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Review

Assembly of the *Escherichia coli* NADH:ubiquinone oxidoreductase (complex I)

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ABSTRACT

The proton-pumping NADH:ubiquinone oxidoreductase is the first of the respiratory chain complexes in many bacteria and the mitochondria of most eukaryotes. In general, the bacterial complex consists of 14 different subunits. In addition to the homologues of these subunits, the mitochondrial complex contains approximately 31 additional proteins. While it was shown that the mitochondrial complex is assembled from distinct intermediates, nothing is known about the assembly of the bacterial complex. We used *Escherichia coli* mutants, in which the *nuo*-genes coding the subunits of complex I were individually disrupted by an insertion of a resistance cartridge to determine whether they are required for the assembly of a functional complex I. No complex I-mediated enzyme activity was detectable in the mutant membranes and it was not possible to extract a structurally intact complex I from the mutant membranes. However, the subunits and the cofactors of the soluble NADH dehydrogenase fragment of the complex were detected in the cytoplasm of some of the *nuo*-mutants. It is discussed whether this fragment represents an assembly intermediate. In addition, a membrane-bound fragment exhibiting NADH/ferricyanide oxidoreductase activity and containing the iron–sulfur cluster N2 was detected in one mutant.

1. Introduction

The proton-pumping NADH:ubiquinone oxidoreductase, also called respiratory complex I, is the least understood of all the respiratory enzyme complexes due to its enormous complexity [1–7]. The eucaryotic complex consists of 45 different subunits, seven of which are encoded by mitochondrial DNA [1,2,8]. The bacterial complex I represents a structural minimal form of the proton-translocating NADH:ubiquinone oxidoreductase generally consisting of 14 different subunits [5,7,9]. In *Escherichia coli* the genes are named *nuoA* to *nuoN* with *nuoC* and *nuoD* fused to one gene [10–12]. Electron microscopy revealed that complex I consists of a peripheral and a membrane arm [13–15]. The six peripheral subunits NuoB, CD, E, F, G, and I build the peripheral arm and contain the known redox groups, namely one flavin mononucleotide (FMN) and nine iron–sulfur (Fe–S) clusters [16–19]. Recently, the structure of this arm of the *Thermus thermophilus* complex I was solved at 3.3 Å resolution [7,17]. The remaining seven subunits are hydrophobic proteins predicted to fold into 61 α-helices across the membrane, a value supported by electron microscopy [15]. These subunits comprise the membrane arm. Little is known about the function of the seven hydrophobic subunits, but they are most likely involved in ubiquinone reduction and proton translocation [9,20].

The assembly of the mitochondrial complex I from eukaryotes has been investigated in *Neurospora crassa* and human cells lines. It was shown that the peripheral arm and the membrane arm assemble independently from each other and are fused *en bloc* to build the holoenzyme [21–23]. The membrane arm itself is assembled from two intermediates with the participation of chaperones [23–25]. However, a different, more complicated model was proposed for the assembly of the human complex I [26]. Nothing is known about the assembly of the bacterial complex. Phylogenetic analyses have shown that the bacterial complex evolved from preexisting modules for electron transfer and proton translocation [3,9,27,28]. The soluble NADH dehydrogenase module comprises the electron input module of complex I. It is connected to the hydrophilic part of an amphipatic hydrogenase module, which is also present in a familiy of multisubunit membraneous hydrogenases [29]. The hydrophobic part of the hydrogenase module is connected to the so-called transporter module containing homologues of multisubunit monovalent cation/proton antiporters [3,28,30]. It is reasonable to assume that assembly intermediates of the bacterial complex possibly resemble these modules.

Fragments of the complex reminiscent to the modules described above have been obtained by splitting the isolated *E. coli* complex [31,32]. The soluble NADH dehydrogenase fragment comprises the subunits NuoE, F and G and harbors the FMN, the binuclear Fe–S clusters N1a and N1b, and the tetranuclear Fe–S clusters N3, N4, N5, and N7 [16–18]. The NADH dehydrogenase fragment corresponds to the above mentioned NADH dehydrogenase module concerning

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subunit and cofactor composition. This fragment is also obtained by overexpression of the genes *nuoB-G* [7,11]. Expression of *nuoB* and *nuoCD* is essential for the assembly of the fragment, but the subunits are not contained in the preparation [11].

