ferredoxins (Adman *et al.*, 1973), it can be proposed that C64, C67, C70, and C113 coordinate the first cubane

cluster, whereas C103, C106, C109, and C74 coordinate the second. By direct gene replacement, we converted cysteines C70, C74, and C106 into serines (mutants C70S, C74S, C106S) and the neighboring aspartate residue E71 into lysine (E71K). The charge inversion at the level of aspartate E71 was introduced to assess the putative contribution of this protonable group in an eventual redox driven proton transport (see above). We also used episomal complementation of a Δ*nuoI* strain to generate mutations C64S, C64R, C67S, and C67R. Western blot analyses indicated that the NUOI subunit was virtually missing in the membranes of mutants C64R, C64S, and C67R, but was present in low amounts in strains C70S and C74S. Finally, normal amounts of this subunit were found in the membranes of strains C67S, E71K, and C106S. These three mutants still presented a NADH-dependent respiration ≥30% of the wild-type bacterium. The ability of mutant E71K to grow under anaerobic conditions and the qualitative demonstration of complex I-dependent energization of the corresponding membranes suggested that the presence of residue E71 is not mandatory for complex I energy coupling. Spectroscopic analyses of the mutants showed a decrease in the N2 EPR signal, relative to the N1 signal (Chevallet *et al.*, manuscript in preparation). This effect reveals an alteration of the N2 center in relation to NUOI modification and thus suggests that the N2 EPR signal may be assigned to the two [Fe–S] clusters of NUOI subunit.

The alternative subunit, which could house the N2 center, NUOB (PSST in mitochondria), is remarkably related to the N-terminal domain of the small catalytic subunit of [NiFe] hydrogenases (Albracht, 1994). This domain can ligate one [4Fe–4S] cluster. In the related NUOB subunit of complex I, only three of the four cysteine residues coordinating this [4Fe–4S] cluster are conserved. Thus far, the putative supplementary ligand required to bind the fourth iron atom of a putative [4Fe–4S] cluster in NUOB is still not characterized (Ohnishi, 1998; Ahlers *et al.*, 2000). Alternatively, from structural comparisons between the N-terminus domain of the small subunit of *Desulfovibrio gigas* [NiFe] hydrogenase and flavodoxin, Albracht and Hedderich recently proposed that the region of NUOB putatively involved in the coordination of a [4Fe–4S] cluster might actually take part in the binding of a second FMN on complex I (Albracht and Hedderich, 2000). In our mind, this would not be compatible with the presence of a [Fe–S] cluster in this subunit. Mutations C64A and C129A, altering two of the conserved cysteines in the *E. coli* NUOB, have been reported to specifically suppress the EPR signal of cluster N2 (Friedrich, 1998). The same authors suggested that the [Fe–S] centers of NUOI are EPR silent (Friedrich *et al.*, 2000). However, the overexpressed NUOI subunit displays clearly identifiable [4Fe–4S] EPR signals (Yano *et al.*, 1999).

From the crystal structure of the phylogenetically related hydrogenases (Volbeda *et al.*, 1995), it can be predicted that the NUOB and NUOI subunits must be in close interaction (see Fig. 4 and discussion below). As discussed by Ahlers *et al.* (2000), any structural alteration of one of these subunits may have drastic side effects on the properties of the prosthetic groups borne by its neighbor. Thus, the definitive location of the N2 center should await a better refinement of the existing data.

Characterization of the Quinone-Binding Sites of Complex I

Interaction studies of quinones with quinone-binding enzymes are often limited by the hydrophobicity, as well as the relative low affinity and poor specificity of these molecules for their binding sites. On the other hand, crystallographic studies of the reaction center (RC) of photosynthetic bacteria and of the bc_1 complex have clearly confirmed the long-made assumption that most inhibitors of these enzymes are actually addressing their quinone-binding sites (Lancaster and Michel, 1997; Xia *et al.*, 1997). This validates the accepted idea that most of the many inhibitors of complex I are reliable tools to probe its quinone-binding site(s) (for review see Degli Esposti, 1998). In the work presented below, we preferentially used three of these inhibitors: rotenone, piericidin, and pyridaben.

