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The Complex I from *Rhodobacter capsulatus*

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Abstract

The NADH-ubiquinone oxidoreductase (type I NDH) of *Rhodobacter capsulatus* is a multisubunit enzyme encoded by the 14 genes of the *nuo* operon. This bacterial enzyme constitutes a valuable model for the characterization of the mitochondrial Complex I structure and enzymatic mechanism for the following reasons. (i) The mitochondria-encoded ND subunits are not readily accessible to genetic manipulation. In contrast, the equivalents of the mitochondrial ND1, ND2, ND4, ND4L, ND5 and ND6 genes can be easily mutated in *R. capsulatus* by homologous recombination. (ii) As illustrated in the case of ND1 gene, point mutations associated with human cytopathies can be reproduced and studied in this model system. (iii) The *R. capsulatus* model also allows the recombinant manipulations of iron–sulfur (Fe–S) subunits and the assignment of Fe–S clusters as illustrated in the case of the NUOI subunit (the equivalent of the mitochondrial TYKY subunit). (iv) Finally, like mitochondrial Complex I, the NADH-ubiquinone oxidoreductase of *R. capsulatus* is highly sensitive to the inhibitor piericidin-A which is considered to bind to or close to the quinone binding site(s) of Complex I. Therefore, isolation of *R. capsulatus* mutants resistant to piericidin-A represents a straightforward way to map the inhibitor binding sites and to try and define the location of quinone binding site(s) in the enzyme. These illustrations that describe the interest in the *R. capsulatus* NADH-ubiquinone oxidoreductase model for the general study of Complex I will be critically developed in the present review. © 1998 Elsevier Science B.V.

Keywords: Complex I; Iron–sulfur cluster; NADH-ubiquinone oxidoreductase; *nuo* Operon; Quinone binding site; Piericidin; Rotenone

1. Introduction

The high complexity of mitochondrial NADH-ubiquinone oxidoreductase (Complex I) has prompted some authors to look for related but simpler bacterial enzymes. Bacterial NADH-ubiquinone oxidoreductases (also called NDHs for NADH dehydrogenases) can be classified into at least three categories: (i) the H^+-pumping NADH-ubiquinone oxidoreductases or type I NDHs [1], (ii) the uncoupled NADH-ubiquinone oxidoreductases or type II NDHs [2] and (iii) the Na^+-pumping NADH-ubiquinone oxidoreductases [3]. Only type I NDHs display high structural and functional similarities with the mitochondrial Complex I and can thus be used as a model system for the study of this enzyme. Purple non-sulfur bacteria such as *Rhodobacter capsulatus* are considered to be phylogenetically related to the ancestor from which mitochondria derived [4]. The presence

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of a type I NADH-dehydrogenase activity in *R. capsulatus* has been reported quite a long time ago by Baccarini-Melandri et al. [5] who demonstrated the presence of an energy coupling site I in the membranes of this bacteria. They also showed that the NADH-dehydrogenase activity associated with this energy coupling site was 95% sensitive to rotenone. In this regard *R. capsulatus* type I NDH is very similar to the eukaryote Complex I and thus constitutes an interesting model for the study of this enzyme. More recently, the authors of the present review identified an *nuo* operon encoding this multisubunit enzyme [6,7]. As will be discussed below, the genes clustered in this *nuo* operon code for 14 proteins homologous to 14 major subunits of the mitochondrial enzyme. Like the type I NDHs of *Escherichia coli*, *Paracoccus denitrificans* and *Thermus thermophilus* HB-8 [8–10], the enzyme of *R. capsulatus* appears to be a kind of minimal system in comparison to mitochondrial Complex I. Furthermore, compared to the mitochondrial Complex I and to the other bacterial models, the NADH-ubiquinone oxidoreductase of *R. capsulatus* has specific advantages that will be illustrated and discussed in this review. For the sake of simplicity, *R. capsulatus* NADH-ubiquinone oxidoreductase will be called *R. capsulatus* Complex I in the following.

2. Structure of *R. capsulatus* Complex I

2.1. Structural features of the subunits of the *R. capsulatus* Complex I

Historically, two different strategies were followed to clone the operons encoding the bacterial NADH-ubiquinone oxidoreductases. In the case of *P. denitrificans*, the isolation and biochemical characterization of a partial Complex I preceded the identification of the *nqo* operon [9]. In contrast to this, the identification of the *R. capsulatus nuo* operon really constituted the first step towards the molecular characterization of *R. capsulatus* Complex I. Based on the assumption that the TYKY subunit identified in bovine Complex I must be highly conserved among organisms, part of a gene of *R. capsulatus* potentially encoding a peptide homologous to TYKY was amplified by PCR [6]. This PCR product was used as a probe for screening a lambda genomic library. From the sequence of the isolated clones it has been possible to generate a nearly 20-kbp contig containing at least 23 open reading frames (orfs). Fourteen out of these orfs code for subunits of the *R. capsulatus* Complex I (Table 1). The identity of these *nuo* genes has been mainly assigned by comparing their sequences or that of their products with the bovine mitochondrial sequenced cDNA or proteins or with the *P. denitrificans* genes. In *R. capsulatus* as in all other known bacterial systems, the *nuo* genes are clustered in the bacterial chromosome (Fig. 1). The *R. capsulatus* gene cluster has been located on a high resolution genetic map in a region also containing the *puc* cluster (encoding the polypeptides of the light harvesting complex II) and the *fru* cluster (encoding the component of the fructose transport and uptake system) [12]. At its 5′ end, the *nuo* cluster is bordered by one unidentified reading frame located nearly 300 bp upstream of *nuoA*. On the other extremity, *nuoN* gene is immediately followed by the *urfR242* which potentially encodes a protein homologous to the catalytic domain of the *E. coli* biotin(acetyl-CoA carboxylase) ligase [13]. Definitive demonstration that all the clustered *nuo* genes belong to the same transcriptional unit has not been obtained so far. Nevertheless, we assume that these genes are part of an operon coding for all the components of the Complex I. Indeed, upstream the *nuoA* gene, three putative consensus promotor motifs can be spotted. At the opposite boundary of this gene cluster, about 400 bp downstream of the *nuoN* gene, the 3′ part of the sequenced fragment contains a motif which is able to form a very stable stem and loop structure at the level of the RNA [7]. This structure includes a GC rich motif and a T_5 sequence and is similar to sequences believed to play the role of a *rho*-independent termination signal for transcription [14,15]. As a consequence, the *urfR242* must not be considered as part of the *nuo* operon which is probably transcribed as a single polycistronic RNA potentially encoding up to 21 polypeptides. The size of this *R. capsulatus* operon (from the *nuoA* ATG start codon to the *nuoN* stop codon) is 18 345 bp. It is the longest NADH-ubiquinone oxidoreductase bacterial operon sequenced so far. The G + C content of the corresponding genes is in the range of 58 to 72%. As stated above, 14 out of the 21 genes identified in *nuo*

Table 1
Characteristics of the polypeptides potentially encoded by *R. capsulatus nuo* operon

	Nucleotide position of gene in *nuo* operon	Amino acid number	Molecular masses (Da)	Bovine equivalent	Putative membranous domains	Conserved cysteines[a]	% Sequence homologies Bov/Rc	% Sequence homologies Pd/Rc
nuoA	353–730	126	14 235	ND3	3		60	94
nuoB	724–1254	177	19 507	20	1?	5	90	97
nuoC	1269–1865	199	23 089	30			73	85
nuoD	1914–3152	413	46 446	49		1	79	94
nuoE	3155–4321	389	41 257	24		4	67	86
urf1	4424–4864	147	14 796					
nuoF	5060–6352	431	47 130	51		9	86	95
urf2	6362–6835	158	17 012					
urf3	6838–7119	94	9612					
urf4	7119–7889	257	28 910					
nuoG	7950–9944	665	71 258	75		12	70	92
urf5	9951–10 355	135	13 629					
nuoH	10 392–11 426	345	37 850	ND1	8–9		64	91
urf6	11 432–11 710	93	10 035					
nuoI	11 713–12 201	163	18 846	23		8	90	96
urf7	12 201–12 608	136	15 214					
nuoJ	12 608–13 213	202	21 732	ND6	5		18	87
nuoK	13 260–13 565	102	10 983	ND4L	3		27	99
nuoL	13 573–15 708	712	77 527	ND5	15–16		57	90
nuoM	15 711–17 246	512	55 753	ND4	14		52	93
nuoN	17 262–18 695	478	50 124	ND2	13–14		25	88

Genes of the *R. capsulatus nuo* operon and features of the expressed subunits are presented in the first four columns. Comparisons of the *R. capsulatus* subunits with the bovine and *P. denitrificans* equivalent subunits were performed by the clustal method (matrix PAM250) using DNA star multialignment software.

[a] Conserved cysteines in bovine, *P. denitrificans* and *R. capsulatus* subunits. Bov = bovine, Rc = *R. capsulatus*, Pd = *P. denitrificans*.

