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Disruption of individual *nuo*-genes leads to the formation of partially assembled NADH:ubiquinone oxidoreductase (complex I) in *Escherichia coli* ☆

Heiko Erhardt [1], Stefan Steimle [1], Vera Muders, Thomas Pohl [2], Julia Walter [3], Thorsten Friedrich *

Albert-Ludwigs-Universität Freiburg, Institut für Organische Chemie und Biochemie and Spemann Graduate School of Biology and Medicine, Albertstr. 21, 79104 Freiburg i. Br., Germany

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ABSTRACT

The proton-pumping NADH:ubiquinone oxidoreductase, respiratory complex I, couples the electron transfer from NADH to ubiquinone with the translocation of protons across the membrane. In *Escherichia coli* the complex is made up of 13 different subunits encoded by the so-called *nuo*-genes. Mutants, in which each of the *nuo*-genes was individually disrupted by the insertion of a resistance cartridge were unable to assemble a functional complex I. Each disruption resulted in the loss of complex I-mediated activity and the failure to extract a structurally intact complex. Thus, all *nuo*-genes are required either for the assembly or the stability of a functional *E. coli* complex I. The three subunits comprising the soluble NADH dehydrogenase fragment of the complex were detected in the cytoplasm of several *nuo*-mutants as one distinct band after BN-PAGE. It is discussed that the fully assembled NADH dehydrogenase fragment represents an assembly intermediate of the *E. coli* complex I. A partially assembled complex I bound to the membrane was detected in the *nuoK* and *nuoL* mutants, respectively. Overproduction of the ΔNuoL variant resulted in the accumulation of two populations of a partially assembled complex in the cytoplasmic membranes. Both populations are devoid of NuoL. One population is enzymatically active, while the other is not. The inactive population is missing cluster N2 and is tightly associated with the inducible lysine decarboxylase. This article is part of a Special Issue entitled: Biogenesis/Assembly of Respiratory Enzyme Complexes.

1. Introduction

The proton-pumping NADH:ubiquinone oxidoreductase, also called complex I, is the main entry point for electrons from NADH into the respiratory chains of most mitochondria and many bacteria [1–7]. Its mechanism is not understood due to its enormous complexity. The eucaryotic complex consists of about 45 different subunits, seven of which are encoded by mitochondrial DNA [1, 2, 8]. The bacterial complex I is generally made up of 14 individual subunits, named NuoA to NuoN in *Escherichia coli* [3, 4, 9, 10]. They are encoded by the genes *nuoA* to *nuoN* [9]. As in a few other bacteria, the *E. coli* genes *nuoC* and *nuoD* are fused leading to a complex consisting of 13 subunits [10, 11]. X-ray crystallography and electron microscopy revealed that complex I is made up of a peripheral arm, which is built up by the hydrophilic subunits NuoB, CD, E, F, G, and I, and a membrane arm containing the polytopic subunits NuoA, H, J, K, L, M, and N [12–15]. The peripheral arm contains the redox groups of the *E. coli* complex I, namely one flavin mononucleotide (FMN) and nine iron-sulfur (Fe/S) clusters [6, 7, 16–19]. The structure of this arm of the *Thermus thermophilus* complex I was resolved at molecular resolution revealing the electron pathway from the FMN to the most distal Fe/S cluster N2 discussed as direct electron donor to the substrate ubiquinone [18, 20]. Recently, the structure of the entire complex from *T. thermophilus* and *Yarrowia lipolytica* was determined at 4.5 and 6.3 Å resolution, respectively, revealing the two-part structure of the complex and the arrangement of 64 transmembranous-helices as well as one large 'horizontal' helix on NuoL [14, 15]. This helix is most likely used to couple the energy released by the electron transfer reaction in the peripheral arm with proton translocation in the membrane arm [6, 14, 21].

While the assembly of the mitochondrial complex I from eucaryotes has been investigated in detail [22,23] and see contribution by M. Ryan, this BBA special issue, 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, 10, 14, 24, 25]. The soluble NADH dehydrogenase module, also present in soluble NAD-dependent hydrogenases and dehydrogenases, comprises the electron input part of the complex. A preparation of this module has been obtained in *E. coli* either by splitting the complex or overexpressing the corresponding genes [11, 26, 27]. It comprises the subunits

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* Corresponding author. Tel.: +49 761 203 6060; fax: +49 761 203 6096.
E-mail address: tfriedri@uni-freiburg.de (T. Friedrich).
[1] These authors contributed equally to this study.
[2] Present address: SuppreMol GmbH, Am Klopferspitz 19, 82152 Martinsried, Germany.
[3] Present address: Freie Universität Berlin, Institut für Immunologie und Molekularbiologie, Philippstraße 13, 10115 Berlin, Germany.

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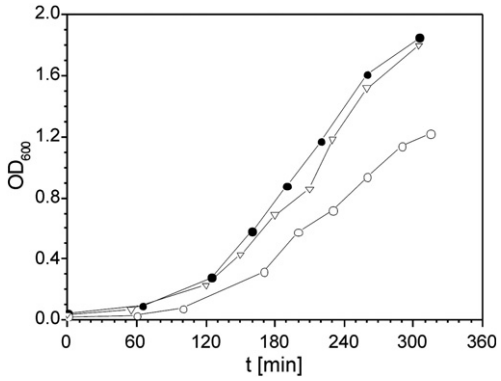

Fig. 1. Growth characteristics of various *E. coli nuo*-mutant strains in LB-medium. The *nuo*-mutant strain BW25113*nuoF::nptI* (open circles) grows slower and to a lower cell density than the parental strain BW25113 (full circles). The growth curves of the other *nuo*-mutant strains were virtually identical to that of the *nuoF* mutant strain. Episomal complementation of the disrupted *nuo*-gene on the chromosome as examplified by strain BW25113*nuoF::nptI*/pCA24N*nuoF* (open triangles) led to a growth curve similar to that of the parental strain.

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 [11, 17, 19, 28]. The NADH dehydrogenase module delivers the electrons from NADH via the FMN and the Fe/S clusters to the amphipathic hydrogenase module, which is also a part of a family of multi-subunit membranous [NiFe] hydrogenases [29]. The reduction of ubiquinone most likely takes place in the interface between this and the third module, the transporter module. The transporter module contains subunits homologous to some subunits of multi-subunit monovalent cation/proton antiporters [24, 25, 30, 31]. So far, neither a cofactor has been detected in the module nor does it contain a typical sequence motif for binding a cofactor. It is reasonable to assume that assembly intermediates of the bacterial complex possibly resemble these modules.