Two pathogenic mutations of the mitochondrial DNA affecting, respectively, residue Ala52 of ND1 subunit (mutation 3460G>A, A52T) and residue Arg340 of ND4 subunit (mutation 11778G>A, R340H) were reported to have a marginal effect on the NADH-dependent respiration and to slightly affect the sensitivity of human complex I to rotenone (Majander *et al.*, 1996; Degli Esposti *et al.*, 1994). These effects, although controversial, were taken as genetic indications for the involvement of the ND1 and ND4 subunits in the binding of ubiquinone. To check their relevance, we reproduced the two mutations in the corresponding *nuoH* and *nuoM* genes of *R. capsulatus*. Comparative studies did not show any alteration of the growth phenotype of mutant NUOH/"3460" on carbon sources-such as lactate or malate (Dupuis *et al.*, 1998b), while mutant NUOM/"11778" displayed a limited, but reproducible, decrease of its growth efficiency on malate (Lunardi *et al.*, 1998). When activity was measured on isolated membranes, only for mutant NUOH/"3460" did we observed a significant decrease in the NADH oxidase activity (80+/−5 % of the wild-type NADH oxidase

activity). On the contrary, we could not evidence any effect of mutation NUOH/"3460" on piericidin or rotenone sensitivity, whereas the sensitivity of the complex I of mutant NUOM "11778" was decreased only by a 1.5 factor. An independent reproduction of mutation ND1/R340H in *P. denitrificans* led to very limited effects consistent with the above observations (Zickermann *et al.*, 1998). However, in this case, alterations of other amino acid residues in the vicinity of the corresponding arginine induced significant alterations of the kinetic properties of complex I.

To obtain more information about the quinone-binding site of complex I, we directly isolated mutants resistant to piericidin via a classical ethyl-methanesulfonate mutagenesis. Simple screening on piericidin-supplemented medium was not stringent enough to directly select for *R. capsulatus* mutants resistant to piericidin. We thus designed an original "double inhibitor" screening using a medium supplemented by both piericidin and myxothiazol (a specific inhibitor of bc_1 complex). About 10% of the isolated colonies appeared sensitive to myxothiazol and resistant to piericidin (Darrouzet and Dupuis, 1997). By testing the sensitivity of NADH-dependent respiration to piericidin on porous cells, four mutants displaying the highest resistance to piericidin (mutants Pi-A, -B, -C, -D) were selected for further characterization. These mutants displayed a remarkable cross-resistance to rotenone, but remained fully sensitive to rolliniastatin-2 and capsaicin, two other inhibitors of complex I.

Several observations pointed to the NUOH or NUOM subunits as candidates to harbor a quinone-binding site: (1) the hydrophobic ubiquinone Q10 was expected to interact with the membrane module of the complex I; (2) human mutations on NUOH (ND1) and NUOM (ND4) affect quinone and/or rotenone binding (see above); and (3) photoaffinity analogs of rotenone specifically labeled the mitochondrial ND1 subunit (Earley and Ragan, 1984; Earley *et al.*, 1987). However, attempts to complement the Δ*nuoH* and Δ*nuoM* disrupted mutants with the *nuoH* and *nuoM* genes originating from the above four piericidin-resistant mutants resulted in the reconstitution of a normal, "wild-type," complex I activity. The same was true for genes *nuo-I, -J, -K, -L, and -N*. Thus, we systematically sequenced the *nuo* operon of PiC mutant. To our surprise, the identified PiC point mutation altered a valine residue (V407) in the very C-terminal strand of the NUOD subunit of the connecting module (Fig. 2, panel A). Using site-directed mutagenesis, we reconstructed the PiC mutation in an independent *R. capsulatus* strain. This unambiguously confirmed that the mutation was directly associated with the observed piericidin resistance of mutant PiC (Darrouzet *et al.*, 1998).