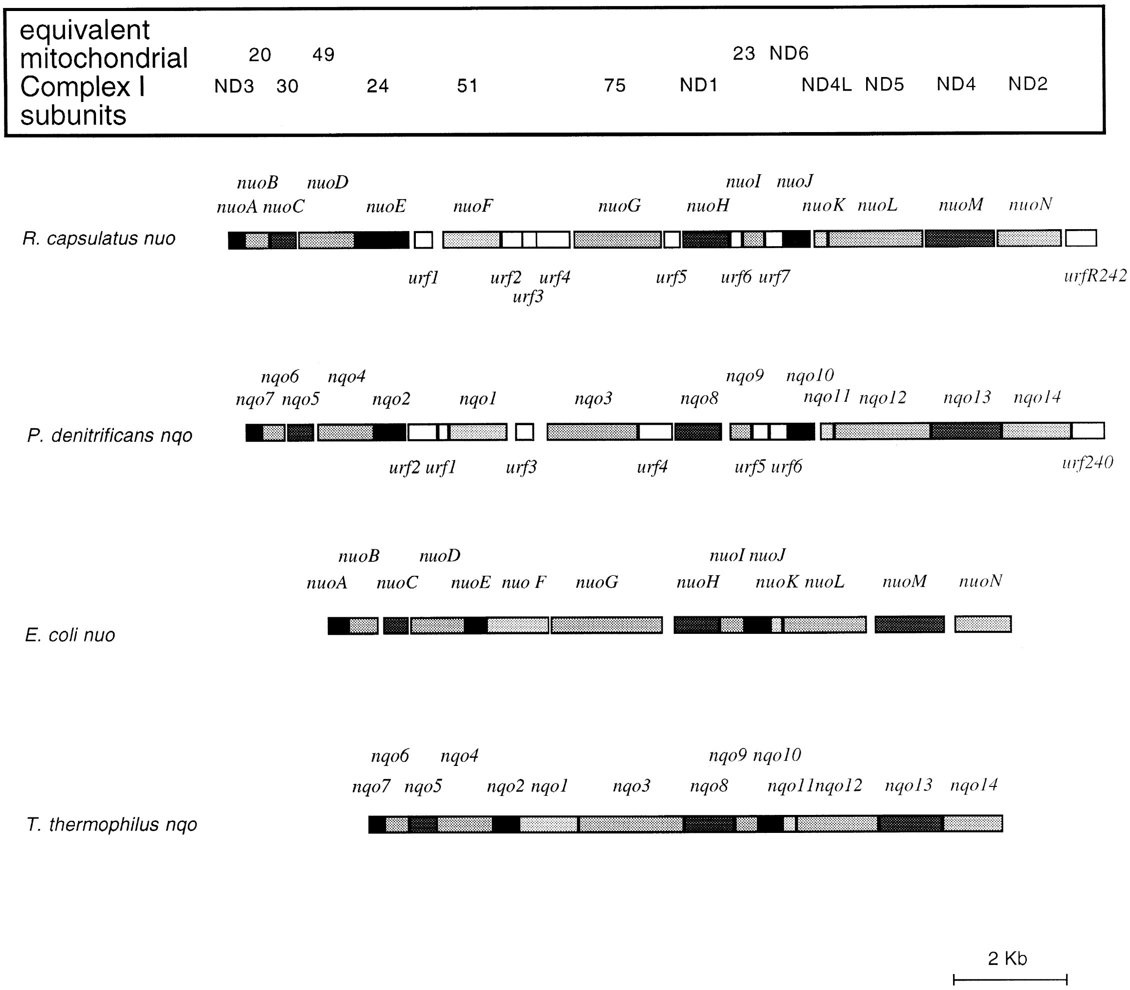

Fig. 1. Comparison of the different bacterial *nuo/nqo* operons. *R. capsulatus* operon (Refs. [6,7,11,21] and this issue; GenBank accession number: AF029365) differs from the other known operons by the presence of seven orfs (*urf1* to *urf7*). Only *R. capsulatus urf5* and *urf7* display a significant homology with *P. denitrificans urf4* and *urf6*, respectively. Another particularity of *R. capsulatus nuo* operon is the atypical length of the *R. capsulatus nuoE* gene leading to a 150 amino acid extension at the C-terminus of NUOE subunit. (*P. denitrificans nqo* operon [9], *E. coli nuo* operon [8], *T. thermophilus nqo* operon [10]).

operon exhibit clear homologies with their mitochondrial counterparts. The seven remaining orfs have been called *urf1-7* and might potentially code for polypeptides with molecular masses in the range of 9.6 to 28.9 kDa. The *P. denitrificans* equivalent *nqo* operon contains six orfs [9]. The orfs are located in five different intergenic regions in *R. capsulatus* but in only four intergenic regions in *P. denitrificans*. Interestingly, four orf-containing intergenic regions, located between the *nuoE* and *nuoF* genes (*nqo2–nqo1*, respectively in *P. denitrificans*); between *nuoF* and *nuoG* (*nqo1–nqo3*); between *nuoG* and *nuoH* (*nqo3–nqo8*) and between *nuoI* and *nuoJ* (*nqo9–nqo10*) are common to *P. denitrificans* and *R. capsulatus*. However, the number of orfs in these regions varies from one to three depending on the species. The additional orf-containing intergenic region of *R. capsulatus* is located between *nuoH* and *nuoI*. Only the putative products of the *R. capsulatus urf5* and *urf7* show significant homologies with the products of the *P. denitrificans urf4* and *urf6*, respectively (*urf5* and *urf6* of *P. denitrificans* are actually related to each other). All the other *urfs* are poorly or not at all similar to the ones found in *P. denitrificans*. The role of the putative products of these *urfs*, if they are expressed, is unknown. They may be related to the metabolic specificity of *R. capsulatus*: the difference with *P. denitrificans* is that *R. capsulatus* is a photosynthetic bacteria. On this regard, Herter et al. [11] noted that *R. capsulatus urf4* putative gene product displays similarities with subunit M of *Rhodopseudomonas viridis* photosynthetic reaction center. According to these authors, this putative peptide present an ATP/GTP binding domain and might be part of a regulatory system.

When expressed, the 14 *nuo* genes give rise to polypeptides whose features are reported in Table 1. In the absence of polypeptide maturation or modification and assuming that only one copy of each of the 14 expressed proteins (NUOA to NUON) is assembled into Complex I, it can be calculated that *R. capsulatus* Complex I is a multimeric enzyme composed of 4914 amino acids exhibiting a molecular mass of nearly 536 kDa. Sequence similarities of the subunit of Complex I with related enzymatic systems suggest that this system emerged from the association of different preexisting enzymes. It has been put forward that *nuoB*, *nuoD*, *nuoH*, *nuoI*, *nuoL*, *nuoM* and *nuoN* genes are affiliated to the *hyc* operon which encodes the formate hydrogenlyase of *E. coli* and the central part of the *nuo* operon including *nuoE*, *nuoF* and *nuoG* has been related to the NAD-dependent soluble hydrogenase encoded by the *hox* operon in *Alcaligenes eutrophus* [16,17]. Actually, the comparison can be further extended to the NUOC subunit (equivalent to the 30 kDa mitochondrial subunit) which displays a striking similarity to the N-terminal part of the *hycE* product. This subunit of the formate hydrogenlyase is thus equivalent to a NUOC–NUOD chimera. Such a chimeric NUOC–NUOD subunit was found in the Complex I of the *Buchnera aphidicola* bacteria [18].

2.1.1. ND-like subunits of the R. capsulatus Complex I

Seven *nuo* genes are equivalent to the mitochondrial *nd* genes. These are *nuoA*, *nuoH*, and *nuoJ* to *nuoN*. Like the other bacterial *nuo/nqo* operons known so far, the *R. capsulatus* operon is characterized by the fact that six out of the seven genes, equivalent to the mitochondrial genes coding for the ND subunits of the Complex I, are clustered at the 3′ end of the operon. Only *nuoA*, the *nd3* equivalent gene, is located at the very beginning of the operon. All the ND subunits are hydrophobic in nature and constitute the membranous domain of the Complex I or at least one major subdomain of it. Their role in the proton pumping activity of Complex I is probably prominent. If we assume that a complex made up of only the protonophoric part of Complex I could work as an uncoupled proton channel when freely assembled, the existence of such an hydrophobic domain may be highly toxic for the cells. It is possible to hypothesize that the sequential expression of the operon associated with a sequential assembly of the hydrophillic subunits as well as the NUOA subunit at the level of the membrane could prevent the assembly of a free transmembranous domain made up of ND-like subunits alone. It can be noted that, in the case of the *unc* operon encoding the bacterial H^+-ATPase, a similar organization clustering the genes *uncA*, *uncB* and *uncC*, that encodes the hydrophobic part of the enzyme is also observed. However, in this case, *uncA*, *uncB* and *uncC* are found either in the 5′ part of the *nuo* operon or at a different locus, depending on the bacterial species [14]. The *R. capsulatus* subunits NUOJ, NUOK and NUON are the least conserved subunits of the enzyme and are less than 30% homologous to their mitochondrial equivalents (ND6, ND4L and ND2 subunits, respectively). *R. capsulatus* ND equivalent subunits individually contain between three to up to 16 putative transmembranous domains (Table 1). Thus it can be predicted that the total number of transmembranous domains which are part of the *R. capsulatus* Complex I is potentially in the range of 61 to 64 per enzyme complex.

