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Respiratory complex I from *Escherichia coli* does not transport Na^+ in the absence of its NuoL subunit

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ABSTRACT

We investigated H^+ and Na^+ transport by complex I from *Escherichia coli* devoid of the NuoL subunit, which is probably part of the ion translocating machinery. We observed that complex I devoid of the NuoL subunit still translocates H^+, although to a smaller extension than the complete version of complex I, but does not transport Na^+. Our results unequivocally reinforce the observation that *E. coli* complex I transports Na^+ in the opposite direction to that of the H^+ and show that NuoL subunit is involved in the translocation of both ions by complex I.

1. Introduction

Respiratory complex I (E.C.1.6.5.3) performs energy transduction by coupling ion translocation to NADH:quinone oxidoreduction. In this way it contributes to the establishment and maintenance of the transmembrane difference of electrochemical potential, which is vital for synthesis of ATP, exchange of solutes/nutrients across the membrane and motility. Recently, the investigation on complex I gained a new enthusiasm due to the extensive data obtained in its study, including its crystallographic structure. Still, complex I is the least understood enzyme of the respiratory chain. Bacterial complex I is in general constituted by 14 subunits designated NuoA to N (NADH:ubiquinone oxidoreductase) or Nqo1 to 14 (NADH:quinone oxidoreductase), accounting for a molecular mass of ~550 kDa. The subunits are arranged in an L-shaped structure, composed of a peripheral arm (NuoB-G and I), in which the catalytic activity takes place and a membrane arm (NuoA, H and J-N), which harbors the ion translocating machinery [1]. The seven subunits composing the membrane arm are almost linearly arranged as NuoH, A, J, K, N, M and L. The NuoL, M and N proteins, located at the distal end of the arm in relation to the base of the peripheral arm, are homologous to each other and to subunits of the multidrug resistance proteins (Mrp) Na^+/H^+ antiporters [1–3]. Based on the structural information [4] and taking into account the accepted stoichiometry of $4H^+/2e$ [5–7], the presence of four ion translocation sites was suggested (Fig. 1). Three of these translocation sites were hypothesized to be present in each antiporter-like subunit and the fourth site was proposed to be formed by a consortium composed of subunits NuoH, J and K [4].

Complex I is considered to be a H^+ pump [5–8] and in the case of complexes I from *Bos taurus* [6] and *Yarrowia lipolytica* [7] a stoichiometry of $4H^+/2e$ was obtained. Complex I from *Klebsiella pneumoniae* [9] was the first one suggested to be a Na^+ pump. Na^+ was proposed to be a coupling ion also in the case of complexes I from *Escherichia coli* [10] and *Y. lipolytiva* [11]. These results were highly debated also because H^+ translocation by *K. pneumonia* complex I was later observed and Na^+ transport was attributed to another enzyme [12].

We addressed the relation of complex I with Na^+ using complex I from *Rhodothermus marinus* as a model system. We investigated the translocation of ions by this complex I developing an innovative method using ^{23}Na-NMR spectroscopy, which allowed us to take the advantage of directly monitoring changes in Na^+

Abbreviations: HL, long amphipathic α helix; Mrp, multidrug resistance proteins; EIPA, 5-(N-ethyl-N-isopropyl)-amiloride; BN-PAGE, blue native polyacrylamide gel electrophoresis; NBT, nitroblue tetrazolium; EPR, electron paramagnetic resonance; DMN, 2,3-dimethyl-1,4-naphthoquinone; DUQ, decylubiquinone; DDM, n-dodecyl-β-D-maltoside; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; ACMA, amino-6-chloro-2-methoxyacridine; deamino-NADH, reduced nicotinamide hypoxanthine dinucleotide

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concentration [13–14]. Our results showed for the first time that complex I translocates Na^+ ions to the direction opposite to that of the establishment of the positive membrane potential, being the H^+ the coupling ion. To test if Na^+ translocation was a general property of complexes I, we extended our studies to other enzymes. We observed that complex I from *E. coli* also presents the antiporter activity but that from *Paracoccus denitrificans* does not [15]. The result obtained in the case of *E. coli* agreed with the previous observation that the magnitude of the pH gradient generated by the oxidoreduction activity of reconstituted complex I from *E. coli* increased with the raising of Na^+ concentration [16]. Moreover, we also observed that catalytic and transport activities of complex I from *R. marinus* could be decoupled in the presence of certain amounts of 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) (a Na^+/H^+ antiporter typical inhibitor) and that Na^+ and H^+ transports were differently affected by EIPA concentrations. The data indicated that complex I had two types of coupling sites, one dependent on the presence of Na^+ and the other independent of the presence of this ion, i.e. one working as Na^+/H^+ antiporter and the other as a proton pump, respectively [17]. Recently, Na^+/H^+ antiporter activity was detected in reconstituted complex I from *B. taurus* heart mitochondria in its so called "deactive" form [18].