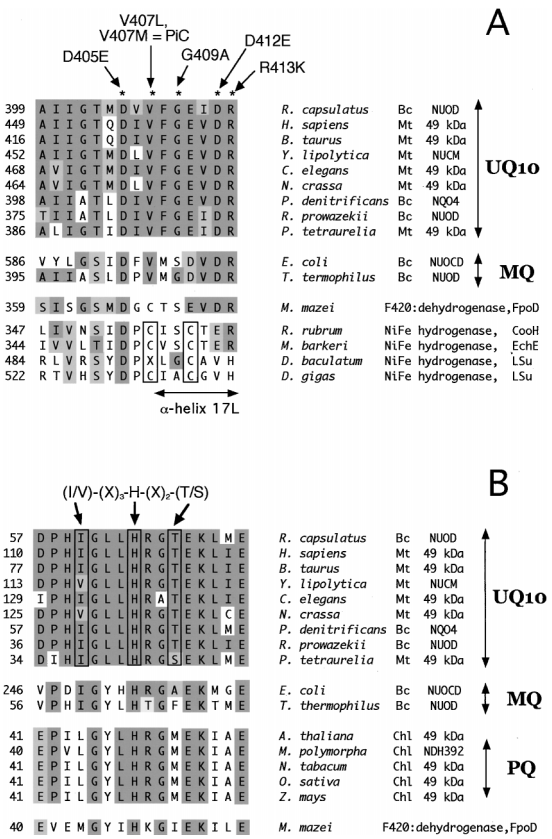
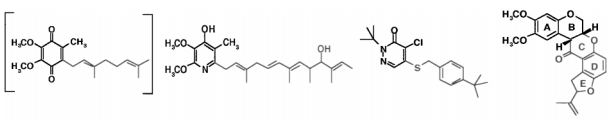


Fig. 2. Conservation of the sequence motifs related to quinone binding in the NUOD subunit of complex I. This figure presents only a summary of larger alignments of thirty mitochondrial (Mt), eighteen bacterial (Bc), one archaebacterial, ten chloroplastic (Chl), a cyanobacterial NUOD subunits, and thirteen hydrogenases large-subunit sequences. (A) Conservation of NUOD C-termini. The first nine sequences aligned correspond to subunits NUOD of ubiquinone-10 reducing complex I (accession number for *R. capsulatus*: O07310, *Homo sapiens*: NP-004541, *Bos taurus*: P17694, *Yarrowia lypolytica*: CAB65521, *Caenorhabditis elegans*: CAB01886, *Neurospora crassa*: P22142, *P. denitrificans*: F42573, *Rickettsia prowazekii*: Q9ZDH4, *Paramecium tetraaurelia*: P15689). The mutations, which we introduced in the *R. capsulatus* NUOD subunit, are indicated above the sequence. The following two sequences (*E. coli*, D65000, and *Thermus thermophilus*, Q5620) are from menaquinone-reducing complex I. The lower part of this panel displays the similarity of the C-terminus of the NUOD subunit to the C-termini of related large subunits of [NiFe] hydrogenases (*Rhodospirillum rubrum*, AAC45121; *Methanosarcina barkeri*, CAA76121; *Desulfomicrobium baculatum*, AA23375; *D. gigas*, P12943). The cysteines liganding the [NiFe] center are boxed. The F420:dehydrogenase of *Methanosarcina mazei* is highly related to complex I, but reduces methanophenazine instead of ubiquinone-10. The C-terminus of its NUOD equivalent subunit (FpoD) is given for comparison (AAF65734). (B) The "Fisher and Rich" quinone-binding motif of the NUOD subunit of complex I. This panel is constructed mainly as is (A), except that the lower part presents the sequence of the corresponding 49-kDa subunits of the chloroplastic plastoquinone-reducing enzymes (*Arabidopsis thaliana*, P56753; *Marchantia polymorpha*, CAA28140; *Nicotiana tabacum*, CAA77398, *Oryza sativa*, CAA33911; *Zea mays*, CAA60359).