2.1.2. The other subunits of the R. capsulatus Complex I

Among the seven other subunits (NUOB–NUOG and NUOI), three have extremely well-conserved sequences in *R. capsulatus* in comparison to the bovine subunits. These are the NUOB subunit (PSST equivalent), the NUOF subunit (51 kDa equivalent) and the NUOI subunit (TYKY equivalent). All three subunits are more than 85% homologous to their bovine counterpart. More details concerning NUOI are given in Section 3.2. Previous structural analyses have predicted NUOB to be involved in the binding of a putative iron–sulfur (Fe–S) cluster as it contains conserved cysteine residues. Indeed, four cysteines (C56, C57, C121 and C151, according to the *R. capsulatus* numbering) are invariably conserved in the *E. coli*, *P. denitrificans* and bovine equivalent subunits. No histidine residues which could be involved in iron coordination are conserved among these sequences. The arrangement of these cysteines does not look like any other consensus motif known to be involved in the coordination of Fe–S cluster. Secondary structure prediction indicates that at least one region of the NUOB *R. capsulatus* subunit has a high probability to be located in the membranous domain. This hydrophobic region would encompass the first two well-conserved C56 and C57 cysteines. A transmembranous location of these cysteines could probably impair Fe–S coordination or else might lead to the generation of an unusual redox group which might play an important role in the activity of the whole enzyme.

The *R. capsulatus* NUOF subunit contains two motifs that are predicted to be involved in the binding of NADH and FMN [16]. Multiple alignments made with the sequences of the 51-kDa subunit equivalents from the mammalian mitochondria, lower eukaryotic mitochondria and *P. denitrificans* show that these subunits contain a total of nine conserved cysteines. However, the total number of conserved cysteines drops down to five when the sequences of the NUOF equivalent subunits of *E. coli* and *T. thermophilus* are taken into consideration. It is not known at present if the four additional and well-conserved cysteines in the *R. capsulatus* NUOF subunit and in the other mitochondrial equivalent subunits take part in Fe–S coordination.

When compared to their bacterial and mitochondrial equivalents, NUOC and NUOD subunits exhibit no specific features. The *R. capsulatus* NUOG subunit is closely related to the mitochondrial equivalent subunit 75 kDa. A total of 12 cysteine residues are strictly conserved in the *R. capsulatus* NUOG subunit, *P. denitrificans* NQO3 subunit and 75 kDa mitochondrial subunits and are probably involved in the ligation of three Fe–S clusters. The first of these 12 cysteines is not conserved in the *T. thermophilus* equivalent subunit (Fig. 2). Surprisingly, the *E. coli* and *T. thermophilus* subunits contain an additional conserved motif of four cysteines [19,10]. The number of clusters bound to the enterobacterial and archaebacterial NUOG subunits has not yet been defined but it is clear that these subunits are structurally distinct from the *R. capsulatus* and mitochondrial equivalent subunits.

Finally, the NUOE subunit is the most peculiar subunit of *R. capsulatus* NADH-ubiquinone oxidoreductase. The N-terminal part of this subunit is highly conserved between *R. capsulatus* and other species especially at the level of the four cysteine residues which are liganding one [2Fe–2S] cluster (either N1a or N1b) [17,20]. However, this subunit has a very unusual size in *R. capsulatus*. In comparison to the bovine subunit, the NUOE subunit is extended at the C-terminus by more than 150 amino acids leading to a molecular mass of 41.3 kDa while the bovine subunit is close to 24 kDa [11,21]. This extension displays some similarities with *P. denitrificans urf2* [11].

2.1.3. The missing subunits

The bacterial NADH-ubiquinone oxidoreductase, composed of only 14 subunits, has a proton pumping efficiency similar to that of mitochondrial Complex I [22]. This raises the question of the function of the supernumerary subunits of mitochondrial Complex I. It seems most probable that these supernumerary subunits are not involved in the fundamental enzyme mechanism but would rather add extra properties such as an increased stability, an adaptation of the Complex I biogenesis to mitochondrial compartmentation, or a finer regulation of the enzymatic activity. However, there are indications that some of these supernumerary polypeptides such as the 9.5-kDa subunit of *N. crassa* Complex I and the 14-kDa subunit of the IP fraction of beef heart Complex I might be directly involved in quinone binding. The purified 14 kDa subunit contains equistoichiometric amounts of quinone [23]. As for the 9.5-kDa subunit, it was labelled with a photoaffinity analog of quinone and is thus considered to potentially bear an ubiquinone binding site [24]. However, these subunits are absent in the bacterial enzyme although quinone binding sites are obviously essential features of Complex I. Two hypotheses can be put forward to explain the photoaffinity labelling of the 9.5-kDa subunit: (i) mitochondrial Complex I has evolved a more sophisticated enzymatic mechanism involving extra quinone

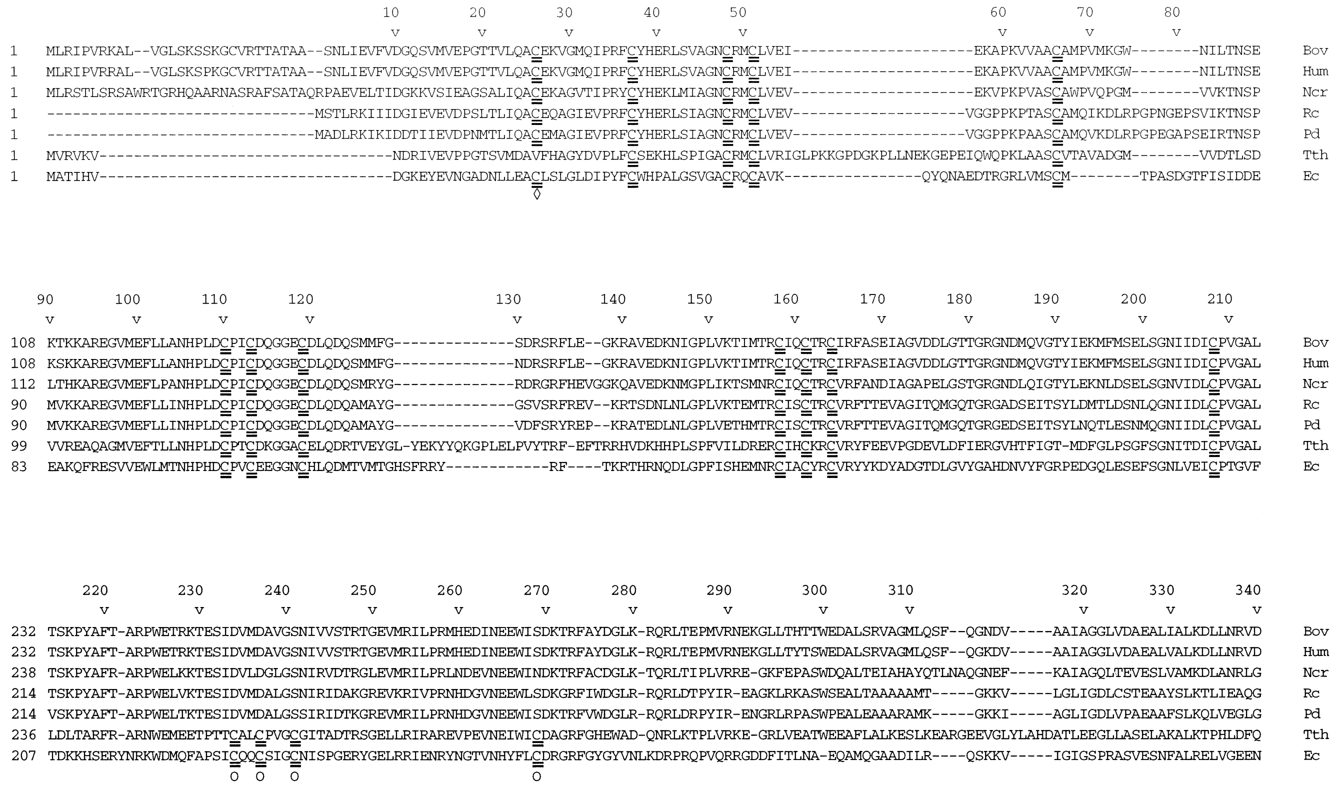

Fig. 2. Sequence comparison between mitochondrial and bacterial subunits equivalent to the bovine 75-kDa subunit. Only the first 340 amino acids of the *R. capsulatus* subunit have been aligned to the homologous segments of the equivalent proteins. Numbering corresponds to the *R. capsulatus* sequence. Bov: bovine 75 kDa premature subunit (Swiss-Prot accession number: P15760); Hum: human 75 kDa premature subunit (Swiss-Prot accession number: P28331); *Ncr.*: *Neurospora crassa* premature subunit (Swiss-Prot accession number: P24918); *Rc*: *R. capsulatus* NUOG subunit (GenBank accession number: AF029365); *Pd*: *P. denitrificans* NQO3 subunit (Swiss-Prot accession number: P29915); *Tth*: *T. thermophilus* NQO3 subunit (Swiss-Prot accession number: Q56223); *Ec*: *E. coli* NUOG subunit (Swiss-Prot accession number: P33602). Invariably conserved cysteine residues are double underlined. The first cysteine which is not conserved only in *T. thermophilus* is marked by a diamond. The additional four cysteine cluster found only in *E. coli* and *T. thermophilus* is indicated by open circles.

site(s), (ii) alternatively, the azido photoreactive group of the probe which is quite remote from the quinone ring may react at some distance from the actual quinone binding site.