The Na^+/H^+ antiporter-like subunits, NuoL, M and N, are strong candidates to perform proton and/or sodium translocation. The crystallographic structural data revealed that NuoL, M and N subunits present a highly similar structural core of 14 transmembrane (TM) helices, with two inverted structural repeats of five TM helices (TM4–8 and TM9–13) in a face-to-back arrangement [1,19]. These inverted structural repeats are typical characteristics of antiporters [20]. Recently, subunits NuoL, M and N were observed to complement the growth of *Bacillus subtilis* cells in which the genes coding for MrpA or MrpD have been deleted [21–22]. In this work we investigated for the first time Na^+ transport by *E. coli* complex I devoid of NuoL, in addition to H^+ translocation. Our results unequivocally reinforce the observation that the entire complex I from *E. coli* transports Na^+ ions in the opposite direction to the transport of H^+ and show that NuoL subunit is involved in both in H^+ and Na^+ transport by complex I.

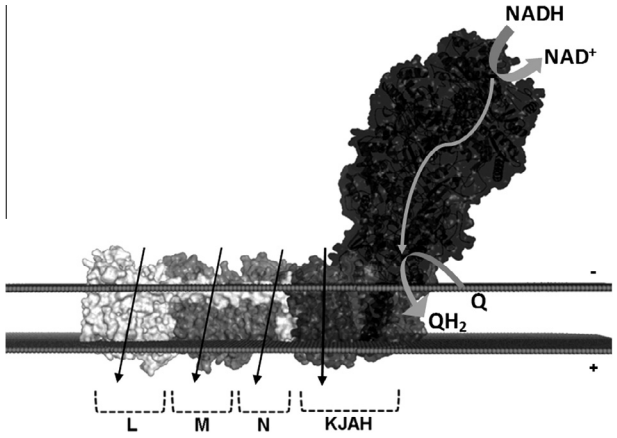

Fig. 1. Schematic representation of respiratory complex I and its ion translocation sites (Adapted from PDB: 3M9S). Bacterial complex I is in general constituted by 14 subunits designated NuoA to N. The subunits are arranged in an L-shaped structure, consisting of a peripheral (NuoB-G and I) and a membrane (NuoA, H and J-N) arms. The peripheral part contains the catalytic sites where NADH is oxidized and quinone reduced. The membrane domain includes the ion translocating machinery composed of seven subunits, almost linearly arranged as NuoH, A, J, K, N, M and L. Complex I contains four proposed ion translocating sites, NuoL M, N (light gray and white subunits) and the consortium composed of NuoH, J and K subunits (dark gray subunits) [4]. The black arrows indicate the approximate sites of ion(s) translocation. In this work the ion transport by complex I devoid of NuoL (white subunit) was investigated.

2. Materials and methods

2.1. PCR

We use an *E. coli* strain devoid of NuoL obtained from Keio collection [23]. The absence of *nuoL* gene from *E. coli* genome was verified by PCR. The selected primers were complementary to the DNA sequence located upstream and downstream of the *nuoL* gene. The amplified fragments were sequenced and their sizes were verified by electrophoresis in an agarose (1%) gel. In this way the absence of the *nuoL* gene and the presence of the kanamycin cassette were confirmed.

2.2. Cell growth and membrane vesicles preparation

E. coli K12-MG1655 and *E. coli* containing complex I devoid of NuoL [23] were grown microaerophilically in LB medium at pH 7.0 and 37 °C. The cells were harvested in late exponential phase, to maximize the expression of complex I [24], suspended in 2.5 mM HEPES–Tris pH 7.5, 5 mM K_2SO_4, 25 mM Na_2SO_4 and disrupted in a French Pressure cell at 6000 psi. The membrane vesicles were obtained by ultracentrifugation of the broken cells (200 000×*g*, 2 h, 4 °C) followed by re-suspension in the previous buffer. Protein concentration was determined using the Biuret method modified for membrane proteins [25].

2.3. Evaluation of complex I assembly

Assembly of complex I was evaluated by blue native polyacrylamide gel electrophoresis (BN-PAGE) using a gradient gel (5–13%). BN-PAGE was carried out as described in [26]. *In-gel* complex I activity was analyzed by NADH:NBT oxidoreductase activity. The gel was incubated in 50 mM potassium phosphate pH 7.0 containing 0.2 mg/ml nitroblue tetrazolium (NBT) and 0.1 mg/ml NADH for 15–20 min, room temperature.