Mutants			
D405E	No	No	No
V407M = PiC	+++	++	+++
V407L	No	No	very weak
G409A	+	++	+
D412E	++	+++	++
R413K	1,5 time more sensitive	No	No

Fig. 3. Effects of mutations of the C-terminus NUOD on the sensitivity to complex I inhibitors. The resistance of the NADH-oxidase activity of NUOD mutants to piericidin, pyridaben, and rotenone was measured in membrane preparations. The structure of piericidin, pyridaben, rotenone, and the ubiquinone-2 are given for comparison in the upper part of the figure.

Considering the striking conservation of the C-terminal part of NUOD between different species (see Fig. 2, panel A), we decided to mutagenize some of the most conserved residues in this stretch. We obtained six-point mutants targeting residues D405 (D405E), V407 (V407L and V407M), G409 (G409A), D412 (D412E), and R413 (R413K) (Prieur *et al.*, 2001). The NADH—oxidase and the NAD–quinone oxidoreductase activities were significantly decreased in all the mutants. Out of these six mutants, V407M and D412E displayed clear resistance to the three inhibitors tested (piericidin, pyridaben, and rotenone; Fig. 3). Thus the three inhibitors must target the same quinone-binding site involving the C-terminus of NUOD. A third mutant, G409A, displayed a strong resistance to pyridaben, but was only slightly resistant to piericidin. On the other hand, mutant R413K proved to be more sensitive to piericidin than the wild-type bacterium. Moreover, V407M proved to be more resistant to piericidin and less resistant to pyridaben than D412E. There is consequently a clear discrimination between these two inhibitors by two mutations distant only of five residues. This fits well with the idea that the different complex I inhibitors and ubiquinone bind to overlapping, but not identical, binding sites (Okun *et al.*, 1999).

DISCUSSION: EXISTENCE OF AN INTERFACIAL QUINONE-BINDING SITE IN THE CONNECTING MODULE OF COMPLEX I

Quinone-Binding Sites Are Located in the Hydrophobic to Polar Transition Zone

The crystal structures of the RC of photosynthetic bacteria and of the fumarate reductase (QFR) of *E. coli* show that quinone-binding sites are located at the interface between the membranous domain and the cytosolic domain of the quinone-binding enzymes (Lancaster and Michel, 1997; Iverson *et al.*, 1999). The "quinone ring pocket" of the Q_B site of RC, as well as the menaquinone Qp site of the QFR, have clear polar characteristics. In the case of bc_1 complex, the inhibitors myxothiazol and antimycin, which are assumed to address, respectively, the Q_P and Q_N ubiquinone-binding sites of bc_1 complex, bind close to the interface between the membrane and the peripheral parts of the enzyme (Xia *et al.*, 1997). Moreover, the N-terminal part of the peripheral Rieske subunit contributes to the Qp site of the bc_1 complex (Brasseur *et al.*, 1997). It is well known that an isolated fragment corresponding to the protruding arm of mitochondrial complex I is able to catalyze NADH–quinone oxidoreduction, especially with short-tailed quinones (Friedrich *et al.*, 1989; Finel *et al.*, 1992). In fact, ubiquinone Q10 is an amphiphatic molecule presenting a polar cyclic head and a hydrophobic isoprenyl tail. We consequently proposed that the ubiquinone binding site of complex I probed by inhibitors would consist of a polar "quinone ring" subsite and a membranous "isoprenyl tail" subsite (Darrouzet *et al.*, 1998).

As noted previously (Yoshida *et al.*, 1980), 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 akin to the isoprenyl motif of ubiquinones. Similar to stigmatellin in the RC and bc_1 complexes, piericidin must essentially address the "quinone ring" subsite in complex I. Structure–function studies of rotenone analogs suggest that the A–B cycles of rotenone mimic the quinone ring of ubiquinone (Ueno *et al.*, 1996), while the C, D, and E cycles can be assimilated to the hydrophobic "isoprenyl tail" of ubiquinone. The chemically reactive groups of dihydrorotenone and arylazidomorphigenin are located at the level of the E cycle of these rotenone analogs. They must, therefore, react essentially with the peptides lining the "isoprenyl-tail" binding subsite. Thus photolabeling of the ND1 subunit by these two analogs (Earley and Ragan, 1984; Earley *et al.*, 1987) suggests that ND1 (NUOH) must harbor the "isoprenyl-tail" binding part of the quinone site discussed in these lines.