2.2. Fe–S clusters of the Complex I of R. capsulatus

Complex I contains at least five EPR detectable Fe–S clusters: two binuclear clusters, known as N1a and N1b, and three tetranuclear clusters called N2, N3 and N4. The physical properties of these clusters as well as putative additional others have been reviewed by others [17,25]. In the bovine mitochondrial Complex I, a number of nuclear-encoded subunits are believed to contain Fe–S clusters. They are called PSST, 24 kDa, 51 kDa, 75 kDa and TYKY [16]. The first attempt to define the Fe–S clusters associated with *R. capsulatus* Complex I was reported by Zannoni and Ingledew [26]. By comparative potentiometric titrations of a mutant deprived of NADH-ubiquinone oxidoreductase activity (mutant M1 see below) these authors observed that two EPR signals at $g = 1.94$ could be associated with Complex I. These signals were characterized by a mid-point potential of -115 and -370 mV. By comparison with the midpoint potential of mitochondrial and *P. denitrificans* Complexes I [25], it can be proposed that the first signal corresponds to cluster N2 and the lower potential signal might be similar to the midpoint potential of cluster N1a. Actually, EPR and thermodynamic parameters for the FeS clusters of Complex I of *R. sphaeroides*—a bacteria closely related to *R. capsulatus*—are remarkably similar to those of *P. denitrificans* and mitochondrial Complexes I [25]. In the case of *R. capsulatus*, the M1 mutant has not been genetically characterized and the specific alteration of Complex I remains unclear. EPR studies of the membranes of different *nuo*-disruption mutants or of mutants altered in the Fe–S protein NUOI (equivalent to the mitochondrial TYKY subunit) evidenced the clear association of clusters equivalent to mitochondrial clusters N1 ($g_{xy} = 1.935$), N2 ($g_{xy} = 1.92$, $g_z = 2.05$) and N3 ($g_x = 1.86$, $g_y = 1.933$) with *R. capsulatus* Complex I [27,28]. Cluster N4 (g values for the bovine enzyme: $g_x = 1.88$, $g_z = 2.08$) was difficult to observe due to large overlaps with cluster N3 and with the intense Rieske signal ($g_y = 1.89$) present in *R. capsulatus* membranes [27,28].

2.3. Attempts to purify the Complex I of R. capsulatus

Most of the genetic studies developed with *R. capsulatus* Complex I would greatly benefit from the use of a preparation of pure Complex I. However, the purification of this membranous enzyme is limited by major drawbacks: firstly, the amount of Complex I appears to be low compared to other respiratory complexes in *R. capsulatus*. Secondly, most enzymatic assays used to characterize Complex I related NADH oxidase activity can reveal other independent NADH oxidase activities. Along this line, Oshima and Drews [29] isolated a single polypeptide with apparent NADH-2,6-dichloroindophenol oxidoreductase activity from *R. capsulatus* membranes. This polypeptide has been assumed to constitute a type II NDH [1]. However, genetic and biochemical data are consistent with the fact that no type II NDH is present in *R. capsulatus*, at least not when the bacteria are grown aerobically [11,21]. Actually, in our hands, use of deamino-NADH in combination with the short chain 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (decylUQ) allows the specific detection of the NADH-ubiquinone oxidoreductase activity of *R. capsulatus* Complex I. The main problem encountered in the purification of the *R. capsulatus* Complex I remains its instability upon detergent solubilization. Such an instability seems to be a general characteristic of bacterial type I NDHs: the procedure for purification of *P. denitrificans* NADH-ubiquinone oxidoreductase leads to a partial complex [30] and to date, only *E. coli* type I NDH enzyme has been reportedly purified to homogeneity in a stable form [19]. Even in this last case, most of the detergents promote the dissociation of the enzyme and one has to use acidic conditions and an alkylglucoside mixture (APG225 provided by Henkel, Germany) to prevent this effect. In the case of *R. capsulatus*, the instability of the enzyme upon solubilization has been consistently observed by three independent teams [11,21,31]. Two different strategies were used to evaluate the integrity of the solubilized complex: one way is to use centrifugation on sucrose gradient and to look for the distribution of NADH-decylUQ activity [11]. Alternatively, we have used subunit specific antibodies such as anti-NUOE and anti-NUOI antibodies to immunopurify associated subunit(s) [21]. Using both strategies, independent screening of many different detergents including glucopon 215 and 225 (two commercial equivalents of APG225) did not allow the isolation of a stable form of *R. capsulatus* Complex I (Refs. [11,21] and Dupuis et al. unpublished results). Alteration of the pH between 6.5 and 8.5, as well as addition of many stabilizers such as reducing agents, alcohols and polyols do not lead to any clear stabilization of the enzyme. As APG225 proved adequate for *E. coli* NADH-ubiquinone oxidoreductase isolation, further efforts were pursued to try and isolate *R. capsulatus* Complex I with its glucopon 215 and 225 equivalents. Two independent attempts using different technical approaches, i.e., immuno-purification in one case [21] and classical chromatographic purification in the other case [11], led to the isolation of the same small subcomplex composed of subunits NUOE and NUOF [11,21]. Like mitochondrial FP fraction, *R. capsulatus* NUOE–NUOF subcomplex contains 0.7 mol of FMN per mol and is able to catalyze NADH-ferricyanide oxidoreduction [11]. The difficulties encountered in *R. capsulatus* Complex I purification illustrate the great difference of stability between the mitochondrial and the bacterial NADH-ubiquinone oxidoreductases. Such an instability of the bacterial type I NDH compared to its mitochondrial equivalent might give a key to understand the relative complexity of the mitochondrial enzyme: some of its ancillary subunits may have been added to stabilize the mitochondrial Complex I enzyme.

3. *R. capsulatus*, a powerful system for the genetic study of Complex I

Although interesting models have been proposed to explain the mechanism of Complex I (Refs. [32,33] and Ohnishi's contribution in this issue) there is an urgent need for the design of new experimental approaches to validate these models. The studies of the mitochondrial respiratory Complexes III and IV have largely benefited from fast spectroscopic techniques compatible with the time scale of electron transfers. Unfortunately, in the case of Complex I, the absence of appropriate chromophores have precluded this kind of approach and the time constants of procedures such as rapid freezing and EPR do not allow the dissection of the electron transfer process. There is evidence for the existence of different inhibitor binding sites but we still have no clear evidence for the existence of associated 'intermediate states'. Genetic approaches might be a way to solve these difficulties: mutants altered at different points of the enzyme can be expected to display altered enzymatic reactions (partial redox reaction, partial proton pumping or uncoupling between redox reactions and proton pumping...) thus allowing a dissection of the Complex I mechanism. In this regard, the existence of powerful genetics techniques to manipulate this bacteria [34] makes *R. capsulatus* an attractive model for the genetic study of Complex I. This can be illustrated here by four different genetic approaches. (i) First *R. capsulatus* mutants deficient at the level of Complex I can be generated by interposon mediated gene disruption (Section 3.1). Although these genetic alterations are very coarse, they compare somewhat to mitochondrial deletions associated with human pathologies [35]. These mutants were helpful to define the growth phenotype of Complex I-deficient mutants and constitute a valuable starting material for further refined genetic studies. (ii) Such a refined strategy is illustrated by the site directed mutagenesis study of the putative Fe–S cluster insertion motifs of the NUOI subunit (Section 3.2). (iii) Alternatively, *R. capsulatus* can be used as a model system to study point mutations of the mitochondrial ND genes associated with pathologies such as the Leber's disease (Section 3.3). (iv) Finally, classical random mutagenesis constitutes a powerful approach to map the different inhibitors and quinone binding sites of the Complex I (Section 3.4).