Electron paramagnetic resonance (EPR) spectra of solubilized membranes, reduced with 5 mM K_2-NADH in the presence of 5 mM KCN, were acquired on a Bruker EMX spectrometer equipped with an Oxford instruments ESR-900 continuous-flow helium cryostat, at 10 K, microwave frequency: 9.38 GHz and microwave power 2.4 mW.

Complex I activity, NADH:quinone oxidoreductase, was monitored at 340 nm ($\varepsilon = 6220\ M^{-1}\ cm^{-1}$) and 25 °C, on a Shimadzu UV-1800 spectrophotometer inside of an anaerobic chamber. The reaction mixture contained membrane vesicles in 2.5 mM HEPES–Tris pH 7.5, 5 mM K_2SO_4, 25 mM Na_2SO_4, 100 μM quinone (2,3-dimethyl-1,4-naphthoquinone (DMN) or decylubiquinone (DUQ)) and 100 μM K_2-NADH or reduced nicotinamide hypoxanthine dinucleotide (deamino-NADH). DMN was synthesized as described in [27]. The obtained data are the average of at least three independent assays.

2.4. Characterization of the membrane vesicles

The orientation of membrane vesicles was investigated by the deamino-NADH:$K_3[Fe(CN)_6]$ oxidoreductase activity, in the absence or in the presence of detergent (n-dodecyl-β-D-maltoside (DDM)). The activity was monitored, at 420 nm ($\varepsilon = 1020\ M^{-1}\ cm^{-1}$), 25 °C, on a Shimadzu UV-1800 spectrophotometer. The reaction mixture contained membrane vesicles or solubilized membrane vesicles in 2.5 mM HEPES–Tris pH 7.5, 5 mM K_2SO_4, 25 mM Na_2SO_4, 250 μM $K_3[Fe(CN)_6]$ and 250 μM deamino-NADH. Solubilized membranes were obtained by stirring an aliquot of membrane vesicles with 2 g DDM/1 g membrane protein for 2 h, 4 °C. The internal volume of the membrane vesicles was determined by EPR spectroscopy,

using 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) in the external medium and potassium chromium (III) oxalate as a quencher [13]. The NADH oxidase activity was monitored at 340 nm (ε = 6220 M^{-1} cm^{-1}) and 25 °C, on an OLIS upgraded Aminco DW2 dual wavelength spectrophotometer. The reaction mixture contained membrane vesicles in 2.5 mM HEPES–Tris pH 7.5, 5 mM K_2SO_4, 25 mM Na_2SO_4 and 100 μM K_2-NADH. Oxygen consumption was monitored at 25 °C on a Clark type oxygen electrode (Oxygraph from Hansatech instruments). The reaction was started by the addition of 4 mM K_2-NADH. When referred, KCN (2 mM) was added.

Membrane potential ($\Delta\Psi$) generation was monitored following oxonol VI absorption ($A_{628nm} - A_{587nm}$) at 25 °C, on an OLIS upgraded Aminco DW2 dual wavelength spectrophotometer [28]. The integrity of the vesicles was verified by NADH-driven $\Delta\Psi$ formation and was performed by addition of 4 mM K_2-NADH to the vesicles. When referred, 100 μM carbonyl cyanide m-chlorophenyl hydrazone (CCCP), 2 mM KCN, 200 μM DMN or 200 μM DUQ were added.

2.5. Detection of ΔpH

ΔpH generation was investigated by fluorescence spectroscopy, following the quenching in amino-6-chloro-2-methoxyacridine (ACMA) fluorescence intensity ($\lambda_{excitation}$ = 410 nm, $\lambda_{emission}$ = 480 nm) at 25 °C, on a Varian Cary Eclipse spectrofluorimeter. The reaction mixture contained membrane vesicles in 2.5 mM HEPES–Tris pH 7.5, 5 mM K_2SO_4, 25 mM Na_2SO_4 with 5 mM $MgSO_4$, 100 μM DMN, 5 μM valinomycin, 2 μM ACMA and 400 μM K_2-NADH. When referred, 10 μM CCCP or 2 mM KCN were added prior to the addition of the substrate. The output was calculated with a moving average of 15 points.

2.6. Na^+ transport

Na^+ transport was monitored by ^{23}Na-NMR spectroscopy. NMR spectra were recorded on a Bruker Avance II 500 MHz spectrometer, 18 °C, operating at 132 MHz for ^{23}Na. Experiments were performed as described in [13]. Thulium (III) 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis (methylenephosphonate) (4.5 mM) was used as a shift reagent for the Na^+ signal of the suspension medium. On average, 400 μl of membrane vesicles containing 27 mg of membrane protein were used in each NMR experiment in a 5 mm diameter tube in a total volume of 500 μl. A capillary tube containing the shift reagent dysprosium (III) tripolyphosphate (22 mM), was used in all experiments as external reference. Sodium concentration outside the membrane vesicles was determined by integrating the resonance frequency peak using the integration of the resonance frequency peak of Na^+ in the presence of $Dy(PPPi)_2^{7-}$ (inside the capillary) as reference. Spectra were obtained upon addition of 4 mM K_2-NADH to membrane vesicles or to membrane vesicles which were previously incubated with CCCP (10 μM), KCN (10 mM) or DMN (200 μM).