A Consensus Motif for Quinone-Binding Sites Is Present on NUOD

Following the study of the different known structures of quinone–quinol-binding enzymes, Fischer and Rich (2000) described a "consensus quinone-binding motif" corresponding to an α-helical structure of the

form: aliphatic-$(X)_3$-H-$(X)_{2/3}$-(L/T/S). This motif is actually present in the N-terminal part of NUOD. All the thirty mitochondrial 49-kDa subunits found in the Genbank, as well as the three NUOD subunits of bacterial complex I using ubiquinone, presented the quinone-binding motif: (I/V)60-$(X)_3$-H64-$(X)_2$-(T/S)67. Interestingly, the last position of this consensus motif is not conserved in the C-terminus of NUOD from menaquinone-reducing complex I. Similarly, Fisher and Rich observed that their motif was present in the SdhC of *E. coli* (a ubiquinone-reducing enzyme), but not conserved in the closely related fumarate dehydrogenase (a menaquinol-oxidizing enzyme). Along the same line, the F_{420}:dehydrogenase of *Methanosarcina mazei* shows strong phylogenetic similarities with complex I, but reduces methanophenazine instead of ubiquinone (Bäumer *et al.*, 2000). It is thus interesting to note that the quinone-binding motif is not conserved in subunit FpoD that is the equivalent of NUOD in this enzyme. These different observations further strengthen the idea that the polar head pocket of the quinone-binding site addressed by rotenone and piericidin is borne by the NUOD subunit.

Schuler *et al.* (1999) recently observed that (trifluoromethyl)diazirinyl-[^{3}H]pyridaben, a photoaffinity analog of pyridaben, specifically labeled subunit NUOB. These authors thus proposed to locate the quinone-inhibitor binding site on the NUOB subunit in apparent contradiction with our experiments and our proposed model. However, this apparent controversy can be easily explained in the light of a structural model based on phylogenetic comparisons between complex I and [NiFe] hydrogenases.

The [NiFe] Hydrogenases: A Structural Model for the Connecting Module of Complex I

As discussed above, the NUOB subunit displays striking similarities to the N-terminal part of the small subunit of [NiFe] hydrogenases. This protein is associated in a functional dimer with the large subunit that bears the catalytic [NiFe] site (see Albracht, 1994). The catalytic site can either receive electrons from the [Fe–S] clusters located on the small subunit in order to reduce protons or give electrons back to the same clusters in the reverse reaction. As noted by Albracht and Hedderich (2000), the large subunits of type-3 hydrogenases share five common sequence motifs with NUOD. Two of them encompass the two regions highlighted by our studies (see above). The first one is overlapping with the "Fisher and Rich" consensus motif for quinone binding in NUOD (Fig. 2, panel B) and is located close to the interface with the small subunit in hydrogenases. The second one comprises the C-terminal part of the NUOD subunit that we identified by mutagenesis in complex I (Fig. 2, panel A). The sequence of this C-terminus of NUOD is reminiscent of the [Gx_4Dx_8**D**P**C**x**C**ST(**D**/**E**)**Rcooh**] motif present at the C-terminal position of mature type-3 [NiFe] hydrogenases large subunits HycE and EchE (Böhm *et al.*, 1990; Künkel *et al.*, 1998). This sequence is directly contributing to the coordination of the NiFe center. However, the two cysteine residues of this motif that coordinate the nickel atom are not conserved in the NUOD protein family. In these proteins, the first of these two cysteines is actually replaced by the valine residue V407, which was first highlighted by our mutagenesis studies (Darrouzet *et al.*, 1998). Similarly, only the first of the two cysteines is conserved in the FpoD subunit of the F_{420}:dehydrogenase of *M. sarcina*. It can thus be hypothesized that, in the NUOD subunit of complex I (or in the FpoD subunit of the F_{420}:dehydrogenase), the proton reduction site of the [NiFe] large subunit might have evolved in a quinone-reduction site (or methanophenazine-reduction site), using the preexisting protons and electrons pathways. The homology between type-3 hydrogenases and the soluble [NiFe] hydrogenase of *D. gigas* is rather weak (about 20%). Nevertheless, interesting structural predictions concerning the NUOD–NUOB module of complex I can be drawn from the crystal structure of this hydrogenase (Volbeda *et al.*, 1995) (Fig. 4). The C-terminus of the *D. gigas* large subunit (corresponding to the end of the second conserved motif presented above), is organized as a six amino acid α-helix (helix 17L), which is buried in the very heart of the protein. The generation of this C-terminus by cleavage of a precursor sequence is a prerequisite for the final folding and activation of [NiFe] hydrogenases. Thus, the two terminal helixes (16 and 17L) are central to the packaging of the active large subunit (see interesting discussion of these points in Volbeda *et al.*, 1995). Using a Robson–Garnier structure algorithm, the C-terminus of NUOD subunit is predicted to be organized in a helix–coil–helix secondary structure, equivalent to helixes 16L and 17L of the [NiFe] hydrogenase. If we assume a partial conservation of the overall structure of the large subunit of [NiFe] hydrogenases in NUOD, the stringent termination of all known NUOD subunits on the motif (D/E)R must reflect the strong structural pressure put on this C-terminus by its precise positioning in the heart of the mature protein (Fig. 4). A most interesting prediction of this structural model is that the distance of closest contact between the C-terminal helix of NUOD and the putative [4Fe–4S] cluster attributed to subunit NUOB would be lower than 10 Å. This structural prediction casts a bridge between our genetic observation and the report of Schuler *et al.* (1999): (trifluoromethyl)diazirinyl-[^{3}H]pyridaben trapped in the