Here, we report that the deletion of any of the *nuo*-genes resulted in a complete loss of complex I activity in the membrane and the loss of a structural intact complex. However, the cytoplasm of several *nuo*-mutants contains the fully assembled NADH dehydrogenase fragment equipped with its cofactors. In addition, membrane-bound fragments of complex I were found in the *nuoK* and *nuoL* mutants. Overproduction of the ΔNuoL variant led to the accumulation of two partially assembled complex I populations in the membrane, one being tightly associated with the inducible lysine decarboxylase. A possible role of the lysine decarboxylase for the assembly of the complex is discussed.

2. Characterization of the *nuo*-mutants

E. coli mutants with individually disrupted *nuo*-genes were obtained from the 'Keio collection' of the Nara Institute of Science and Technology, Japan [32]. *E. coli* strain BW25113, a derivative of *E. coli* K-12 BD792, was used as parental strain [33]. Gene disruption was accomplished by the insertion of a kanamycin resistance cartridge. The correct insertion of the resistance cartridge was checked by PCR techniques [31]. Strains were grown in 400 mL LB medium containing 50 mg/L kanamycin in a 1 L baffled conical flask at 37 °C. Under these conditions the growth rate of the *nuo*-mutants was approximately 1.5 times less than that of the parental strain and reached approximately half of the final optical density (Fig. 1). No differences were detected among the mutant strains. This indicated a loss of complex I activity in all mutants.

The presence of a functional complex I was examined by measuring the physiological NADH oxidase activity of the parental strain and the *nuo*-mutants (Table 1). Membranes were prepared as described [19] and resuspended in 50 mM MES/NaOH, 50 mM NaCl, pH 6.0. The NADH and succinate oxidase activity of cytoplasmic membranes were measured with a Clarke-type oxygen electrode at 30 °C as described [34]. *E. coli* contains two membrane-bound NADH dehydrogenases, the energy-converting complex I and a non-energy-converting, alternative NADH dehydrogenase both contributing to the NADH oxidase activity of the cytoplasmic membranes [35, 36]. The activities of the two NADH dehydrogenases were distinguished by their sensitivity to annonin VI, a specific inhibitor of complex I [36, 37]. Strain BW25113 exhibited a specific NADH oxidase activity of 0.55 U/mg, which was inhibited to 45% by annonin VI (Table 1). Thus, nearly half of the NADH oxidase activity of strain BW25113 is mediated by complex I, which is in agreement with data obtained from other *E. coli* strains [37–41]. The inhibitor-insensitive activity derived from the alternative NADH dehydrogenase. The specific NADH oxidase activity of the *nuo*-mutants varied between 0.30 and 0.35 U/mg (Table 1). This activity was completely insensitive to annonin VI, demonstrating that it derived entirely from the alternative NADH dehydrogenase. The succinate oxidase activity of the parental and the *nuo*-mutant strains was not influenced by the mutations and varied between 0.20 and 0.25 U/mg. The NADH and succinate oxidase activity of the mutant membranes demonstrated that the disruption of the *nuo*-genes resulted exclusively in a loss of complex I activity and did not influence other components of the respiratory chain.

To consider a possible polar effect caused by the insertion of the resistance cartridge, the *nuoB* and *nuoF* mutants were complemented with the corresponding wild type *nuo*-gene *in trans*. Expression of the cloned genes was induced by an addition of 0.1 mM isopropyl-ß-D-thiogalactopyranoside or 0.2% arabinose. Cells were grown in the presence of 43 mg/L chloramphenicol. The *nuoB*- and *nuoF*-mutant strains complemented with either plasmids pBAD*nuoB* [42] or pCA24N*nuoF* [43] exhibited a growth curve like that of the parental strain (Fig. 1). They showed an NADH oxidase activity of 0.48 U/mg similar to the parental strain. This activity was inhibited to 42 and 45% by annonin VI, respectively. Thus, the integration mutagenesis

Table 1
E. coli strains used and the NADH-derived activities of the cytoplasmic membranes.

Strains	NADH oxidase activity	Inhibition by annonin VI	NADH/ferricyanide oxidoreductase activity	NADH oxidase activity/NADH/ferricyanide oxidoreductase activity
	[U/mg]	[%]	[U/mg]	
BW25113	0.55 ± 0.1	45	2.1 ± 0.3	0.26
BW25113 *nuoA::nptI*	0.35 ± 0.03	0	1.2 ± 0.3	0.29
BW25113 *nuoB::nptI*	0.31 ± 0.03	0	1.2 ± 0.3	0.26
BW25113 *nuoCD::nptI*	0.32 ± 0.03	0	1.0 ± 0.3	0.32
BW25113 *nuoE::nptI*	0.32 ± 0.03	0	1.1 ± 0.3	0.29
BW25113 *nuoF::nptI*	0.31 ± 0.03	0	1.1 ± 0.3	0.28
BW25113 *nuoG::nptI*	0.31 ± 0.03	0	1.1 ± 0.3	0.28
BW25113 *nuoH::nptI*	0.32 ± 0.03	0	1.2 ± 0.3	0.27
BW25113 *nuoI::nptI*	0.32 ± 0.03	0	1.2 ± 0.3	0.27
BW25113 *nuoJ::nptI*	0.30 ± 0.03	0	1.2 ± 0.3	0.25
BW25113 *nuoK::nptI*	0.31 ± 0.03	0	1.4 ± 0.3	0.22
BW25113 *nuoL::nptI*	0.30 ± 0.03	0	1.5 ± 0.3	0.20
BW25113 *nuoM::nptI*	0.31 ± 0.03	0	1.2 ± 0.3	0.26
BW25113 *nuoN::nptI*	0.33 ± 0.03	0	1.2 ± 0.3	0.28

seems to have only a marginal polar effect on the transcription of the downstream genes.