3. Results and discussion

3.1. Functional assembly of complex I devoid of NuoL

The assembly of complex I devoid of NuoL was verified by BN-PAGE using solubilized membranes. For comparison, membranes from *E. coli* K12 containing the entire complex I were subjected to the same protocol. Bands compatible with the respective expected molecular masses for each complex I were observed in the two membrane preparations. The bands from both preparations presented NADH:NBT oxidoreductase activity, indicating the presence of a NADH dehydrogenase (Supplementary Fig. 1). The mass of the bands and their reactivity with NADH evidence the presence of complex I in the membranes of *E. coli* containing complex I devoid of NuoL.

The membrane vesicles from *E. coli* containing complex I devoid of NuoL showed NADH:DMN, deamino-NADH:DMN, NADH:DUQ and deamino-NADH:DUQ oxidoreductase activities of 254.5 ± 15.5 nmol NADH $mg^{-1}_{protein}$ min^-, 38.7 ± 3.7 nmol dNADH $mg^{-1}_{protein}$ min^{-1}, 395.2 ± 4.5 nmol NADH $mg^{-1}_{protein}$ min^{-1}, 47.1 ± 1.2 nmol dNADH $mg^{-1}_{protein}$ min^{-1} which compares, under the same conditions, to 219.8 ± 21.9 nmol NADH $mg^{-1}_{protein}$ min^{-1} 52.5 ± 8.7 nmol dNADH $mg^{-1}_{protein}$ min^{-1}, 428.5 ± 13.6 nmol NADH $mg^{-1}_{protein}$ min^{-1}, 119.7 ± 18.0 nmol dNADH $mg^{-1}_{protein}$ min^{-1} for membrane vesicles containing the entire complex I. The assembly of complex I devoid of NuoL was further supported by the observation that solubilized membrane vesicles containing this complex I presented EPR signals upon reduction with NADH in the presence of KCN compatible with the presence of the N2 iron-sulfur cluster (Supplementary Fig. 2).

3.2. Characterization of the membrane vesicles from E. coli containing complex I devoid of NuoL

Membrane vesicles containing complex I devoid of NuoL and those containing the entire complex I showed deamino-NADH: $K_3[Fe(CN)_6]$ oxidoreductase activities of 438.8 ± 25.4 nmol dNADH $mg^{-1}_{protein}$ min^{-1} and 418.2 ± 32.2 nmol dNADH $mg^{-1}_{protein}$ - min^{-1}, respectively. The formed presented an inside-out orientation of 85% and the latter of 82%. Both membrane vesicles had an internal volume of 1 μl mg^{-1}, in agreement with previous results [15]. NADH oxidation by the respiratory chain was 165.0 ± 16.6 nmol NADH $mg^{-1}_{protein}$ min^{-1} and 160.9 ± 22.9 nmol NADH $mg^{-1}_{protein}$ min^{-1} for the membrane vesicles containing complex I devoid of NuoL and those containing the entire complex I. The activity was 63% and ~55% inhibited, respectively, in the presence of 0.5 μM piericidin A, the typical inhibitor of *E. coli* complex I. Both preparations contained tight vesicles allowing a stable formation of $\Delta\Psi$, when K_2-NADH was added, in the presence of oxygen. The establishment of $\Delta\Psi$ was abolished in the presence of the protonophore CCCP or inhibited in the presence of KCN (Fig. 2, panel A and B). Establishment of $\Delta\Psi$ was also observed in the presence of KCN, for both preparations, upon addition of K_2-NADH when DMN or DUQ was present (Fig. 2, panel C and D). This observation indicates the establishment of $\Delta\Psi$ by the activity of complex I.

3.3. H^+ and Na^+ transport by Complex I from E. coli containing complex I devoid of NuoL

H^+ transport by complex I was monitored by following the quenching in ACMA fluorescence intensity, using the two preparations of membrane vesicles, one containing the entire complex I and another containing complex I devoid of NuoL. H^+ transport by complex I, upon addition of K_2-NADH was observed for both preparations (Fig. 3). H^+ transport was not observed in the presence of CCCP. Considering an equivalent relative amount of complex I in the two preparations based on their deamino-NADH:DMN oxidoreductase activity, a smaller H^+ translocation by complex I devoid of NuoL can be estimated. We observed that the ACMA quenching in the presence of the membranes containing complex I devoid of NuoL upon addition of NADH is less than the ACMA quenching in the presence of membranes with entire complex I, under the same conditions. This result is in agreement with other reported works, in which the NuoL subunit was deleted, C-terminal truncated or point mutated. In all those cases, complex I presented less H^+ translocation activity [29–33].