Here, we report that the deletion of any of the *nuo*-genes resulted in a loss of complex I activity in the membrane. However, the NADH dehydrogenase fragment was assembled and equipped with the cofactors in the cytoplasm of several *nuo*-mutants. The possible role of this fragment as an assembly intermediate is discussed in the light of the evolution of the bacterial complex I. A membrane-bound fragment with a yet unknown subunit composition was found in one mutant.

2. Loss of a functional complex I in *nuo*-mutants from strain AN387

To exemplarily determine whether the *nuo*-genes that code for subunits of the hydrogenase module and the transporter module are necessary for the assembly of a functional complex I in *E. coli*, the genes *nuoB*, *H*, *I*, and *N* on the chromosome of *E. coli* strain AN387 [33] were disrupted by insertion of a kanamycin cartridge. In brief, a fragment from the *E. coli nuo*-operon containing *nuoA* and *B* and one containing *nuoE* to *N* [10] were cloned in pT7T3 19U using the PstI and the SmaI/PstI restriction sites, respectively. For the construction of the plasmid pT*nuoB*::*Km*^{r} the Km cartridge was cut from pCHK20 and inserted into *nuoB*, leaving 1.3 kb and 1.0 kb flanking regions from the *E. coli* DNA, respectively. For the construction of the plasmid pT*nuoH*::*Km*^{r} the Km cartridge was inserted into *nuoH*, leaving 3.1 kb and 3.6 kb flanking regions from the *E. coli* DNA, respectively. For the construction of the plasmid pT*nuoI*::*Km*^{r}, a BamHI/ClaI fragment was ligated in pT7T3 19U and the Klenow-treated Km cartridge cut with BamHI was inserted into the blunt ended KpnI restriction of *nuoI*, leaving 1.0 kb and 1.1 kb flanking regions, respectively. For the construction of pT*nuoN*::*Km*^{r} a HincII/PstI fragment was ligated in pT7T3 19U and the Km cartridge was inserted into the BamHI restriction site of the *nuoN* gene, leaving 1.1 kb and 1.6 kb flanking regions, respectively. Restriction endonuclease mapping of the plasmids confirmed the same direction of the Km^R gene and of the *nuo*-genes (data not shown). *E. coli* strain JC7623 was transformed with these plasmids individually and chromosomal mutants were selected on kanamycin plates. Double crossovers replacing the corresponding chromosomal *nuo*-gene by the respective *nuo*::Km constructions were identified by screening Km transformants for Ap^S. The chromosomal *nuo*::Km^r constructions were transferred from strain JC7623 into strain AN387 by bacteriophage P1 transduction. Transductants were selected for Km^R and screened for Ap^S. The correct integration into the chromosome was confirmed by southern blot analysis (data not shown). The resulting mutant strains were named ANN021 (*nuoB*::Km), ANN081 (*nuoH*::Km), ANN091 (*nuoI*::Km) and ANN141 (*nuoN*::Km).

The growth rate of the *nuo*-mutant strains was approximately 1.5 times less than that of the parental strain in M9-minimal medium supplemented with mannitol. To examine the presence of a functional complex I, the d-NADH:decyl-ubiquinone oxidoreductase activity of the cytoplasmic membranes from the parental strain and the *nuo*-mutants was determined. The titration was performed with the artificial substrate d-NADH due to the presence of the alternative NADH dehydrogenase, which has a low affinity to but a high turnover with NADH. Complex I has the same affinity to d-NADH and NADH, but d-NADH is a poor substrate for the alternative NADH dehydrogenase [34]. Membranes from the parental strain revealed biphasic kinetics as reported [34]. At high d-NADH concentrations the activity of the alternative NADH dehydrogenase was measured marked by its low affinity of approximately $K_M^{\text{d-NADH}}$ of 100 μM (Fig. 1). At lower d-NADH concentrations the activity of complex I is measured as indicated by the extrapolated $K_M^{\text{d-NADH}}$ of approximately 10 μM (Fig. 1). The biphasic kinetics resulted from the overlap of the activities of the two NADH dehydrogenases. The membranes of the *nuo*-mutant strains exhibited a monophasic titration curve with an $K_M^{\text{d-NADH}}$ of approximately 100 μM, indicating that complex I is not active in the mutant membranes.