3.1. Genetic disruption of the nuo operon

The general procedure to alter *R. capsulatus* genome makes use of bacterial conjugation with an *E. coli* donor strain such as S17-1 [36]. This allows the transfer of either replicative plasmids such as pRK415 [37] for protein expression and complementation or non-replicative (suicide) plasmids such as pPHU281 [38] for homologous recombination. In the present case, this last strategy was used to individually disrupt *nuo* genes by insertion of KIXX interposon [39]. KIXX interposon presents two resistance genes for kanamycin and bleomycin. It has no transcription termination signal and its interposition in a given gene is reputed to induce no or limited polar effect on cotranscribed genes. In practice, gene disruption mutants are generated by legitimate double cross-over between a circular suicide plasmide bearing the targeted gene disrupted by KIXX and *R. capsulatus* genome. Thus, the screening for such mutants has to be conducted in two steps: first, all the recombinants carrying the interposon (constructed either by single or by double cross-over) are isolated on a kanamycin-containing medium. Then, true gene disruption mutants resulting exclusively from double cross-over events are characterized by the loss of a plasmid born marker (tetracyline-resistance in the case of pPHU281). In our hands an efficient gene disruption requires flanking regions at least equal to 500 bp. Actually, we experienced double cross-over frequencies between 0.5 and 5% of the total recombinants using suicide constructions with interposon flanking regions ranging from 450 bp to 2.2 kbp, respectively.

In bacteria where only a type I NADH-ubiquinone oxidoreductase is present, such as *R. capsulatus* and *P. denitrificans*, genetic alteration of the type I NADH-ubiquinone oxidoreductase is expected to alter their ability to grow aerobically. In this respect, *R. capsulatus* is a very versatile bacteria able to grow under numerous conditions including photoheterotrophic anaerobiosis [40]. Under these last conditions energization of the bacterial membrane is realized by the photosystem whereas organic carbon source and reducing equivalents are provided to the bacteria in the form of a substrate such as lactate or malate. Thus, photoheterotrophy looks well-suited to isolate and to maintain respiratory-deficient mutants. Along this line, Marrs et al. [41] designed a negative screening strategy to isolate *R. capsulatus* respiratory-deficient mutants: following exposure to nitrosoguanidine, *R. capsulatus* was cultured aerobically in the presence of penicillin. This allowed the counterselection of *R. capsulatus* strains unable to grow under dark aerobic conditions. After suppression of the antibiotic, the still viable cells were allowed to grow under photoheterotrophic conditions. Using this screening strategy they successfully isolated several mutants of the respiratory chain. One of those (M1) exhibited an altered NADH-ubiquinone oxidoreductase activity. This M1 mutant was unable to grow under aerobic conditions but exhibited normal growth in strictly anaerobic photoheterotrophic cultures. Thus, to isolate *nuo*-disruption mutants generated by KIXX interposon specific insertions in different *R. capsulatus nuo* genes, we first chose photoheterotrophic growth conditions. The different genes individually targeted in this study were *nuoH*, *nuoI*, *nuoJ*, *nuoK*, *nuoL*, *nuoM* and *nuoN* corresponding respectively to ND1, TYKY, ND6, ND4L, ND5, ND4 and ND2 in the mitochondria. No mutant could be isolated under anaerobic photoheterotrophic growth conditions using lactate, malate or succinate as a carbon source. However, for all the genes targeted, *nuo*-disruption mutants could be isolated under aerobic conditions [42]. The difference of phenotype between these mutants and M1 mutant is puzzling. This observation has now been confirmed by extensive growth studies: *nuo*-disruption mutants are able to grow aerobically in the dark on lactate or malate but are unable to grow photoheterotrophically on these carbon sources under anaerobiosis. However, *R. capsulatus* Complex I-deficient mutants can grow photoheterotrophically under anaerobiosis, provided DMSO is added to the culture [42]. This phenotype can be extended to point mutants, such as the C74S mutant of the NUOI subunit (see below and Ref. [27]). To explain the phenotype of *R. capsulatus* *nuo*-disruption mutants, it can be noted that this phenotype appears remarkably similar to the phenotype of *R. sphaeroides* Rubisco-deficient mutants [43]. In this last case, screening for compensatory mutations led to the isolation of nitrogenase-deregulated mutants. The simplest explanation for this compensatory effect is to consider that nitrogenase drains the excess of reducing equivalents accumulating in the absence of carbon dioxide fixation. Actually, the common characteristic of nitrogenase and DMSO reductase pathways is that they drain electrons from ubiquinol. *R. capsulatus* is known to be very sensitive to redox unbalance [44]. This might be associated with the fact that cyclic photosynthesis requires oxi-

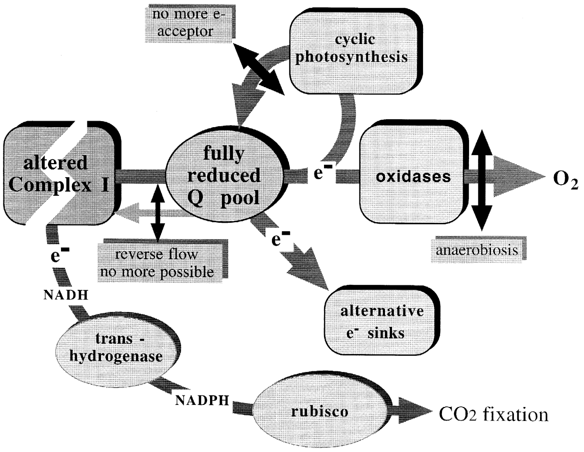

Fig. 3. Tentative metabolic rationalization of the photoheterotrophy negative phenotype of *nuo*-disruption mutants. In *R. capsulatus*, unpoised redox state is known to alter the efficiency of photosynthetic electron chain [44]. Under photoheterotrophic conditions, lactate or succinate oxidation can lead to an overreduction of the quinone pool. In the wild type strain, this excess of reducing equivalents can be transferred from the ubiquinone to NAD^+ by reverse flow at the level of the Complex I. These reducing equivalents are then normally dissipated by carbon dioxide fixation with the participation of the Rubisco. Thus, *R. capsulatus nuo*-disruption mutants appear to be unable to poise the redox state of ubiquinone pool via carbon dioxide fixation. This control of the quinol pool redox state can be achieved by alternative electron sinks such as the DMSO reductase, thus rescuing the *nuo*-disrupted mutants. Vertical black arrows are figuring blockade of anaerobic metabolism resulting from Complex I deficiency.

dized ubiquinone as an acceptor for the electrons of the bacteriochlorophyll (Fig. 3). Thus maintaining all the ubiquinone pool in a reduced state might glut the photosynthetic pathway. Under photoheterotrophic anaerobic conditions, *R. capsulatus* normally down regulates the redox poise of its nicotinic cofactor pool (NAD/NADH and NADP/NADPH) by carbon dioxide fixation with the participation of the Rubisco. Transfer of reducing equivalents from the ubiquinone to NAD^+ by reverse flow at the level of the Complex I might extend this pathway of redox regulation (Fig. 3). In such a case, *R. capsulatus nuo*-disruption mutants would be unable to poise the redox state of ubiquinone pool via carbon dioxide fixation. A reverse flow of electrons at the level of *R. capsulatus* Complex I has been demonstrated in vitro [45]. The phenotype of the *nuo*-disruption mutants brings the first direct in vivo evidence for such a major involvement of *R. capsulatus* Complex I in photosynthesis metabolism.

As noted above, although clearly deficient in their NADH-ubiquinone oxidoreductase activity, *R. capsulatus nuo*-disruption mutants do grow aerobically on energy and carbon sources like malate or lactate. The efficiency of the different carbon sources used for the cultures in dark aerobic conditions calls for some comments. Under these conditions malate appears to be a poor carbon source for the mutants whereas in the presence of lactate, the growth characteristics of the mutants are very similar to those of the wild type strains. This high capacity of the *nuo*-disruption mutants to grow specifically on lactate was explained by the demonstration of the existence of an NAD-independent lactate dehydrogenase (iLDH) in the membrane of *R. capsulatus* [42]. This activity is insensitive to all the classical inhibitors of Complex I and allows transfer of reducing equivalents to the respiratory chain directly at the level of the quinone pool. This iLDH activity in *R. capsulatus* is very fortunate: indeed, in *P. denitrificans*, Complex I appears to be a vital enzyme. Although the insertion of *E. coli* type II NDH was a very smart way to overcome the apparent impossibility to obtain Complex I altered mutants [46], the presence of a type II NDH activity in the resulting mutants could lead to complications in complementation experiments and for enzymatic studies of the different mutants thus constructed. In this respect, *R. capsulatus* constitutes a better model for mutagenesis and complementation studies of the type I NDH.

At the biochemical level, disruption of any of the seven *R. capsulatus nuo* genes targeted so far abolishes both NADH-oxidase and deamino-NADH-ferricyanide activities at the level of *R. capsulatus* membranes. Furthermore, these activities cannot be evidenced in concentrated cytosolic fractions of the *nuo*-disruption mutants. This suggests that partially active subcomplexes are not present either in chromatophores or in cytosol concentrates of our mutants. In *N. crassa*, chloramphenicol inhibition of the biosynthesis of the mitochondrially-encoded ND subunits, led to the assembly of a catalytically active subcomplex composed of about 16 subunits and presenting NADH-ubiquinone oxidoreductase activity [47]. This contrasts with the enzymatic properties of our *nuo*-bacteria disruption mutants. Actually, immunochemical characterization of these mutants shows that noticeable amounts of the peripheral subunits NUOC, NUOD, NUOE, as well as subunits NUOI and NUOJ can still be found in the membranes of the different *nuo*-disruption mutants [28]. However, the apparent variability in the amount of these subunits suggests that they are not stoichiometrically-associated in a single inactive subcomplex. Similarly, absence of assembly of Complex I was also noticed in human cell line mutants lacking the mitochondria-encoded subunits ND4 and ND5 due to frameshift mutations [48,49].