3. Detection of the NADH dehydrogenase fragment in the cytoplasm of the *nuo*-mutants

Recently, we detected the EPR signals of the binuclear Fe/S clusters of the soluble NADH dehydrogenase fragment in the cytoplasm of the Δ*nuoN* mutant of the *E. coli* strain AN387 [44]. From this, we concluded that the cytoplasm of this strain contains the fully assembled NADH dehydrogenase fragment [44]. To investigate whether the proteins comprising the fragment are also detectable in the cytoplasm and whether this fragment can be identified in other *nuo*-mutant strains, the cytoplasm of the parental BW25113 strain and the individual *nuo*-mutants was analyzed by means of BN-PAGE. The cytoplasm was diluted to 3.5 mg/mL and 100 μg cytosolic proteins were applied to a continuous gradient BN-gel from 6 to 13% acrylamide, pH 7.0 [45] (Fig. 2). In order to detect proteins with NADH dehydrogenase activity, the gel was stained with NADH and NBT [46]. However, this staining is not specific for complex I. To specifically detect the position of the NADH dehydrogenase fragment in the gel, the purified fragment and the cytoplasm of strain BL21 (DE3)/pET-11a/*nuoB-G*/$NuoF_C$ overproducing the fragment [47] were also applied on the gel. As control the cytoplasm of the parental strain BW25113 was used. A sharp and strongly stained band with an apparent molecular mass of approximately 200 kDa as judged from the position of protein markers was detected in the lanes loaded with the purified fragment and the cytoplasm of strain BL21 (DE3)/pET-11a/*nuoB-G*/$NuoF_C$ as well as in the lanes loaded with the cytoplasm from the *nuoH*-, *nuoI*-, *nuoJ*-, *nuoK*-, *nuoM*, and *nuoN*-mutant strains (Fig. 2). A faint band at the identical position was detected in the *nuoA*-, *nuoB*-, and *nuoCD*-mutant strains. The molecular mass of the NADH dehydrogenase fragment is 170 kDa, which is in good agreement with the mass determined by BN-PAGE. To prove that this band corresponds to the NADH dehydrogenase fragment consisting of the subunits NuoE, F, and G, the cytoplasm of the *nuoN*-mutant strain was loaded onto three lanes and after BN-PAGE the proteins were transferred to a nitrocellulose membrane and incubated with antibodies specifically directed against NuoE, F, and G [48–50]. Subunits NuoE (18.6 kDa), NuoF (49.3 kDa), and NuoG (100.2 kDa) were immunologically detected at the same position in the gel and this position was identical to the one of the bands stained with NBT (Fig. 2). No signals were obtained using antibodies against NuoB and NuoI (data not shown).

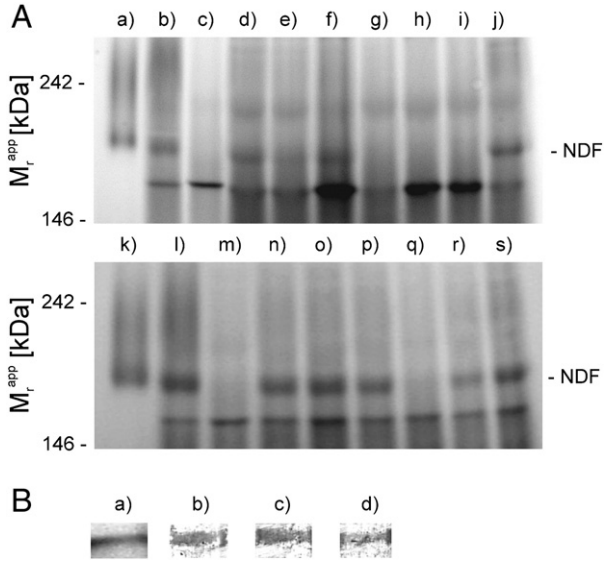

Fig. 2. Detection of the NADH dehydrogenase fragment in the cytoplasm of various *E. coli nuo*-mutant strains. (A) BN-PAGE of the cytoplasm of the *nuoA* d), *nuoB* e), *nuoCD* f), *nuoE* g), *nuoF* h), *nuoG* i), *nuoH* j), *nuoI* n), *nuoJ* o), *nuoK* p), *nuoL* q), *nuoM* r), and *nuoN* s) mutant strains after activity staining with NADH and NBT. As controls the purified NADH dehydrogenase fragment was applied to lanes a) and k) and the cytoplasm of *E. coli* strain BL21(DE3)/pET-11a/*nuoB-G*/$NuoF_C$ overproducing the NADH dehydrogenase fragment was loaded on lanes b) and l). For comparison, the cytoplasm of the parental strain BW25113 was applied to lanes c) and m). The apparent molecular masses are indicated as well as the position of the NADH dehydrogenase fragment ('NDF'). (B) Identification of the subunits comprising the NADH dehydrogenase fragment by immunological means. Four aliquots of the cytoplasm of strain BW25113*nuoN::nptI* were run on a BN-PAGE and the position of the NADH dehydrogenase fragment was determined by staining with NADH and NBT a). Subunits NuoE b), NuoF c), and NuoG d) were identified by western blot analysis using specific antibodies. The position of the subunits in the gel was identical to that of the NADH dehydrogenase fragment.

The staining of bands by NADH and NBT indicated that the NADH dehydrogenase fragment contained the FMN cofactor. To determine whether the fragment in the cytoplasm of the *nuo*-mutants also contained the Fe/S clusters, the cytoplasm was investigated by means of EPR spectroscopy [40]. It was concentrated 10-fold by ultrafiltration using an Amicon Ultra-15 centrifugal filter unit (MWCO 100 kDa, Millipore) and divided in two aliquots. One aliquot was reduced by NADH, the other diluted with an equivalent volume of buffer. The EPR spectra of the NADH-reduced and air-oxidized cytoplasm from the parental strain and the *nuo*-mutant strains were recorded at 40 K and difference spectra were calculated (Fig. 3). Using this approach the binuclear Fe/S clusters N1a and N1b of the overproduced NADH dehydrogenase fragment were detected in the cytoplasm when the fragment was fully assembled [11, 17, 47]. The signals of the clusters N1a ($g_{x,\,y,\,z} = 1.92$, 1.94, and 1.99) and N1b ($g_{//,\perp} = 2.03$ and 1.94; Fig. 3) were clearly present in the difference spectra of the *nuoH*, *nuoI*, *nuoJ*, *nuoK*, *nuoM*, and *nuoN* mutant strains. They were detected in slightly different concentrations in the individual mutants possibly due to differences in their stability or a variance in the treatment of the cytoplasm during concentration. In some cases, the g_z-signal of N1a at $g = 1.99$ overlaps with a radical signal at $g = 2.00$. Only very faint signals of these clusters were detectable in the cytoplasm of strains *nuoA, nuoB* and *nuoCD* (Fig. 3), in accordance with the smaller amount of the fragment in these mutants detected by BN-PAGE (Fig. 2).

It was not possible to detect the tetranuclear Fe/S clusters N3 and N4 of the fragment in the cytoplasm due to a spectral overlap with larger signals deriving from Fe/S clusters of other cytoplasmic enzymes [11]. However, the presence of the FMN cofactor and the binuclear Fe/S clusters clearly indicate that the fully assembled NADH dehydrogenase fragment is accumulated in the cytoplasm of the *nuoH*-, *nuoI*-, *nuoJ*-, *nuoK*-, *nuoM*-, and *nuoN*-mutant strains. It is reasonable to assume that the fragment is also fully assembled with its cofactors in the cytoplasm of the *nuoA*-, *nuoB*-, and *nuoCD*-mutant strains, but that the Fe/S clusters are hardly detectable due to the low abundance of the fragment.