The proteins of the cytoplasmic membrane of the parental strain and the strains ANN021, ANN081, ANN091, and ANN141 were solubilized with dodecyl-maltoside and were applied to sucrose gradient centrifugation as described [31]. The artificial NADH/ferricyanide oxidoreductase activity of the fractions of the gradient mediated by the NADH-binding domain of complex I was measured. It is well known that complex I sediments through two thirds of the gradient under the given conditions [31,35,36]. While this was the case with the detergent extract of the membranes from the parental strain, no activity peak was detected at the corresponding position in the extract from the *nuo*-mutant strains (data not shown). Neither was an additional peak at a different position observed. Thus, a structurally intact complex I or a fragment of the complex exhibiting NADH/ferricyanide oxidoreductase activity was not detectable in detergent extracts of the *nuo*-mutant strains.

Cytoplasmic membranes of strains ANN021, ANN081, ANN091, and ANN141 were adjusted to 50 mg/ml protein and investigated by EPR spectroscopy to detect whether Fe–S clusters were possibly present in fragments of the complex assembled in the mutant membranes. The samples were reduced by NADH and spectra were recorded at 13 K. Under these conditions, the $g_{x,y}$ signal of Fe–S cluster N2 located on subunit NuoB [17,37] was detected at g=1.91 in the EPR spectrum of the membranes from the parental strain (Fig. 2). This signal was missing in the membranes obtained from the *nuo*-mutant strains (Fig. 2). This was in agreement with the fact that we did not detect subunit NuoB in the cytoplasmic membranes of the *nuo*-mutant strains by immunological means (data not shown).

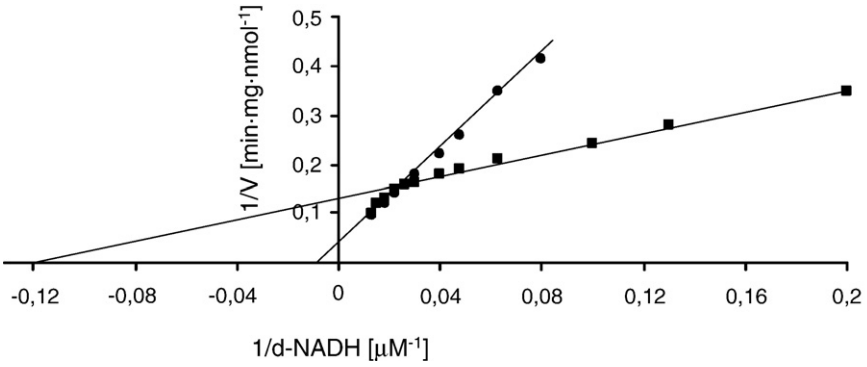

Fig. 1. Double-reciprocal plots of the kinetic data of complex I and the alternative NADH dehydrogenase in *E. coli* cytoplasmic membranes. Plots of 1/V against 1/[d-NADH] using membranes from parental strain (square) and strain ANN141 (circle). Titrations with membranes from strains ANN021, ANN081, and ANN091 gave similar data as derived from the strain ANN141. 150 μg protein were applied and the concentration of decyl-ubiquinone was 20 μM.

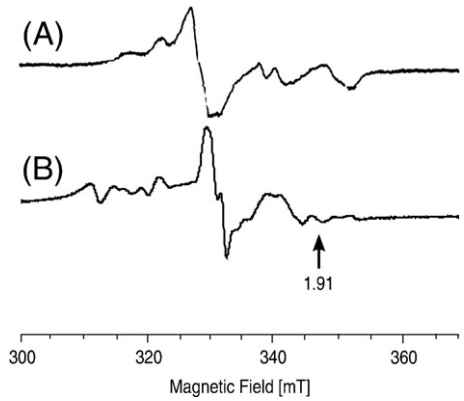

Fig. 2. EPR spectra of NADH-reduced cytoplasmic membranes from *E. coli* strains ANN141 (A) and AN387 (B). The protein concentration was adjusted to 50 mg/mL. Spectra similar to (A) were obtained from the membranes of strains ANN021, ANN081, and ANN091. The signal attributed to cluster N2 at $g = 1.91$ is indicated by an arrow. The spectra were recorded at 13 K and 10 mW. Other EPR conditions were: microwave frequency, 9.44 GHz; modulation amplitude, 0.6 mT; time constant, 0.124 s; scan rate, 17.9 mT/min.