Gene disruption is a powerful way to address the function (if any) of the orfs identified in *R. caspulatus nuo* operon. We thus constructed mutants NU6K1 and W1, respectively, disrupted in *R. capsulatus urf6* and *urf7* [28,27]. These orfs are located between *nuoH* and *nuoI* and between *nuoI* and *nuoJ*, respectively (Fig. 1). They are not present in *E. coli* operon [8] and only *urf7* has an equivalent (*urf6*) in *P. denitrificans* [9,50]. In this last case, based on its similarity with the gamma muconolactone decarboxylase, Yagi proposed that the protein encoded by *P. denitrificans urf6* might be involved in some regulation dependent on lactone metabolism. In such a case, the presence of this orf in *P. denitrificans* and *R. capsulatus* might correspond to a specific metabolic adaptation of these bacteria which is absent in *E. coli*. In a similar way, the specific presence of *urf1*, *urf2*, *urf3*, *urf4* and *urf6* in *R. capsulatus* with no homologous equivalent even in *P. denitrificans* might be related to the peculiarity of *R. capsulatus* photosynthetic metabolism, i.e., the physiological functioning of type I NDH in reverse flow. However, mutants NU6K1 and W1 have a phenotype identical to wild type in all the growth conditions tested. Furthermore, contrary to the other disruption mutants, NU6K1 and W1 display, respectively, 51 and 103% of the wild type Complex I activity. For us, the partial decrease of Complex I activity in NU6K1 can be attributed to a moderate polar effect of the KIXX interposon. Thus, the present observations strongly indicate that these *urf6* and *urf7* are not essential for the biosynthesis of Complex I either under heterotrophic aerobic conditions or under photoheterotrophic conditions. On the other hand, several trials to disrupt *urf242* which is located immediately downstream of the *nuo* operon proved unsuccessful. Whereas KIXX insertion in the nearby *nuoN* gene was easily generated, only single recombinants orientated in the same transcription orientation could be isolated (unpublished results). Such a single cross-over allows the reconstruction of a normal copy of *urfR242*. This suggests that *urfR242*, which has a highly conserved homolog in *P. denitrificans* [50], is encoding an essential protein in *R. capsulatus*. The high similarity of the protein encoded by *urfR242* with *E. coli birA* product [13] suggests that this protein must be involved in the biotinylation process in *R. capsulatus*.

We took advantage of the innocuity of *urf7* disruption on the Complex I biogenesis and activity to develop a strategy to generate point mutants altered at the level of the two putative Fe–S cluster insertion motifs of the NUOI subunit.

3.2. Generation of point mutants altered in the NUOI subunit of R. capsulatus Complex I

The unambiguous assignment of all the Fe–S clusters to the different subunits of Complex I has not yet been completed. Due to its rather high midpoint potential and to the dependance of this potential upon pH, the N2 center is considered to play a key role in the coupling between electron and proton fluxes. The two most probable candidates to harbor cluster N2 are the PSST (NUOB) subunit and the TYKY (NUOI) subunit. The characteristics of subunit PSST have been discussed in Section 2.1. Highly conserved subunits identical to the bovine TYKY subunit, were also found in human [51], in *N. crassa* [52], in *Trypanosoma brucei* (kinetoplast DNA-encoded subunit) [53], in the cyanobacterium *Synechocystis* [54], in plant mitochondria (nuclear-encoded subunit) [55] and in different bacteria such as *R. capsulatus*, *P. denitrificans*, *T. thermophilus* and *E. coli* [6,8–10]. In an attempt to get more information about the role NUOI plays in the *R. capsulatus* Complex I and to try to define which Fe–S cluster(s) is (are) bound to this subunit, we mutagenized the *R. capsulatus nuoI* gene so as to generate a set of NUOI missense mutants. In the Complex I field, in vivo mutagenesis has been already experienced in a number of species like *N. crassa*, *Aspergillus niger* or *P. denitrificans* [56–60,46]. But invariably, only gene knockouts have been performed in vivo till now. Alternative more refined genetic studies were performed on *P. denitrificans* NQO2 or bovine mitochondrial 24 kDa subunit using site-directed mutagenesis, but these were only conducted on the overexpressed protein [61,62]. In the case of *R. capsulatus*, the authors have developed an in vivo mutagenesis approach: *nuoI* missense mutations were introduced in *R. capsulatus* either by complementation of a mutant strain with deleted *nuoI* gene (Δ*nuoI*) or directly at the level of the bacterial chromosome by gene recombination [27]. A panel of mutants altered at the level of the cysteine residues of NUOI was generated. The mutant C74S produced by replacement of cysteine number 74 by a serine and the C67S mutant generated by replacement of cysteine number 67 are representative of this study. Similar to the *nuo*-disruption mutants presented above, C74S mutant was totally unable to grow under photosynthetic and anaerobic conditions whereas mutant C67S grew normally under these conditions. Photosynthetic capacities of C74S mutant were restored by transcomplementation with a plasmid controlling the expression of the wild type *nuoI* gene only. The difference of C67S mutant membranes with C74S mutant membranes is that C74S mutant membranes catalyzed NADH oxidation coupled with oxygen consumption at a very low rate and the NADH oxidase and deamino-NADH-ferricyanide oxidoreductase activities were nearly abolished. EPR analysis clearly indicated that both the N1 and N2 clusters are not significantly assembled at the level of the membranes in the C74S mutant [27]. By immunodetection, the authors also found that C74S mutant membranes were nearly completely depleted of the major peripheral subunits. However, the total amount of the NUOJ subunit (ND6 equivalent) was found to be comparable to that detected in the wild type strain membranes. This loss of peripheral subunits was concomitant with the disappearance of the Complex I Fe–S clusters from the cytoplasmic membranes and clearly indicated that proper assembly of the *R. capsulatus* Complex I strictly requires the presence of an intact NUOI subunit. The presence of noticeable amounts of NUOI subunit in the C74S membranes in the absence of the NUOC, D, E, F shows that NUOI is indeed able to bind directly to the membrane or to interact with the Complex I subdomain assembled in the membrane. These observations on C74S mutant support the idea that NUOI is located at the junction between the membranous domain and the matricial domain where it may play a critical role in the assembly process between the two main parts of Complex I [27]. Probable insertion of the NUOI (or equivalent subunits) into the membrane has been postulated in two species: in *E. coli*, where the N-terminal part of the subunit has been hypothesized to be a transmembranous domain [8] and in *T. brucei*, where both the hydrophobic N and C-termini have been reported as putative membrane-spanning domains [53]. Unlike these two proteins, *R. capsulatus* NUOI homolog subunit does not exhibit similar hydrophobic features. The possibility still remains, however, that in the C74S NUOI mutant, the mutated NUOI subunit still has some affinity for the ND-like membranous anchoring proteins.

NUOI displays a marked similarity with ferredoxins bearing two [4Fe–4S] cluster-binding motifs. Thus, it is worthwhile making a comparison between our mutagenesis experiments and those performed with the 2 × [4Fe–4S] ferredoxin of *Clostridium pasteurianum* where a number of mutations at the level of the fourth cysteine of the first cysteine canonical motif (corresponding to Cys74 in *R. capsulatus* NUOI) have been performed [63]. Expression of this mutated gene in *E. coli* led to either the absence of the ferredoxin or else to an unstable or poorly active ferredoxin. According to the determined 3D structures of the 2 × [4Fe–4S] bacterial ferredoxins [64], while being part of the first cysteine sequence cluster, the fourth cysteine in this first cysteine motif actually participates in the binding of the second Fe–S cubane with three remote cysteines (Fig. 4). Consequently, the cysteine 74 of *R. capsulatus* NUOI subunit may greatly contribute to the overall tertiary structure of the protein by bridging the two cubanes. Substitution of this residue probably leads to the expression of an unstable protein, which is most likely rapidly degraded in the cell.

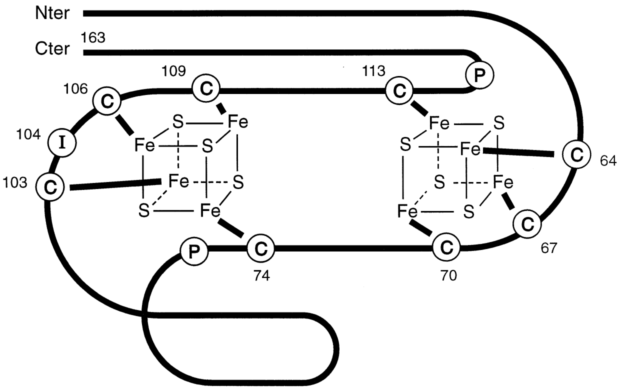

Fig. 4. Schematic structure of the NUOI subunit. The position of the eight highly conserved cysteine residues (C64, C67, C70, C74, C103, C106, C109, C113) and their role as ligands of the two probable [4Fe–4S] clusters is shown. Assignment of the positions of the cysteine groups to the Fe–S clusters was made by comparison of the NUOI subunit to that of 2× [4Fe–4S] bacterial ferredoxins.