We have shown previously that complex I from *E. coli* has Na^+/H^+ antiporter activity [15]. Thus Na^+ transport by complex I

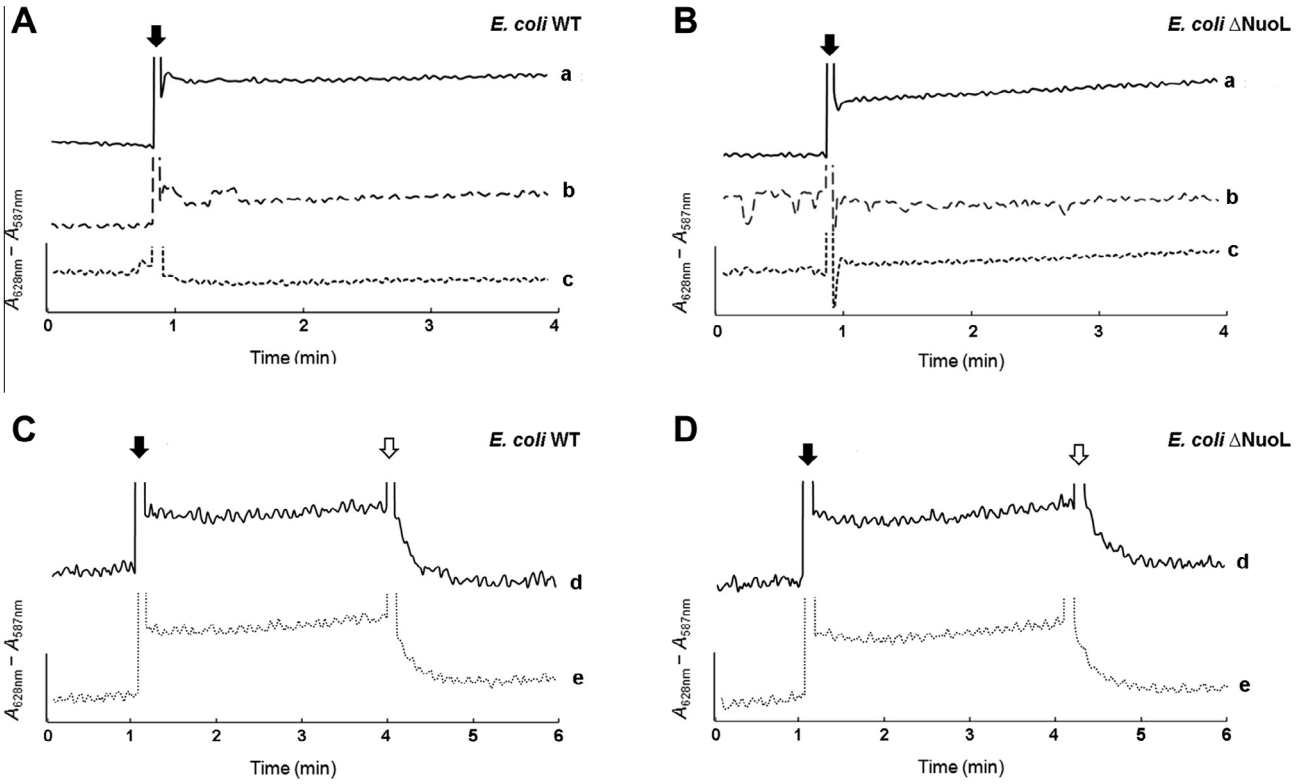

Fig. 2. NADH-driven $\Delta\Psi$ generation by *E. coli* membrane vesicles. $\Delta\Psi$ generation was detected following oxonol VI absorption ($A_{628} - A_{587}$) at 25 °C using membrane vesicles of *E. coli* containing the entire complex I (Panel A and C) and *E. coli* containing complex I devoid of NuoL (Panel B and D) upon addition of 400 μM K_2-NADH (indicated by a black arrow) and 100 μM CCCP (indicated by an white arrow). (a) NADH-driven $\Delta\Psi$ generation of respiratory chain; (b) NADH-driven $\Delta\Psi$ generation of respiratory chain inhibited by 2 mM KCN; (c) NADH-driven $\Delta\Psi$ generation of respiratory chain in the presence of 100 μM CCCP; (d) NADH-driven $\Delta\Psi$ generation of respiratory chain in the presence of 2 mM KCN and 200 μM DMN; (e) NADH-driven $\Delta\Psi$ generation of respiratory chain in the presence of 2 mM KCN and 200 μM DUQ. The represented data are the average of at least three independent assays.