The cytoplasm of the *nuo*-mutant strains was searched for the presence of soluble complex I subunits by immunological means. Using antibodies directed against NuoB, CD and I, we were not able to detect the subunits in the cytoplasm of any of the mutants (data not shown). Therefore, we attempted to detect the Fe–S clusters of the NADH dehydrogenase fragment by means of EPR spectroscopy in the cytoplasm of the *nuo*-mutants. We have shown that the binuclear Fe–S clusters N1a and N1b are readily detected in the cytoplasm of *E. coli* strains containing this fragment, which is made up of the subunits NuoE, F, and G [11,16]. The cytoplasm of the mutant and parental strains was concentrated 10-fold and one aliquot was reduced by NADH while another aliquot was diluted with the same volume buffer. As expected, the EPR difference spectra of the NADH-reduced minus the air-oxidized cytoplasm of the parental strain did not show the signals attributed to clusters N1a and N1b. The signals were also not present in the EPR difference spectra of the strains ANN021, ANN081 and ANN091, but the clusters N1a and N1b were detected in the cytoplasm of strain ANN141 (Fig. 3). It was impossible to detect the tetranuclear Fe–S clusters N3 and N4 of this fragment due to their spectral overlap with Fe–S clusters of other proteins (data not shown). The presence of N1a and N1b in the cytoplasm of strain ANN141 indicated that the NADH dehydrogenase fragment is fully assembled in this mutant.

3. Detection of complex I fragments in *nuo*-mutants from strain BW25113

While we constructed the chromosomal *nuo*-mutants from strain AN387, the 'Keio collection' of the Nara Institute of Science and Technology, Japan [38] was open to the public. This splendid and most helpful collection provides researchers with *E. coli* strains containing an insertion of a kanamycin resistance cartridge in every known gene and ORF. This collection is based on *E. coli* strain BW25113, a derivative of *E. coli* K-12 BD792 [39]. For a systematic investigation of the effect of disrupting the *nuo*-genes on the presence of a functional complex I, we obtained all 13 *nuo*-mutants from the Keio collection.

As with AN387 derivatives, the growth rate of *nuo*-mutant strains of *E. coli* BW25113 was decreased by approximately 1.5 times and reached approximately half of the final optical density compared to the parental strain. The presence of a functional complex was examined by measuring the physiological NADH oxidase activity of the cytoplasmic membranes. To determine the contribution of complex I to the total NADH oxidase activity, the sensitivity of this activity towards annonin VI, a specific complex I inhibitor not affecting the alternative NADH dehydrogenase, was measured [34]. Approximately half of the specific NADH oxidase activity was inhibited by an addition of annonin VI, indicating that half of the NADH oxidase activity of strain BW25113 is mediated by complex I. The specific NADH oxidase activity of all *nuo*-mutant strains was completely insensitive to annonin VI, demonstrating that it derived entirely from the alternative NADH dehydrogenase. Thus, all *nuo*-genes are needed for the production and assembly of a functional complex I.

The NADH/ferricyanide oxidoreductase activity of the cytoplasmic membranes is an indication of the amount of both NADH dehydrogenases present in the membranes. This artificial activity was approximately halved in the mutant membranes corroborating the loss of complex I. To search for membrane-bound fragments of complex I containing the NADH dehydrogenase fragment, the ratio between the NADH oxidase and NADH/ferricyanide oxidoreductase activity of the mutants membranes was compared. The complete loss of complex I has no significant impact on the ratio. However, if a fragment of the complex exhibiting NADH/ferricyanide oxidoreductase activity but not NADH:ubiquinone oxidoreductase activity is enriched in the mutant membranes, the ratio slightly decreases. The ratio was reduced by nearly one quarter in the membranes of the *nuoL* mutant strain. According to this criterion, a membrane-bound complex I fragment with NADH/ferricyanide oxidoreductase activity was expected to be present in the mutant. To examine this possibility, the cytoplasmic membranes from both the parental and the *nuoL* mutant strain were analyzed by EPR spectroscopy for the presence of Fe–S cluster N2 as described above. Indeed, an EPR signal at $g = 1.91$, which was attributed to N2, was detected in both strains [40].

The stability and approximate molecular mass of the complex I fragment present in the *nuoL* mutant strain was examined by sucrose gradient centrifugation of dodecyl-maltoside extracts obtained from cytoplasmic membranes. The fully assembled *E. coli* complex I of the parental strain sedimented through two thirds of the gradient as observed with other strains (Fig. 4). No activity peak was detected at the corresponding position in the extract from all *nuo*-mutant strains indicating that none of the mutants contained a fully assembled complex I as expected from the insensitivity of their NADH oxidase activity to the specific complex I inhibitor annonin VI. However, a