4. Detection of complex I fragments in the cytoplasmic membrane of the *nuo*-mutants

The two membrane-bound *E. coli* NADH dehydrogenases exhibit NADH/ferricyanide oxidoreductase activity. In cytoplasmic membranes, this activity is an indication of the amount of both NADH dehydrogenases present. In complex I, this artificial activity is conferred by the peripheral NADH dehydrogenase fragment of the complex and does not involve the participation of quinones. The specific NADH/ferricyanide oxidoreductase activity was nearly halved in the *nuo*-mutant membranes compared to that of the parental strain confirming the loss of complex I (Table 1). To search for partially assembled complex I bound to the membrane and containing the NADH dehydrogenase fragment, the ratio of the NADH oxidase and NADH/ferricyanide oxidoreductase activity of the *nuo*-mutants membranes was compared. The loss of complex I has a

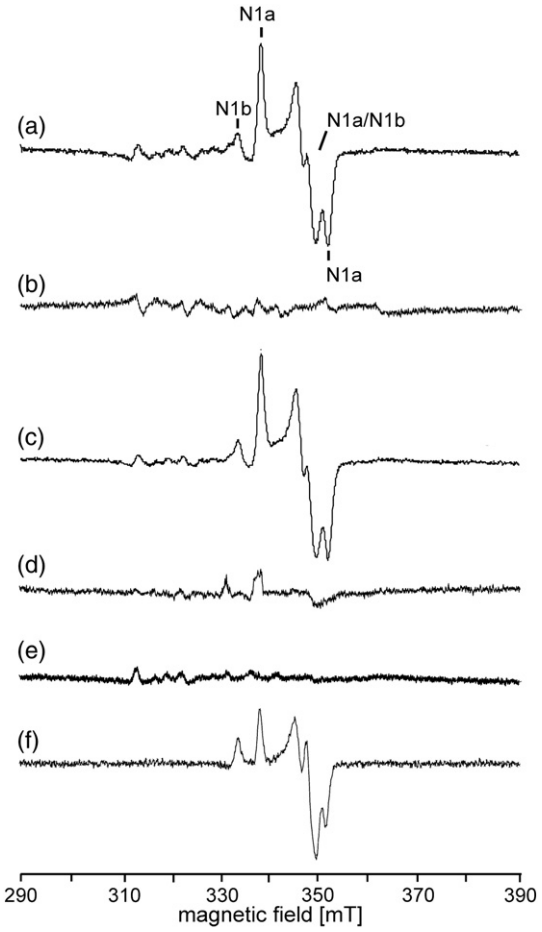

Fig. 3. Detection of the binuclear Fe/S clusters of the NADH dehydrogenase fragment in the cytoplasm of various *E. coli nuo*-mutant strains. EPR difference (NADH-reduced minus air-oxidized) spectra of the cytoplasm from strain BL21(DE3)/pET-11a/*nuoB-G*/$NuoF_C$, overproducing the NADH dehydrogenase fragment (a), the parental strain BW25113 (b), the *nuoN* mutant strain (c), the *nuoCD* mutant strain (d), and the *nuoE* mutant strain (e) are shown. Spectra similar to (c) were obtained with the *nuoH*, *nuoI*, *nuoJ*, *nuoK*, *nuoM*, and *nuoN* mutant strains. Spectra similar to (d) were obtained with the *nuoA* and *nuoB* mutant strains. Spectra similar to (e) were obtained with the *nuoF*, *nuoG* and *nuoL* mutant strains. The spectra were recorded at 40 K and 1 mW. The protein concentration was adjusted to 40 mg/mL. For comparison the spectrum of the isolated NADH dehydrogenase fragment (5 mg/mL) reduced with NADH is also shown (f). Other EPR conditions were: microwave frequency, 9.45 GHz; modulation amplitude, 0.6 mT; time constant, 0.124 s; scan rate, 17.9 mT/min.

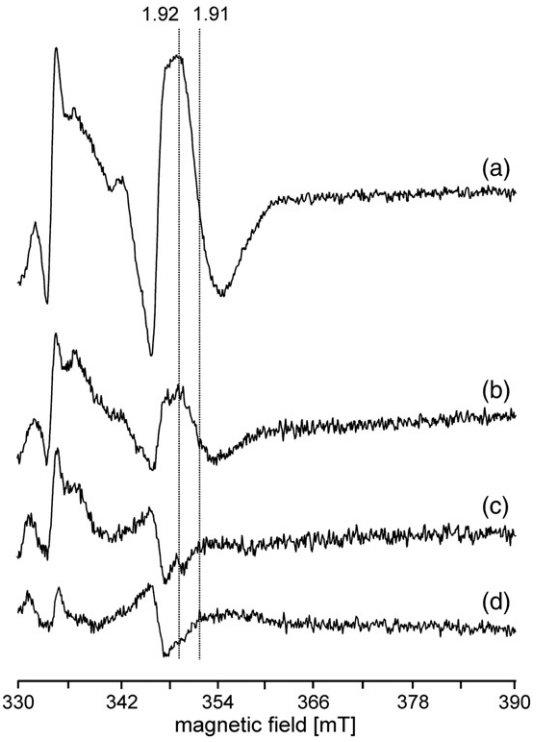

Fig. 4. Detection of Fe/S cluster N2 in *E. coli* cytoplasmic membranes by EPR difference spectroscopy. The membranes of strains BW25113 (a), the *nuoL* (b), the *nuoK* (c), and the *nuoB* (d) mutant were reduced by dithionite and spectra were recorded. From these spectra the spectrum of dithionite-reduced cytoplasmic membranes from the *nuoA* mutant strain was subtracted. The resulting difference spectra are shown. Difference spectra similar to (d) were obtained from all residual *nuo*-mutant strains. The position of the signals at g = 1.91 and 1.92 are indicated by dotted lines. The spectra were recorded at 13 K and 5 mW. Other EPR conditions were as given in Fig. 3.

slightly greater impact on the NADH/ferricyanide oxidoreductase activity than on the NADH oxidase activity leading to a similar or insignificantly higher ratio in the corresponding mutants (Table 1). However, the ratio is expected to decrease when a fragment with NADH/ferricyanide oxidoreductase activity but without NADH:ubiquinone oxidoreductase activity is enriched in the membrane. According to this measure, a membrane-bound complex I fragment was expected in the *nuoK* and *nuoL* mutants (Table 1).