devoid of NuoL was investigated using ^{23}Na-NMR spectroscopy (Fig. 4). For comparison and as a control, Na^+ transport by inverted membrane vesicles containing the entire complex I was monitored under the same conditions. Briefly, upon addition of NADH Na^+ transport to the outside of these vesicles, in the opposite direction of H^+ transport, was observed. Na^+ transport was not observed in the presence of KCN, the inhibitor of heme-copper oxygen reductases, the terminal reductases of the electron transport chain. Na^+ transport was recovered in these conditions by the presence of DMN, i.e. by NADH:DMN oxidoreductase activity. In the presence of CCCP, Na^+ transport by NADH:quinone oxidoreductase activity increased, indicating that the transport is a primary event. These results were equal to those obtained before [13,15]. In the case of *E. coli* membrane vesicles containing complex I devoid of the NuoL, under all experienced conditions, no Na^+ transport was detected (Fig. 4).

3.4. The role of NuoL subunit

In this work we investigated H^+ and Na^+ transports by *E. coli* complex I devoid of NuoL. We aimed at i) reinforcing our previous observations that complex I from *E. coli* translocates Na^+ ions in the opposite direction of the H^+, i.e. that it performs Na^+/H^+ antiporter activity and ii) to investigate the role of NuoL in ion transport.

NuoL subunit is located at the distal end of the membrane arm in relation to the base of the peripheral arm (Fig. 1). It is one of the antiporter-like subunits suggested to contain one ion translocating site. In addition, it contains a unique long amphipathic α helix (HL) parallel to the membrane plan, which may have a determinant role in energy transduction. Complex I devoid of NuoL contains three of the proposed ion translocating sites, NuoM, N and the consortium composed of NuoH, J and K subunits [4].

Four independent studies using complex I devoid of NuoL were carried out before, being apparently contradictory [29–30,32,34]. Two of these studies suggested that complex I devoid of NuoL does not assemble or presents a low expression and/or activity [30,34]. On the other hand the two other works showed that complex I without NuoL can assemble and is active [29,32]. The experiments performed were differently designed, including the use of different strains, which may explain first sight discrepancies respecting the assembly of complex I devoid of NuoL. Belevich et al. used *E. coli* GR70N and evaluated the production/assemble of complex I by the presence of deamino-NADH:HAR (hexammineruthenium (III)chloride) oxidoreductase activity [34]. The authors recognized that the used activity assay may not be always an accurate parameter. Nevertheless, the lack of deamino-NADH:HAR oxidoreductase activity associated to the impaired growth of *E. coli* GRL3 in minimal medium led the authors to conclude that complex I was not assembled if gene *nuoL* was deleted from the chromosome of *E. coli* GRL3 [34]. Torres-Bacete and coworkers used a NuoL knockout mutant from *E. coli* MC4100 [35]. They did not observe deamino-NADH oxidase or deamino-NADH:Q oxidoreductase activities. Still, other subunits of complex I, including NuoM subunit, were present in the membranes as observed by immunobloting assays. The authors suggested that the absence of activities by that mutant could be explained by the incorrect assembly of complex I [35]. Steimle et al. worked with *E. coli* BW25113 and were able to purify complex