The C67S mutant largely differs from C74S mutant. First, C67S strain is able to grow under anaerobiosis. Membranes of C67S display a respiratory activity up to 43% of the wild type membranes. Finally, EPR characterization of C67S membranes demonstrated the presence of N1, N2, N3 and N4 Fe–S clusters. In this case, we reproducibly observed a change in the N1/N2 ratio (unpublished data). These observations were taken as evidence that the NUOI subunit actually harbors two N2 iron sulfur clusters. EPR behavior of two such Fe–S cluster is difficult to predict: ferromagnetic coupling can lead to EPR silent clusters. Alternatively, spin–spin interaction can lead to a splitting of the g lines associated with nearby ferromagnetic centers. Along this line, in the bovine Complex I, van Belzen et al. [65] observed a splitting the g_z line of cluster 2. Multifrequency analysis of this splitting strongly supports the idea that it occurs from spin–spin coupling between two clusters 2. In agreement with our observation on *R. capsulatus* mutant these authors postulated that these two clusters 2 were actually associated with the bovine TYKY subunit [65,17].

3.3. Reproduction of one human pathological alteration of nd1 gene in R. capsulatus

The association of discrete alterations of mitochondrial *nd* genes with human neuropathies such as Leber's disease has been largely documented and is reviewed by Shapira in the present issue. However, the biochemical effects of these genetic alterations on Complex I remain largely uncertain because high mutation rate of mitochondrial genome generates a high degree of heterogeneity and also because mitochondrial Complex I is encoded both by mitochondrial and nuclear genomes. As a consequence, the impact of a mitochondrial mutation can be dependent on the nuclear background. This is illustrated for example, by the large phenotypic variability observed in some Leber's cases (spontaneous recovery, age of disease onset, ...) associated with the same primary mtDNA mutation. As demonstrated in the case of mutation leu207 → arg affecting the subunit a of the human mitochondrial F_1F_0 ATPase, reconstruction of a human mtDNA mutation in a prokaryotic model system can greatly help to further characterize the biochemical effect of the alteration [66]. For these reasons, bacterial NDH appears an interesting model to reproduce, in a genetically homogenous background, mitochondrial pathological point mutations that affect Complex I.

The G → A transition at position 3460 of mtDNA which leads to the replacement of alanine residue at position 52 of human subunit ND1 by a threonine is a major primary mutation associated with Leber's disease. When tested with decylubiquinone [67] or undecylubiquinone [68], the Complex I of patients' mitochondria exhibit a 20–50% decrease of the NADH-ubiquinone oxidoreductase activity [67,68]. The increased inhibitory effect of short chain quinones on the Complex I enzymatic activity of patients' mitochondria suggests that the mutation induces an alteration in the quinone binding site [67]. A small decrease in sensitivity to rotenone was also observed by some authors [68]. On the other hand, the efficiency of oxidative phosphorylation and of proton translocation by Complex I is unaffected in ND1/3460 mutant cells [67]. Likewise, the turnover number of Complex I and respiration rate are not significantly limited during respiration with NAD^+-linked substrates [67]. The discrepancies which can be found in the literature for the biochemical alterations associated with this mutation may have several reasons. Beside the different nature of the biological material used or the enzymatic activities assayed, this can also be due in part to differences in the genetic background of the mutation and to the difficulty to define a matched 'wild' reference.

Alanine residue 52 of human ND1 is conserved in the corresponding *R. capsulatus* NUOH subunit (alanine residue 65). In this regard, *R. capsulatus* NDH can be considered as a reference system since the mutation can be introduced in a controlled genetic background (i.e., mutant and wild type strain differing only by this mutation). Homologous recombination was used to substitute either a Ala65 → Thr mutated or a wild type copy of the *nuoH* gene for the disrupted *nuoH* gene in the mutant NHK1. In both cases, reinsertion of the *nuoH* gene by double crossover led to the recovery of a wild type phenotype and NDH activity. Comparative studies between both recombinants did not show any alteration of the growth phenotype of the resulting NUOH/'3460' mutant on carbon sources such as lactate or malate, under aerobiosis or anaerobiosis. The NADH-ferricyanide oxidoreduction activity was unchanged in the mutant thus indicating that the amount of Complex I was not decreased in mutant membranes. As was observed with mitochondrial mutants, a small but significant decrease in the NADH oxidase activity of the *R. capsulatus* NUOH/'3460' mutant was reproducibly obtained. Mutant activity amounted to $80 +/- 5\%$ of the wild type activity. Furthermore, no difference in the sensitivity of the NADH oxidase to the inhibitors piericidin or rotenone could be evidenced when comparing the mutant and the wild type NADH oxidases in *R. capsulatus*. This study conducted in a controlled genetic background confirms that the effect of the NUOH/'3460' mutation on the overall *R. capsulatus* Complex I function is not very dramatic. This is in agreement with conclusions indicating that Complex I-dependent respiration is modestly reduced in lymphoblastoid cell lines harboring the ND1/3460 mutation [67]. While no definitive conclusions emerged from these experiments as to the precise biochemical defect associated with the ND1/3460 mutation in Leber's disease pathogeny, these results indicate however that *R. capsulatus* Complex I represents an appropriate model which may become even more valuable once this enzyme is obtained in a purified form.

3.4. Isolation of piericidin-resistant mutants

A better knowledge of the structure of the quinone binding sites is mandatory to progress in the elucidation of Complex I mechanism. However, very few experimental approaches are available to tackle this subject. Inhibitors such as rotenone and piericidin seem to bind at or close to the quinone binding sites of Complex I. Thus, characterization of the binding sites for these inhibitors is essential for the elucidation of the proton pumping mechanism. Isolation of inhibitor-resistant mutants constitutes an original approach to characterize directly the inhibitor binding sites of Complex I. Such an approach was first tried in eukaryote systems like human and *Drosophila* cell lines. Human cell lines resistant to rotenone have been isolated by Hofhaus and Attardi [49]. However, these resistant cell lines proved deficient in NADH-dependent respiration and their resistance did not appear related to Complex I. Similarly, resistance to rotenone induced in *Drosophila* cell lines was associated with a protein which binds and neutralizes the inhibitor rather than a true Complex I alteration [69]. As presented above, a slight decrease of rotenone-sensitivity in human Complex I was also observed in patients' samples with pathological mutations on ND1 and ND4 genes [68]. Furthermore, photoaffinity labelling of Complex I with radioactive azidomorphigenin or dihydrorotenone led to the labelling of the ND1 subunit [70,71]. All these data point to ND1 or ND4 as candidates for location of a rotenone binding site. Since in the eukaryotes, the ND genes are not easily amenable to genetic studies, bacterial systems appear especially interesting to develop such studies. Compared to *R. capsulatus* or *P. denitrificans*, *E. coli* displays a rather poor sensitivity to most of the inhibitors targeting mitochondrial Complex I [72]. This can be tentatively related to the fact that the cytoplasmic membrane of *E. coli* contains mainly demethylmenaquinone-8, menaquinone-8 and benzoquinone Q8 [73] whereas the mitochondria, as well as *R. capsulatus* [74] contain exclusively the ubiquinone Q10. Thus, *E. coli* does not seem to constitute the best model for the study of Complex I quinone and related inhibitor binding sites. This prompted us to take advantage of our bacterial system to try and isolate piericidin-A-resistant mutants. The choice of piericidin was dictated by several considerations: (i) piericidin is one of the most potent inhibitors both for mitochondrial Complex I ($I_{50} = 1$ nM) and for *R. capsulatus* NDH ($I_{50} = 0.45$ nM) (Table 2), (ii) both NADH-driven respiration and NADH oxidase activities borne by *R. capsulatus* membranes are at least 95% sensitive to piericidin and (iii) among the different Complex I inhibitors, piericidin displays a striking structural similarity with ubiquinone.

Table 2
Sensitivity of the NADH oxidase activity of wild type strain and piericidin-resistant mutant membranes to different Complex I inhibitors

Inhibitors		Wild type	Mutants	I_{50} ratio
Piericidin	max. inhibition	> 95%	95%	
	I_{50}	0.45 nM	8 nM	18
Rotenone	max. inhibition	> 95%	95%	
	I_{50}	100 nM	1.2 μM	12
Rolliniastatin-2	max. inhibition	> 95%	95%	
	I_{50}	100 nM	100 nM	1
Capsaicin	max. inhibition	80%	80%	
	I_{50}	0.1 mM	0.1 mM	1

NADH oxidase activities were recorded spectrophotometrically with 0.1 mg of membranes.