To detect the most distal Fe/S cluster N2 possibly associated with such a fragment, cytoplasmic membranes of the parental strain and the *nuo*-mutants were analyzed by EPR-spectroscopy. Cluster N2 is located on subunit NuoB, which is closely connected to the membrane arm [14, 18]. Due to its axial symmetry the cluster exhibits a sharp signal at g = 1.91, which is detectable in the membrane [41, 42]. Cytoplasmic membranes of the parental and the *nuo*-mutant strains were adjusted to a protein concentration of approximately 60 mg/mL and reduced with dithionite. To obtain a better signal/noise ratio, difference spectra were calculated. The spectrum of the membranes from the *nuoA* mutant strain was subtracted from the spectra of all other mutant strains. The *nuoA* mutant was chosen as reference because there was no indication that a membrane-bound fragment of complex I was present in the mutant (see below). Difference spectra similar to that shown in Fig. 4 were also obtained by using either the *nuoB* or the *nuoCD* mutant strain as reference (data not shown). The difference of the EPR spectrum of the parental strain minus that from the *nuoA* mutant strain showed a small but significant signal at g = 1.91 (Fig. 4). This signal was attributed to cluster N2 and was missing in the membranes from most other *nuo*-mutants. However, it was detected in the membranes of the *nuoL* mutant strain (Fig. 4) indicating the presence of N2 in this mutant. The membranes from the *nuoK* mutant strain exhibited a small but significant signal around g = 1.92, which was neither present in the parental strain nor in any of the other *nuo*-mutants (Fig. 4). It is possible that the disruption of *nuoK* led to a change in the environment of cluster N2 resulting in a shift of the signal.

To examine the possibility that a stable, but only partially assembled complex I is accumulated in the cytoplasmic membranes of the *nuoK* and *nuoL* mutants, the membrane proteins of the parental strain and the *nuo*-mutant strains were solubilized with dodecyl-maltoside and applied to sucrose gradient centrifugation [26]. The NADH/ferricyanide oxidoreductase activity of the fractions of the gradient was measured (Fig. 5). The fully assembled *E. coli* complex I of the parental strain sedimented through two thirds of the gradient as observed with other strains [26, 51]. No activity peaks were detected at the corresponding position in the extract from all *nuo*-mutant strains (Fig. 5) indicating that none of the *nuo*-mutants contained a fully assembled or stable complex I as expected from the insensitivity of the NADH oxidase activity to annonin VI (Table 1). It is noteworthy that the amount of the alternative NADH dehydrogenase, which sediments at a position around fraction 3 under these conditions, was not enhanced in the *nuo*-mutants derived from strain BW25113.

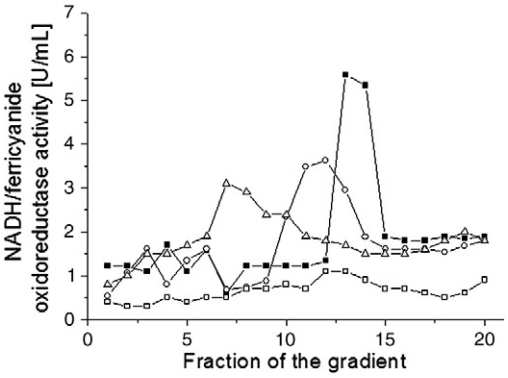

Fig. 5. Detection of fully and partially assembled complex I by sucrose gradient centrifugation of dodecyl-maltoside solubilized cytoplasmic membranes. Extracts from strains BW25113 (closed square), the *nuoB* (open square), the *nuoK* (open triangle), and *nuoL* (open circle) mutant strains were separated 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. Fractions of the gradients (numbered 1–20 from top to bottom) were collected and analyzed for NADH/ferricyanide oxidoreductase activity. The activity was normalized to 30 mg of protein loaded on the gradients for a better comparison. An activity profile similar to that of the *nuoB* mutant strain was obtained with all residual *nuo*-mutant strains.

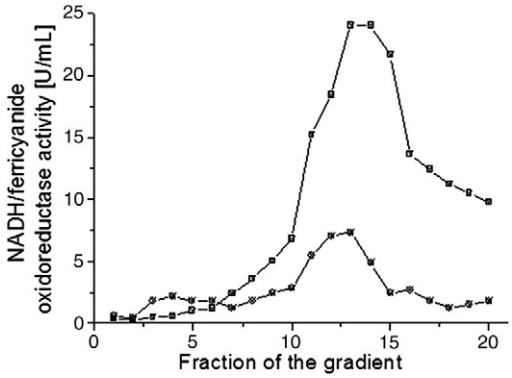

Fig. 6. Detection of overproduced complex I by sucrose gradient centrifugation of dodecyl-maltoside solubilized cytoplasmic membranes. Extracts from strains BW25113Δ*nuo*/pBAD*nuo*$_{His}$ (closed square) and BW25113Δ*nuo*/pBAD*nuo*$_{His}$, *nuoL::ntpI/sacRB* (open diamond) were separated by means of gradients of 5–30% (w/v) sucrose as described in Fig. 5. The activity was normalized to 30 mg of protein loaded on the gradients for a better comparison.

A prominent peak of the NADH/ferricyanide oxidoreductase activity was detected in the extract from the *nuoL* mutant in the middle of the gradient (Fig. 5). The peak most likely stems from complex I missing subunit NuoL, which has a molecular mass of 66.4 kDa [9, 16]. A partially assembled complex I missing NuoL would therefore have a molecular mass of approximately 470 kDa, which is in accordance with the position of the activity peak in the gradient. Due to its low abundance it was not possible to further purify this partially assembled complex to homogeneity.

The NADH/ferricyanide oxidoreductase activity profile of the sucrose gradient of the extract obtained from the *nuoK* mutant showed a small peak around fraction 7, corresponding to the position of the NADH dehydrogenase fragment (Fig. 5). Again, this partially assembled complex was not stable enough to allow further purification. If the activity peak was caused by the NADH dehydrogenase fragment, as indicated by its position in the gradient, it must derive from a larger fragment containing other, hydrophobic complex I subunits. Otherwise it would not be contained within the membrane fraction. It is likely that the NADH dehydrogenase fragment was cleaved from its membrane anchoring subunits by the detergent extraction. The residual membranous subunits were not detected by our assay because they do not exhibit NADH/ferricyanide oxidoreductase activity.