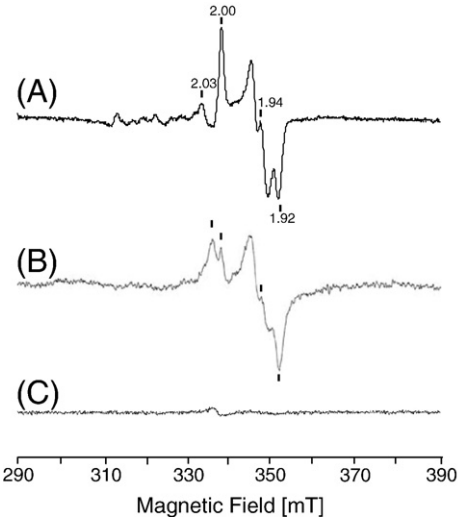

Fig. 3. EPR difference spectra of the cytoplasm from various *E. coli* strains. (A) shows the spectrum of the cytosol from BL21(DE3) pET-24/*nuoB–G*, overproducing the NADH dehydrogenase fragment [11], (B) spectrum from strain ANN141, and (C) spectrum from strain AN387. The spectra were recorded at 40 K and 1 mW. The protein concentration was adjusted to 40 mg/mL. Other EPR conditions were as given in Fig. 2. The signals of cluster N1a ($g_{x,\ y,\ z} = 1.92$, 1.94, and 2.00) and N1b ($g_{//,\perp} = 2.03$, 1.94) are indicated. A radical signal overlaps the signal at $g = 2.00$ in (A). The amount of cluster N1b relative to N1a is reduced in (B).

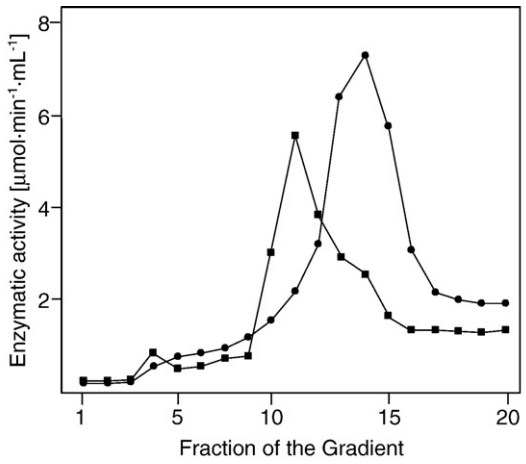

Fig. 4. Sucrose gradient centrifugation from dodecyl-maltoside solubilized cytoplasmic membranes from strains BW25113 (circle) and BW25113 *nuoL*::*nptI* (square). Membrane proteins were separated by means of gradients of 5–30% (w/v) sucrose in 50 mM MES/NaOH, pH 6.0, 50 mM NaCl and 0.1% dodecyl-maltoside. The activities were standardized to 30 mg protein loaded on each gradient. Fractions of the gradients (numbered 1–20 from top to bottom) were collected and analyzed for NADH/ferricyanide oxidoreductase activity.

prominent peak of the NADH/ferricyanide oxidoreductase activity was detected in the extract from the *nuoL* mutant in the middle of the gradient (Fig. 4). According to its position, the peak most likely represents a fragment of complex I missing subunit NuoL, which has a molecular mass of 66.4 kDa, but no other subunits.

As described above, the cytoplasm of the *nuo*-mutants from strain BW25113 was searched for the presence of the binuclear Fe–S clusters N1a and N1b of the NADH dehydrogenase fragment. The signals of these clusters were detected in the difference spectra of the cytoplasm of the *nuoCD*, *nuoH*, *nuoI*, *nuoJ*, *nuoK*, and *nuoN* mutant strains. This indicated that the NADH dehydrogenase fragment is fully assembled not only in the *nuoN* mutant as observed with AN387 strain. It was not possible to isolate the fragment from the mutant strains due to its instability. To prove that subunits NuoE, F, and G comprising the NADH dehydrogenase fragment are assembled in one complex, we separated the cytoplasmic proteins by BN-PAGE and the position of any NADH dehydrogenase was determined by staining the gel with NADH and NBT [41]. A sharp band with an apparent molecular mass of approximately 200 kDa was detected. The subunits NuoE (18.6 kDa), NuoF (49.3 kDa), and NuoG (100.2 kDa) were detected by means of Western blot analysis using specific antibodies at the same position in the gel and at the same height as the band stained with NBT. The molecular mass of the NADH dehydrogenase fragment as derived from the DNA sequence of the individual subunits is 170 kDa, which is in good agreement with the mass determined by BN-PAGE.