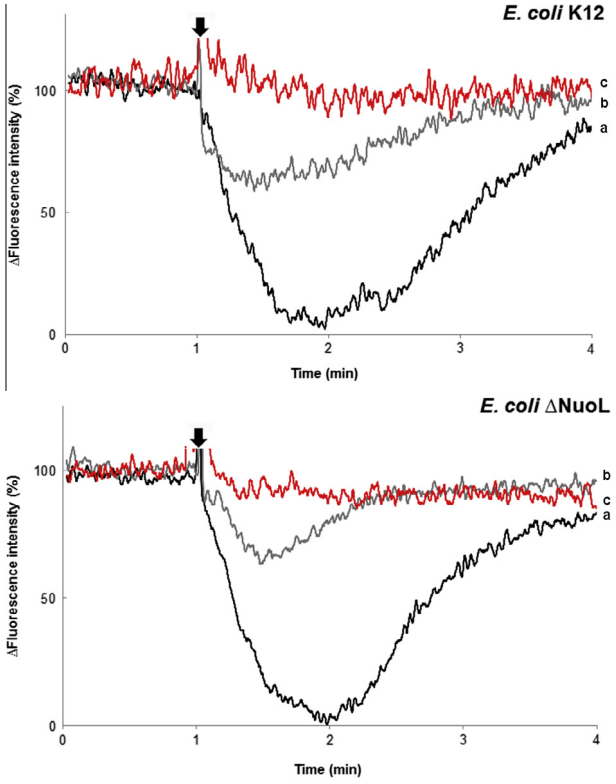

Fig. 3. ΔpH generation by *E. coli* membrane vesicles. Quenching of ACMA fluorescence intensity ($\lambda_{excitation}$ = 410 nm, $\lambda_{emission}$ = 480 nm) at 25 °C using membrane vesicles of *E. coli* containing the entire complex I (Panel A) and *E. coli* containing complex I devoid of NuoL (Panel B) upon addition of 400 μM K_2-NADH (indicated by an arrow). The reaction mixture contained membrane vesicles in 2.5 mM HEPES–Tris pH 7.5, 25 mM Na_2SO_4, 5 mM K_2SO_4 with 5 mM Mg_2SO_4, 150 μM DMN, 5 μM valinomycin and 2 μM ACMA. (a) No further addition; (b) r in the presence of 2 mM KCN and 200 μM DMN (complex I); (c) in the presence of 100 μM CCCP. The represented data are the average of at least three independent assays.

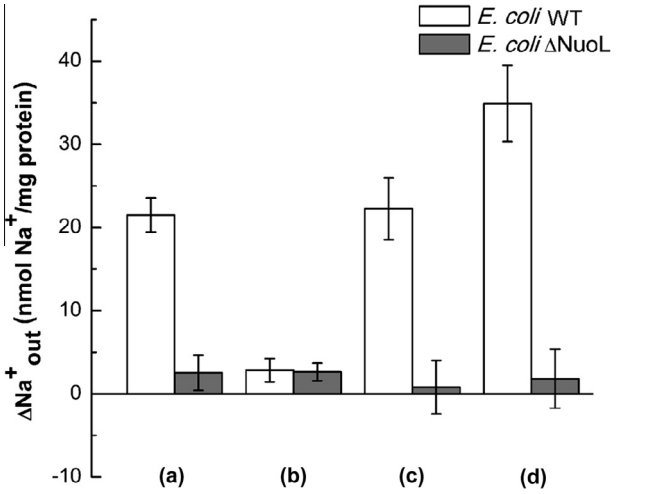

Fig. 4. Na^+ transport by *E. coli* membrane vesicles. ^{23}Na-NMR spectroscopy was used to monitor Na^+ transport, 5 min after K_2-NADH addition, using membrane vesicles of *E. coli* containing the entire complex I (white columns) and *E. coli* containing complex I devoid of NuoL (gray columns). (a) No further addition; (b) in the presence of 10 mM KCN; (c) in the presence of 10 mM KCN and 200 μM DMN; (d) in the presence of 10 mM KCN, 200 μM DMN and 10 μM CCCP. The represented data are the average of at least three independent assays.

I devoid of NuoL subunit [32]. Both the entire complex I and the one devoid of NuoL presented similar EPR spectra, when reduced with NADH. The complex devoid of NuoL was shown to be active and to translocate H^+, although to a smaller extension [32].

We observed that complex I devoid of NuoL subunit from *E. coli* assembles and is active, presenting lower H^+ translocation, when compared to the entire complex. In addition no Na^+ transport was observed. It can thus be hypothesized that (i) NuoL subunit is the site in which Na^+ and part of H^+ transports occur, (ii) NuoL influences ions transport by other subunits or (iii) both.

Subunits NuoL, M and N are homologous to subunits MrpA and MrpD of the so-called Mrp Na^+/H^+ antiporters. Recently, it was observed that cells from *B. subtilis* in which the genes coding for MrpA or MrpD have been deleted, could closely recover the respective growth profiles when expressing NuoL, M or N subunits [21–22]. Previously, membrane vesicles of *E. coli* lacking Na^+/H^+ antiporters were shown to exhibit a higher Na^+ uptake, when a C-terminal truncated version of NuoL subunit is expressed. This C-terminal truncated version of NuoL does not contain HL or the second inverted repeat. Still, Na^+ uptake was also observed when this construct was reconstituted in liposomes [36]. All together these results reinforce that NuoL is involved Na^+ transport, being possibly a Na^+/H^+ antiporter. Na^+ transport may not be exclusive to NuoL, since expression of NuoM or N also recovered the growth profiles of *B. subtilis* devoid of *mrpA* or *mrpD* genes. Thus NuoL may also influence the transport of this ion by other antiporter- like subunit(s) in the entire complex I.