5. Overproduction and characterization of the ΔNuoL variant

As it was not possible to purify the partially assembled complex I from the *nuoL* mutant strain due to its low abundance, all *nuo*-genes but *nuoL* were overexpressed in a strain lacking complex I. A derivative from *E. coli* strain BW25113, in which the *nuo*-operon was deleted by genomic replacement methods was used as host strain (M. Vranas and T. Friedrich, unpublished results). This strain was transformed either with the plasmid pBAD*nuo*$_{His}$ containing all *nuo*-genes [40] or with the plasmid pBAD*nuo*$_{His}$, *nuoL::nptI/sacRB* containing all *nuo*-genes but *nuoL* [21]. This system was used for the overproduction of complex I and the ΔNuoL variant. As described above, the membrane proteins of strains BW25113Δ*nuo*/pBAD*nuo*$_{His}$ and BW25113Δ*nuo*/pBAD*nuo*$_{His}$, *nuoL::nptI/sacRB* were solubilized with dodecyl-maltoside and separated by sucrose gradient centrifugation (Fig. 6). The NADH/ferricyanide oxidoreductase activity of the strain producing the parental complex sedimented through two thirds of the gradient as shown in Fig. 5. Thus, a fully assembled complex was obtained by overexpression of the *nuo*-genes from plasmid pBAD*nuo*$_{His}$. In contrast, the activity sedimented in fractions of lower density in the extract from the strain lacking *nuoL* on the plasmid (Fig. 6). Thus, the activity profile of the sucrose gradient centrifugation obtained from the overproducing strains was virtually identical to that obtained from the chromosomal mutants (Figs. 5,6).

The ΔNuoL variant was purified from the overproducing strain by anion-exchange chromatography and affinity chromatography as reported [40]. As expected, the preparation did not contain subunit NuoL but it contained an additional protein in substoichiometric amounts with an apparent molecular mass of 80 kDa. In order to separate this putative impurity from the variant, an additional anion-exchange chromatography on SQ-15 in 50 mM MES, 50 mM NaCl, 0.1% DDM, pH 6.0 was performed (Fig. 7). Unexpectedly, the elution profile showed two absorption maxima with NADH/ferricyanide oxidoreductase activity of approximately equal size (Fig. 7; Table 2). SDS-PAGE of both peaks revealed the presence of all complex I subunits but NuoL in both peaks. Only the second peak contained the additional 80 kDa band (Fig. 7). This band was identified as the inducible lysine decarboxylase (LdcI or CadA) by N-terminal sequencing (eight residues) and mass spectrometric analysis (sequence coverage of 43%). In some preparations this protein band was hard to identify because it has a similar apparent molecular mass as a proteolytic fragment of NuoG [47]. The second peak containing CadA eluted as a single homogeneous peak from a subsequent size-exclusion chromatography indicating the homogeneity of the preparation. It was not possible to remove CadA from the preparation by chromatographic methods. Even a re-chromatography using the affinity material with extensive washing after binding did not lead to the loss of CadA from the preparation demonstrating its firm attachment to the ΔNuoL variant.

To determine further differences between the two peaks obtained in the second anion-exchange chromatography, the electron transfer and proton translocation activity were measured. For this purpose, the preparations were reconstituted into proteoliposomes as described [21]. In the presence of 150 μM NADH and 60 μM decylubiquinone, the ΔNuoL variant lacking CadA showed an electron transfer rate of 1.5 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, which is 80% of the activity of the complex from the parental strain. Both, wild type and variant, were inhibited to 90% by the complex I specific inhibitor piericidin A. Proton translocation was measured as quench of the ACMA fluorescence. Under the same experimental conditions, the complex from the parental strain showed a 40% quench of the ACMA fluorescence while the ΔNuoL variant quenched the ACMA signal by 18%. Thus, the electron transfer reaction of the ΔNuoL variant present in the first peak after anion-exchange chromatography was only slightly reduced by the loss of NuoL, while its proton translocation activity was

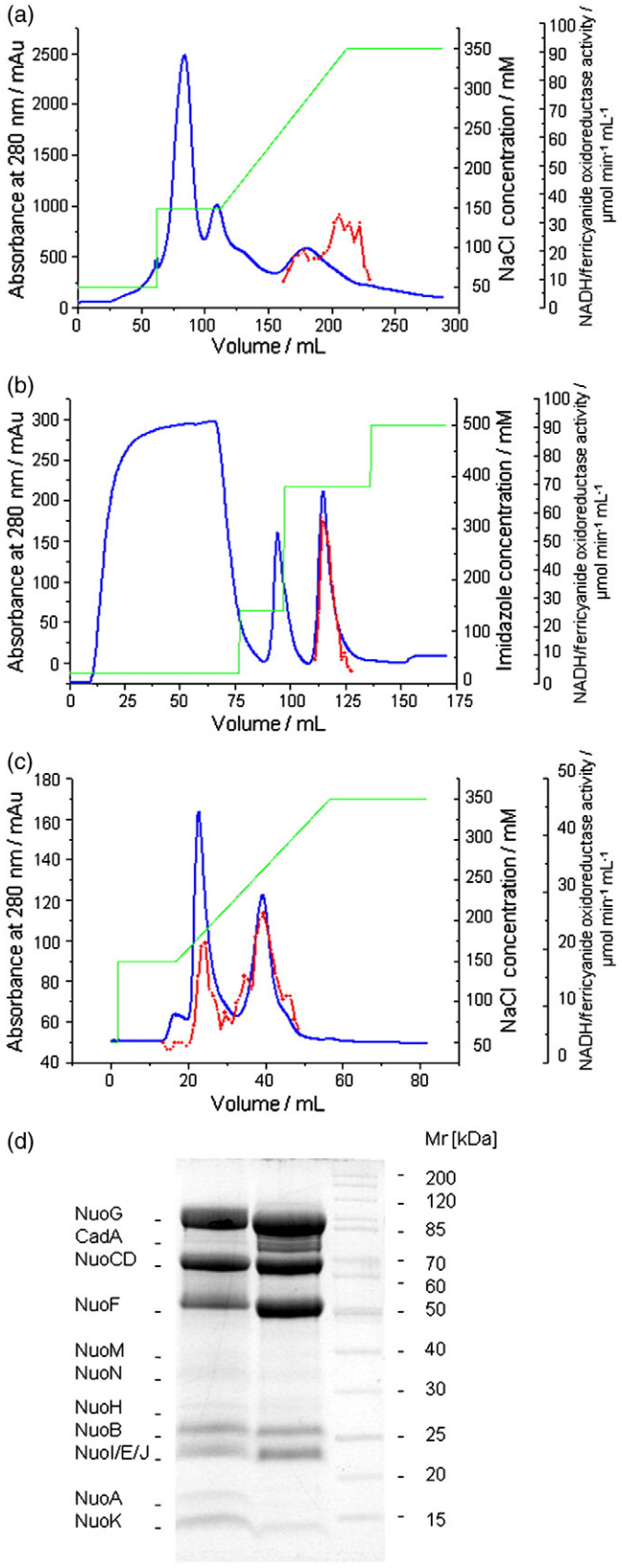

Fig. 7. Isolation of the two ΔNuoL variants produced in strain BW25113Δ*nuo*/pBAD*nuo*$_{His}$, *nuoL::ntpI/sacRB*. Chromatography on Fractogel EMD (a); chromatography on Pro Bond Ni^{2+}-IDA (b); chromatography on Source-Q15 (c); absorbance at 280 nm (blue); NADH/ferricyanide oxidoreductase activity (red); NaCl or imidazole gradient (green). SDS-PAGE of the two peaks after the third chromatographic step (d).