4. Implications for the assembly of the respiratory complex I in *E. coli*

The mechanism(s) of energy conservation by complex I is not known although the structure of the peripheral arm of the complex was recently solved at molecular resolution [17]. Therefore, the investigation of the assembly of this huge and modular machinery may shed light on the function of the individual modules and their interaction within the complex. Our data show that inactivation of any of the *E. coli nuo*-genes by insertion of a resistance cartridge prohibited either the production or the assembly of a functional complex I. This was demonstrated by the loss of any complex I-mediated activity in the mutant membranes and the failure to detect the fully assembled complex in detergent extracts from cytoplasmic membranes. The insertion of the kanamycin resistance cartridge did not cause a strong polar effect because we were able to complement the *nuoB* and *nuoF* mutants with the wild-type gene *in trans* [40]. The slightly reduced complex I activity measured in all these experiments is most likely due to a mild polar transcriptional effect of the integration mutagenesis. The marginal polar effect of the inserted kanamycin resistance cartridge has also been reported for the mutagenesis of the *nuo*-operon in *Rhodobacter capsulatus* [42].

The EPR signals of the binuclear Fe–S clusters N1a and N1b of the NADH dehydrogenase fragment were detected in the cytoplasm of the *nuoCD*, *nuoH*, *nuoI*, *nuoJ*, *nuoK*, and *nuoN* mutants of strain BW25113, but not in the cytoplasm of the *nuoH* and *nuoI* mutants of strain AN387. This might indicate that the stability of the fragment is different in the two strains. Therefore, we cannot completely rule out the possibility that the fragment is also present when an abundance is lacking in other *nuo*-mutants, although we were neither able to detect the binuclear Fe–S clusters nor the subunits NuoE, F, and G in the cytoplasm of the *nuoA*, *nuoB*, *nuoL*, and *nuoM* mutant strains. The absence of the NADH dehydrogenase fragment in the *nuoE*, *nuoF*, and *nuoG* mutants is expected as these subunits comprise the fragment. After separating the cytoplasmic proteins of the *nuoN* mutant by BN-PAGE, a single band was detected consisting of the subunits NuoE, F, and G and showing NADH dehydrogenase activity [40]. The position of the band corresponds to a protein of a molecular mass of approximately 200 kDa indicating that no other complex I subunits are associated with the fragment. Thus, the NADH dehydrogenase fragment with the FMN and at the least two binuclear Fe–S clusters is assembled in the cytoplasm of the *nuoN* mutant strain. We assume that the fragment also contains the tetranuclear clusters, which were, however, not detectable in the cytoplasm due to the spectral overlap with the Fe–S clusters from other proteins. We propose that the NADH dehydrogenase fragment might represent an assembly intermediate of complex I in *E. coli*, which would be compatible with both evolutionary schemes that have been proposed for the bacterial complex I [3,9,28]. In both models, the NADH dehydrogenase fragment is the latest acquisition of the progenitor of today's complex I of bacteria and eukaryotes. However, we cannot exclude the possibility that it is a stable fragment resulting from the decay of a larger assembly intermediate present in the corresponding *nuo*-mutant strains. To discriminate between these two possibilities, we are currently studying the assembly of the *E. coli* complex by pulse-labeling methods.

Most surprisingly, a membrane-bound complex I fragment without a detectable NADH oxidase activity was found in the *nuoL* mutant strain [40]. Due to its instability we were not able to characterize its subunit composition. As this fragment exerts NADH/ferricyanide oxidoreductase activity and contains cluster N2 it should at least comprise subunits NuoB, E, F, and G. From its position after sucrose gradient centrifugation it should be made up of all complex I subunits with the exception of NuoL. It was shown by electron microscopy that NuoL and NuoM are located at the distal part of the membrane arm of the complex and can be individually removed from the entire complex while retaining the other complex I subunits [20,43]. It is discussed that NuoL is involved in proton translocation, due to its homology to subunits of multisubunit monovalent cation/proton antiporters. Thus, NuoL is of central importance to the enzyme's mechanism [3,15]. In order to understand the function of the complex, it will be crucial to determine, whether this fragment exerts any electron transfer or proton translocation activity at all. Due to its lacking abundance in the mutant membrane, it is possible that there is residual electron transfer activity in the fragment, which is obscured by the activity of the alternative NADH dehydrogenase. Currently, our aim is to overexpress the *nuo*-operon lacking *nuoL* from the *nuo*-expression plasmid developed recently in our laboratory [44] and to characterize the properties of the purified fragment.

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