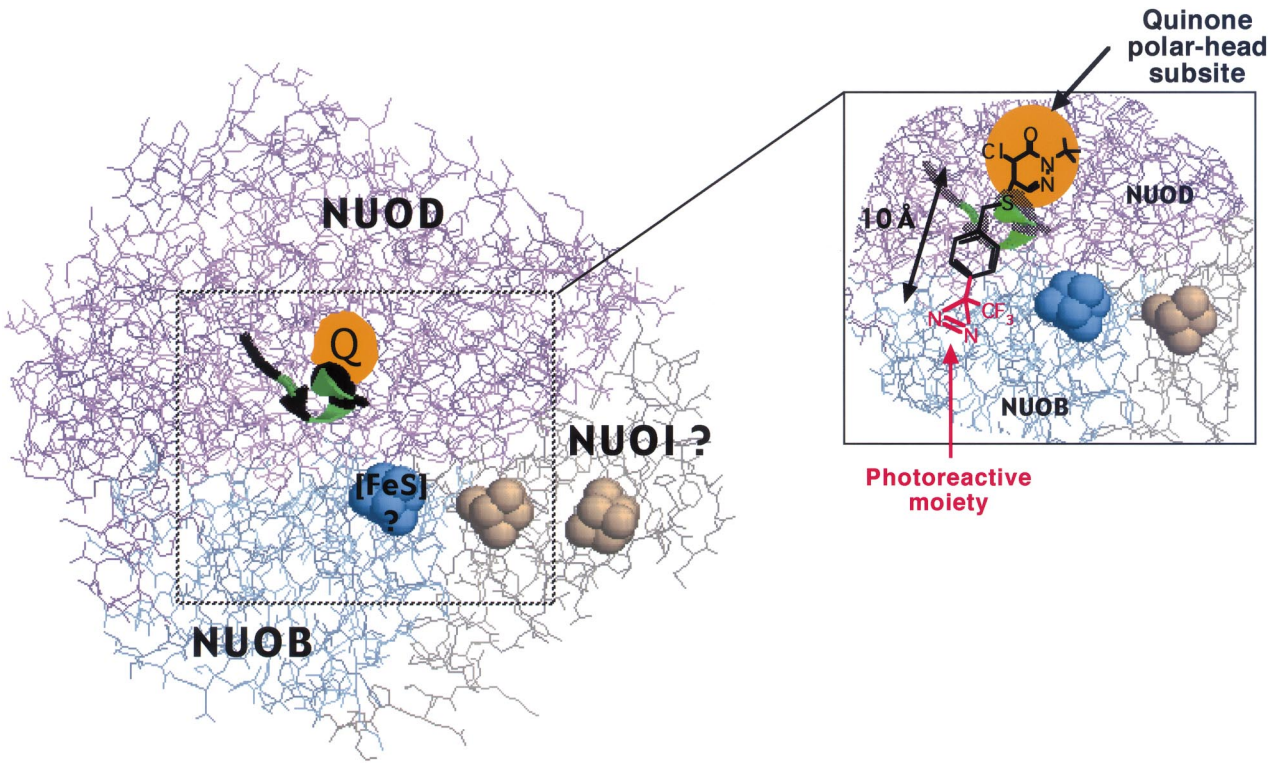


Fig. 4. Possible localization of a quinone-binding site in the connecting module of complex I. The crystallographic structure of the [NiFe] hydrogenase of *D. gigas* (Volbeda *et al.*, 1995) is given here as a speculative basis for the organization of the NUOB-D-I module. Although the overall structure is only indicative, the location and the structure of the C-terminal α-helix of the [NiFe] large subunit must be conserved in NUOD subunit (see text). This helix is represented here as a dark ribbon. Putative positions of residues V407, G409, and D412, which are involved in inhibitors interactions, are colored green. The right side of the figure illustrates how the photoreactive analogu of pyridaben {(trifluoromethyl)diazirinyl-[^{3}H]pyridaben)}, bound on NUOD would label the NUOB subunit.

quinone-binding site homed by the NUOD subunit would clearly be at labeling distance from the NUOB subunit (Fig. 4).

Nature of the Quinone-Binding Site Associated with the NUOD Subunit

The number of quinone-inhibitor binding sites present on complex I is still a matter of debate. It has long been reported that the inhibitors of complex I could be classified in at least three categories, depending on their effect on complex I kinetic parameters (for review see Degli Esposti, 1998). Along this line, we previously observed that mutant PiC displayed a great difference of sensitivity to different complex I inhibitors (Darrouzet and Dupuis, 1997). This observation can be extended to mutations V407M, G409A, and D412E that alter amino acids in close spatial relation (see above). These data are in good agreement with the idea that, except for the capsaicin family, all the inhibitors of complex I would actually address the same large binding pocket (Okun *et al.