Table 2
Isolation of the *E. coli* complex I variants from strain BW25113Δ*nuo*/pBAD*nuo*$_{His}$ *nuoL::nptI/sacRB* starting from 26 g cells (wet weight).

	Volume	Protein	NADH/ferricyanide oxidoreductase activity		Yield
			Total	Specific	
Preparation	[mL]	[mg]	[µmol·min^{-1}]	[µmol·min^{-1}·mg^{-1}]	[%]
Membranes	63	695	1390	2.0	100
Detergent extract	61	583	1220	2.1	88
Fractogel	54	103	700	6.8	51
Probond-IDA	1.1	3.9	420	109	30
Source 15Q Peak 1	0.2	1.0	120	120	9
Source 15Q Peak 2	0.6	1.1	130	124	9

approximately halved [21]. This is in good agreement with the proposal that the large 'horizontal' helix on NuoL is involved in transmitting energy released by the redox reaction to the proton translocation site [6, 14, 21].

The ΔNuoL variant present in the second peak of the anion-exchange chromatography and associated with CadA was completely inactive with respect to electron transfer and proton translocation. Both peaks from the anion-exchange chromatography were analyzed by EPR spectroscopy after reduction with a 1000-fold molar excess NADH. The spectra of the binuclear Fe/S clusters recorded at 40 K were identical in both preparations (data not shown). However, the spectra recorded at 13 K revealed that the inactive population was lacking the most distal cluster N2 explaining its inactivity (Fig. 8). By western blot analysis it was evident that subunit NuoB containing N2 was present in the preparation (data not shown).

Thus, overexpression of all *nuo*-genes except *nuoL* in an *E. coli* strain lacking complex I led to the production of two populations of a partially assembled complex I. One population was missing NuoL and has retained a nearly unchanged electron transfer activity and a roughly halved proton translocation activity [21]. The other population was lacking NuoL but was tightly associated with CadA. This population is lacking N2 and therefore enzymatically inactive.

6. Discussion and outlook

The mechanism of complex I is not yet fully understood although the recent determination of the structures of the complex from *T. thermophilus* and *Y. lipolytica* [14, 15] was a milestone in complex I research. Complementary to structural studies, the investigation of the assembly of such huge and modularly built machinery may shed light on the function of the individual modules and their interactions within the complex. The first step in studying the assembly of the *E. coli* complex I involves the question whether all genes of the *nuo*-operon are essential for the production and the assembly of a functional complex. Our data with mutants containing individually disrupted *nuo*-genes by insertion of a resistance cartridge show that this is indeed the case. The *nuo*-mutant strains showed a decreased growth rate (Fig. 1) and complex I mediated activity could not be detected in the cytoplasmic membranes (Table 1). In addition, no structurally intact complex could be extracted from the mutant membranes (Fig. 5). Thus, all *nuo*-genes are needed for the production or the assembly of a functional complex I in *E. coli*.

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 corresponding wild-type gene *in trans* (Fig. 1). This is in accordance with reports in the literature describing the successful complementation of mutants with a resistance cartridge inserted in *nuoA, B, CD, G, H, J, K, M, and N* [28, 39–42, 49, 50, 52–56]. The slightly reduced complex I activity measured in all these

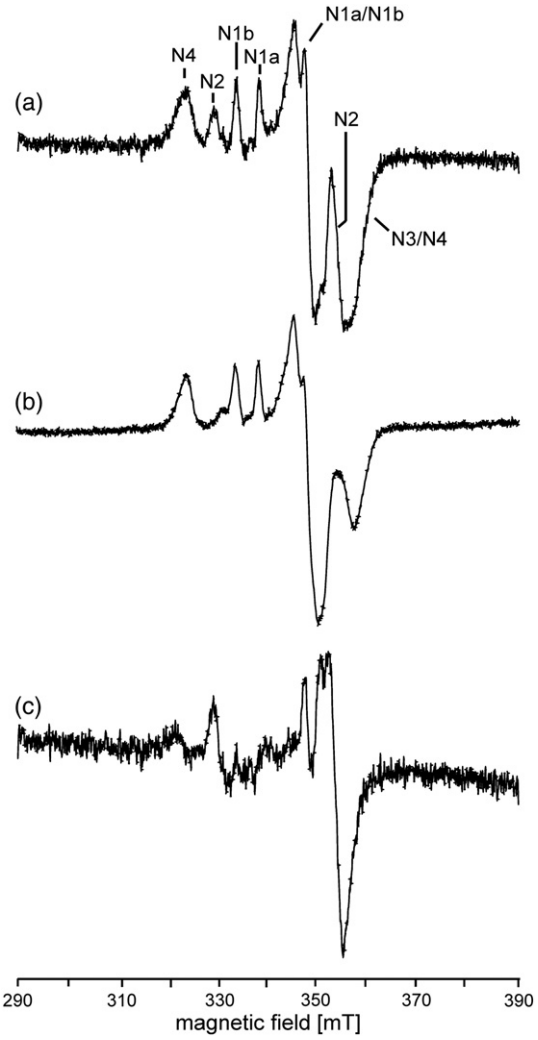

Fig. 8. EPR spectra of the two populations of the ΔNuoL variant produced in strain BW25113Δ*nuo*/pBAD*nuo*$_{His}$, *nuoL::ntpI/sacRB*. The spectrum of the first (a) and the second (b) peak after anion-exchange chromatography is shown. The samples were reduced by NADH and the spectra were recorded at 13 K and 5 mW. Other EPR conditions were as given in Fig. 3. The differences between the two spectra are shown in (c).

experiments is most likely due to a mild polar transcriptional effect of the integration mutagenesis. The fact that the individual *nuo*-genes either overlap, meet each other or are separated by small intergenic sequences in the *nuo*-operon [9] indicates translational coupling of the genes. The progression of the ribosome on the mRNA may be required to create the structures needed at the translational initiation site of the following *nuo*-gene, which may be otherwise trapped in a secondary structure. The marginal polar effect of the inserted kanamycin resistance cartridge has also been reported for the mutagenesis of the *nuo*-operon in *Rhodobacter capsulatus* [57].