We have reported above that *R. capsulatus* Complex I-deficient mutants are unable to grow photosynthetically under anaerobiosis. Similarly, we first thought that blockade of Complex I by piericidin would lead to a similar growth deficiency under photosynthetic growth conditions. Indeed, piericidin concentrations up to 10 μM induce a 3–5-day retardation of the growth on solid medium under photosynthesis. However, under these conditions, piericidin growth inhibition did not look stringent enough to allow an efficient screening for piericidin-resistant mutants. The apparent discrepancy between these observations and the phenotype described above for Complex I-deficient mutants might be explained in different ways; one of which might be that anoxygenic phototrophic bacteria such as *R. capsulatus* are able to photodegrade aromatic toxics [75]. After several other unsuccessful trials, we designed an original double inhibitory screening procedure based on the following rationale (Fig. 5). In aerobic growth conditions, inhibition of Complex III (complex b/c1) by myxothiazol is not sufficient to prevent the bacterial growth. This is mainly due to the presence of an alternative quinol oxidase in the cytoplasmic membrane of *R. capsulatus* [76] (Fig. 5). However, our *nuo*-disruption mutants do not grow aerobically with malate as a carbon source in the presence of 10 μM myxothiazol. This suggests that the alternative oxidase pathway by its own is not sufficient to allow a normal bacterial growth. Wild type strain grown on malate in the presence of piericidin must experience a metabolic stress similar to that experienced by Complex I-deficient mutants. Therefore, in a medium containing both piericidin (2.5 μM) and myxothiazol (10 μM) and malate as a carbon source, only bacteria resistant to one of the two inhibitors can grow. As myxothiazol fully inhibits photosynthetic growth of *R. capsulatus* by blockade of the cyclic photosynthesis, it is easy to identify mutants resistant to this inhibitor due to their ability to grow in the presence of myxothiazol under photoheterotrophy. Actually, about 90% of the colonies isolated in the (myxothiazol + piericidin) screening appeared to be myxothiazol-resistant but the remaining mutants proved clearly sensitive to myxothiazol and resistant to piericidin. Two independent experiments led to two different types of mutants. A first group demonstrating a rather low resistance factor (2–3 times) was not further studied. We concentrated on the other set of mutants which displayed an apparent decrease of piericidin-sensitivity at least 18 times higher ($I_{50} = 8$ nM) (Table 2) [77]. As discussed above, in the mitochondrial system, survey of the literature pointed to subunits ND1 and ND4 as the main candidates for quinone and rotenone binding sites. However, sequencing demonstrated that the mutation responsible for piericidin-resistance in our mutants is not borne by these subunits.

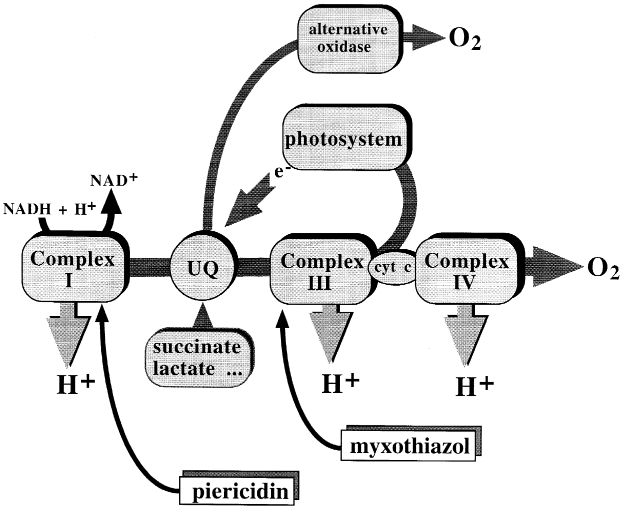

Fig. 5. Metabolic rationale of the piericidin-resistance screening procedure. Under aerobic growth conditions, inhibition of Complex I by piericidin is not sufficient to prevent the bacterial growth. Under these conditions, membrane energization results from proton pumping by Complex III and IV. Similarly, inhibition of Complex III by myxothiazol alone is not sufficient to prevent the bacterial aerobic growth. Under these conditions, the electron flow through Complex I and alternative quinol oxidase generates a vital membrane potential. Since the *nuo*-disruption mutants do not grow aerobically in the presence of myxothiazol, most of the energization of the cytoplasmic membrane under such conditions must result from proton pumping at the level of Complex I. Accordingly, when Complex I and Complex III are blocked by the combination of piericidin and myxothiazol, energization of the bacterial membrane is prevented and only mutants resistant to either of the two inhibitors are able to grow. Also, as myxothiazol blocks cyclic photosynthesis, mutants resistant to this inhibitor can be discriminated by their ability to grow photosynthetically in the presence of this inhibitor.

Enzymatic studies have demonstrated the existence of at least three classes of Complex I inhibitor binding sites but this classification remains uncertain and sites appear to somehow overlap (see Degli Esposti's contribution in this issue for a review on the inhibitor binding sites). Furthermore, the relationship between these different types of sites and the ubiquinone binding sites remains unclear. Our genetic study constitutes an original alternative to characterize directly the inhibitor binding sites of Complex I. Piericidin is considered to bind to two different sites, one of these is also the rotenone binding site [78]. The piericidin-resistant mutants are also clearly resistant to rotenone (Table 2) but display an unmodified sensitivity to rolliniastatin-2 or capsaicin [77]. In the light of the present knowledge on Complex I inhibitor binding sites, the simplest explanation of our data is to assume that our mutants would be altered at a site shared by rotenone and piericidin but not by capsaicin and rolliniastatin. The great variability of structure of the different Complex I inhibitors suggests that the quinone and inhibitors binding domains might rather represent a large interactions area shared by different inhibitors rather than a well-defined binding site. Thus, the promising genetic approach presented here is still in its infancy and has to be extended by the isolation and characterization of resistant mutants against other Complex I inhibitors to really get a realistic representation of Complex I inhibitor binding sites.

4. Conclusions

Among bacterial NDH, *R. capsulatus* Complex I constitutes an interesting model for the study of Complex I: compared to *E. coli* NDH, the subunits of *R. capsulatus* Complex I are far more homologous to the mitochondrial enzyme. Similarly, the identified EPR signals associated with *R. capsulatus* as well as *P. denitrificans* and *R. sphaeroides* enzymes compare well with those of mitochondrial Complex I [25,28]. The main limitation of this system is the instability of the complex that has precluded so far the isolation of an intact solubilized enzyme. On the other hand, this system presents several attractive advantages. First, the efficiency of proton pumping of *R. capsulatus* Complex I has been demonstrated for quite a long time [5]. Thus, despite its simplicity, this bacterial NADH-ubiquinone oxidoreductase must function essentially as the mitochondrial enzyme. It constitutes a kind of minimal system to study the fundamental enzymatic mechanism of this type of enzyme. The difference with *E. coli* and *R. capsulatus* is that the absence of a type II NADH-ubiquinone oxidoreductase in *R. capsulatus* simplifies genetic and enzymatic studies. Powerful genetic tools have been developed to manipulate the genome of *R. capsulatus* [34] and this allows the development of genetic approaches hardly accessible with mitochondrial systems especially for the mitochondria-encoded ND subunits. In the case of *R. capsulatus*, its versatile metabolism and the presence of a NADH-independent lactate dehydrogenase bypassing the Complex I allows easy isolation of Complex I-deficient mutants as demonstrated by the construction of *nuo*-disruption mutants and several missense mutants [27,28,42]. Reconstruction of pathological mtDNA mutations in *R. capsulatus* can be easily achieved. As an example, construction of mutation ND1/‘3460’ in the controlled genetic background provided by *R. capsulatus* confirmed the observations reported for the human Complex I. The accessibility to a genetic approach is also of interest for the study of the quinone and inhibitor binding sites. In this regard, again different to *E. coli*, *R. capsulatus* membranes contain the same ubiquinone Q10 as the mitochondrial system. As illustrated by the study of *R. capsulatus* piericidin-resistant mutants, this original and powerful approach can give essential informations on the quinone and inhibitor binding sites of Complex I. Finally, the *R. capsulatus* system presents one other major interest not yet exploited in the enzymatic study of the Complex I: the presence of the bacterial photosystem allows fast reduction of the quinone pool and fast energization of the membranes.

Klemme et al. [45] demonstrated that *R. capsulatus* Complex I is able to reduce NAD$^+$ under such conditions in vitro. Since spectral change of the carotenoid band also allows fast measurements of the membrane potential [79], *R. capsulatus* membranes may be used for fast kinetic reverse flow experiments not possible with other mitochondrial or bacterial systems. This approach will be developed to further refine the study of the different Complex I-deficient mutants already constructed in *R. capsulatus*. All these considerations demonstrate that *R. capsulatus* Complex I has its own specificities complementary to the other studied Complexes I. Integration of all the advantages of the different systems will allow decisive progress to be made in the understanding of this sophisticated enzyme.

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