*, 1999). However, comparison with other quinone-binding enzymes, like the QFR, suggests that the singleness of the complex I inhibitor-binding site does not preclude the existence of other supplementary quinone-binding sites. Biophysical studies provided strong evidence for several quinone binding sites on complex I (Van Belzen *et al.*, 1997; Ohnishi, 1998; Yano *et al.*, 2000). It is generally accepted that the first quinone to be reduced in complex I must obtain its electrons directly from the [Fe–S] center N2. As discussed above, this N2 center must be located either on subunit NUOB or NUOI. In this regard, it should be stressed that the C-terminal domain of the small subunit of soluble hydrogenases is organized in a ferredoxin-like domain structurally related to the NUOI subunit (Volbeda *et al.*, 1995). From the literature, it is clear that the quinone/inhibitor binding site of complex I must be a rather large pocket (Degli Esposti, 1998; Darrouzet *et al.*, 1998; Okun *et al.*, 1999). Depending on its precise location and orientation, a quinone bound at this site might accept electrons from either the putative

[4Fe–4S] center of NUOB or the proximal [4Fe–4S] center of NUOI. In both cases, a quinone-binding site located close to the C-terminal helix of NUOD would be in a good position to be the first quinone reduction site in complex I. Based on the extensive study of the relaxation characteristics and thermodynamical properties of these radicals, Ohnishi and collaborators discriminated three semiquinone species associated with complex I (called SQ_{Nf}, SQ_{Ns}, and SQ_x). Among these, SQ_{Nf} is postulated to be bound at close distance from center N2 and its presence is prevented by piericidin. The estimated distance between center N2 and SQ_{Nf} (8–11 Å) compares well with the closest distance between the nickel bound to the large subunit and the first [Fe–S] center of the small subunit of the [NiFe] hydrogenase of *D. gigas* (Volbeda *et al.*, 1995).