It was shown with *R. capsulatus* that disruption of the homologues of *nuoH*, *J*, *K*, *L*, and *N* led to a complete loss of complex I [57, 58]. No complex I fragments were detected in these mutant strains. A C-terminal deletion of subunit NuoG leads to the loss of a functional *E. coli* complex I [59]. The mutant grew poorly on minimal salt medium containing acetate as the sole carbon source and failed to produce the inner L-aspartate chemotactic band on tryptone swarm plates. This is most likely due to the fact that the NADH/NAD^+ ratio is out of balance in cells lacking a functional complex I [59, 60] and that elevated levels of NADH inhibit the function of the TCA cycle and the glyoxylate shunt [61].

Separation of the cytoplasmic proteins of the *nuoH*, *nuoI*, *nuoJ*, *nuoK*, *nuoM,* and *nuoN* mutant strains by BN-PAGE resulted in a single band consisting of the subunits NuoE, F, and G and showing NADH dehydrogenase activity (Fig. 2). The position of the band corresponds to a molecular mass of approximately 200 kDa indicating that no other complex I subunits were associated with this fragment. The absence of the fragment in the *nuoE*, *NuoF* and *nuoG* mutants was expected as the corresponding subunits comprise the fragment. It is most likely not found in the cytoplasm of the *nuoL* mutant because it is entirely present in the partially assembled complex I detected in the cytoplasmic membranes of this strain (Fig. 5). The same holds true for the *nuoK* mutant, however, as the partially assembled complex of the *nuoK* mutant strain seems to be instable as indicated by sucrose gradient centrifugation (Fig. 5), it is most likely that a part of the NADH dehydrogenase fragment is split off the membrane leading to its presence in the cytoplasm of the strain. The smaller amount of the fragment in the cytoplasm of the *nuoA*, *nuoB*, and *nuoCD* mutant strains might be caused by the mild polar effect due to the insertion of the resistance cartridge leading to a smaller amount of mRNA and subsequently to a lower concentration of the fragment in the cytoplasm. The presence of the FMN (Fig. 2) and of the binuclear Fe/S clusters N1a and N1b (Fig. 3) in the fragment indicates that it is fully assembled.

The fully assembled NADH dehydrogenase fragment present in the cytoplasm of most *nuo*-mutant strains might represent an assembly intermediate of complex I in *E. coli*, which would be compatible with evolutionary schemes that have been proposed for the bacterial complex I [3, 10, 24, 25]. In the models, the acquisition of first NuoG and then NuoE and NuoF is the last step in the evolution of the progenitor of today's complex I of bacteria and eucaryotes. However, it is also conceivable that it is a stable fragment resulting from the decay of a larger assembly intermediate present in the corresponding *nuo*-mutant strains.

Membrane-bound fragments of the complex were detected in the *nuoK* and *nuoL* mutant strains (Figs. 4,5). Neither of the fragments showed any detectable NADH:ubiquinone oxidoreductase activity (Table 1), however, their abundance in the mutant strains is very low and the resulting low activity might be covered up by that of the alternative NADH dehydrogenase. Due to their low abundance we were not able to characterize the subunit composition of the partially assembled complex in the chromosomal mutants. The EPR-signals of N2 were clearly detected in the *nuoL* mutant membrane indicating no disturbances in its environment (Fig. 4). While the partially assembled complex in the *nuoL* mutant seems to be rather stable, the one present in the *nuoK* mutant tends to lose the NADH dehydrogenase fragment (Fig. 5). The EPR spectrum of the *nuoK* mutant membranes showed the signal of an Fe/S cluster reminiscent to that of N2, however, at a slightly different position (Fig. 4). According to the structure of the *T. thermophilus* complex NuoK is located in close proximity to NuoB harboring N2 [14, 18]. The loss of NuoK might lead to a change of the environment of NuoB resulting in a change of the position of EPR-signal of cluster N2.

A biochemical characterization of the partially assembled complex produced in the absence of *nuoL* was possible by overexpression of all *nuo*-genes but *nuoL* in a strain lacking complex I. Two populations of a partially assembled complex I were accumulated in the membranes of this strain. One population is lacking NuoL but contains all EPR-detectable Fe/S clusters. It showed redox-driven proton translocation although with approximately half of the H^+/e^- stoichiometry of the parental strain [21]. This is in agreement with the finding by electron microscopy that NuoL and NuoM are located at the distal part of the membrane arm and can be removed individually while retaining the other complex I subunits [62]. The other population was inactive due to the absence of N2. More surprisingly, the inducible lysine decarboxylase CadA was tightly associated with this preparation.

CadA converts lysine to cadaverine using pyridoxal phosphate as cofactor to decarboxylate the α-carboxyl group of lysine [63, 64].

Together with the lysine/cadaverine antiporter CadB, CadA is expected to play a role in pH homeostasis at weak acidic conditions (pH≈5) by consuming intracellular protons and neutralizing the acidic by-products of fermentation acids [64, 65]. Maximum expression of the *cadBA* operon is achieved under anaerobic conditions at low pH and an excess of lysine [66]. It might be speculated that CadA is fortuitously bound to complex I as it consumes intracellular protons and complex I is a major sink for intracellular protons. However, CadA is neither bound to the fully assembled complex nor to the active ΔNuoL variant. It is tightly attached to the inactive ΔNuoL variant, indicating that this localization is not due to an affinity to a proton-rich environment. It is noteworthy, that CadA forms a distinctive complex with another protein named RavA [67]. RavA is a member of MoxR protein family of AAA^+ ATPases, which are discussed to act as molecular chaperones important for the formation of protein complexes or mediating the insertion of metal cofactors in apoproteins [68]. Indeed, it was demonstrated that CoxD, another member of the MoxR protein family, is involved in the biogenesis of the [$CuSMoO_2$] cofactor of the *Oligotropha carboxidovorans* CO dehydrogenase [69]. Thus, it is tempting to speculate that CadA activates a so far unknown member of MoxR protein family of AAA^+ ATPases, which is involved in the biogenesis of the distal Fe/S cluster N2 of the *E. coli* complex I. However, it cannot be excluded that CadA binds to the partially assembled complex to keep it in an assembly competent state. To gain further insight into the role of CadA for the assembly of complex I, we are currently investigating the assembly state of complex I in *cadA* and *ravA* mutant strains.

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