Relation of the NUOD Quinone-Binding Site with Proton Pumping

The ability of *E. coli* complex I to pump Na^+ ions as well as protons strongly supports the contribution of a "gate and channel" machinery in the proton transport activity of complex I (Steuber *et al.*, 2000). In the reaction center, an important conformational change of the ubiquinone bound to center Q_b can be associated to the reduction reaction (4 Å translation and 180° rotation of the quinone head during catalysis) (Lancaster and Michel, 1997; Graige *et al.*, 1998). Similarly, a quinone movement is proposed to take part in electron transfer in the bc_1 complex (Crofts *et al.*, 2000) and might as well explain the apparent width of the inhibitor–quinone-binding site of complex I. The potential motility of quinone points to it as an ideal cofactor for such a gate mechanism. Involvement of a gate quinone has often been put forward to explain the proton pumping activity of complex I (Vinogradov, 1993; Degli Esposti and Ghelli, 1994; Dutton *et al.*, 1998, Steuber *et al.*, 2000). In light of its sensitivity to membrane $\Delta\mu H^+$, the semiquinone SQ_{Nf} would be a good candidate for such a gating process (Yano *et al.*, 2000). In hydrogenases, the [NiFe] center is buried at 30 Å from the protein surface. Such a buried location would be compatible with the stabilization of a semiquinone at the equivalent location in NUOD subunit. The reorganization of the [NiFe] active site of hydrogenases in a quinone-reduction site would allow the "recycling" of the preexisting proton and electron pathways. From the structure of the *D. gigas* [NiFe] hydrogenase, it has been proposed that the ultimate basic residue of the large subunit of these [NiFe] hydrogenases, corresponding to R413 in the complex I NUOD subunit, contributes to the proton path to the catalytic site. Actually, the specific activity of complex I is drastically affected by mutation R413K (Prieur *et al.*, 2001). Furthermore, DCCD, a chemical reported to probe proton channels, inactivates complex I with concomitant covalent binding to NUOH and NUOD subunits (Yagi, 1987; Yagi and Hatefi, 1988). Recent genetic reports seem to exclude NUOH subunit as the specific target inactivated by DCCD (Kurki *et al.*, 2000). This suggests that the DCCD inhibitory effect is associated with its binding to NUOD.

Comparison with the proton pumping type-3 hydrogenases suggests that, in complex I, a minimal "proton-pumping" module would include subunits NUOB, NUOD, NUOI, NUOH, and NUOL (Friedrich and Scheide, 2000). From the above considerations, it can be proposed that NUOD might provide both the quinone gate and a potential proton channel "entry" from the aqueous phase to the internal face of the membrane. Subunits NUOL, NUOM, and NUON of the complex I apparently evolved by triplication of an ancestor gene related to bacterial H^+/K^+ antiporters (Fearnley and Walker, 1992; Friedrich and Weiss, 1997). Consequently, it is tempting to assume that subunit NUOL would provide the transmembrane channel required to complete a proton pump. The absence of a quinone in the proton-pumping hydrogenases and in the F420:dehydrogenase was considered as evidence against the quinone-gate model (Friedrich and Scheide, 2000). However, different cofactors like the methanophenazine of the F420:dehydrogenase, may similarly act as a proton gate. Furthermore, the stoichiometry of proton pumping of these enzymes appears lower than that of complex I (Bäumer *et al.*, 2000). Thus, the appearance of a quinone-reduction site on the NUOD subunit might have been concerted with an increase of the proton-pumping efficiency. In fact, to take into account the complexity and the high efficiency of coupling characteristic of complex I, the proposed mechanisms often include two distinct proton transport processes (Dutton *et al.*, 1998, Steuber *et al.*, 2000).

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