In this work, complex I devoid of the NuoL subunit was observed to be able to transport H^+, although to a lower extent. These results are in agreement with the observed lower H^+/e stoichiometry in *E. coli* complex I devoid of the NuoL subunit [32] and in *Y. lipolytica* complex I devoid of the NuoL and M subunits [29]. In this case, the observation that the same stoichiometry was obtained irrespectively of the presence or absence of NuoM subunit may suggest that this subunit is not operating in complex I devoid of NuoL. Such interpretation supports the hypothesis that NuoL is also influencing the ion transport by other subunits. In this respect, a cooperative electrostatic coupling between titratable residues at the interface of the antiporter-like subunits has been recently suggested by theoretical calculations [37]. NuoL subunit has a unique C-terminal facing the cytoplasmic side, containing a ~110 Å HL spanning laterally to itself, NuoM and N subunits (Fig. 1). The C-terminal ends in a TM helix (TM16) anchored at the interface of NuoN (TM6 and TM7 helices), NuoK (TM1 helix and C-terminal) and NuoJ (TM2 helix) subunits [1]. Since this anchoring helix is on the side of the membrane domain its removal is not expected to destabilize significantly the domain and thus the complex. The role of the extended C-terminal of the NuoL subunit has been largely discussed from two different perspectives, one suggests its function as a coupling element and the other as a stabilizing component, keeping the three antiporter-like subunits together [7,29,32–35]. We have predicted the presence of an antiporter-like subunit with an extended C-terminal in several complexes related to complex I, independently of the number of the antiporter-like subunits present [38–39]. These complexes include the energy-converting hydrogenases and formate hydrogen lyases-1, which contain only one antiporter-like subunit. Such prediction questions an exclusive stabilizing role for the HL, since only one antiporter-like subunit is present [38].

In conclusion, our transport analyses showed that complex I devoid of NuoL is unable to transport Na^+, reinforcing our previous observation that *E. coli* complex I transports Na^+ ions in the opposite direction to the transport of H^+, the coupling ion. Our H^+ translocation assays indicate that complex I devoid of NuoL is less efficient than the entire complex I, in agreement with previous observations [32]. All together, these observations suggest that

NuoL can work as a Na^+/H^+ antiporter, additionally it may also influence the ion transport activity of other subunits.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.10.030.

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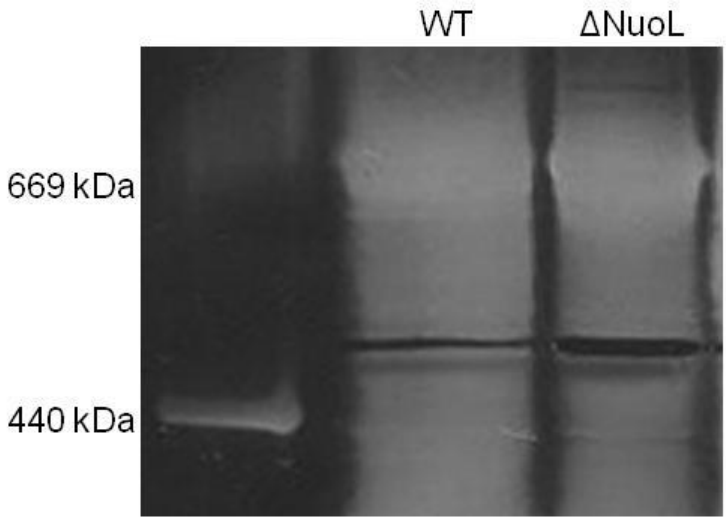
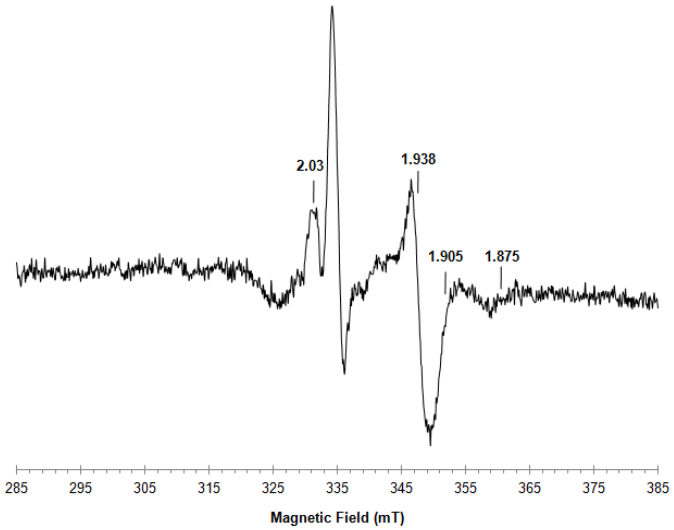
SUPPLEMENTARY MATERIAL



Supplementary Figure 1 – WT and ΔNuoL Complex I assembling and active. Blue Native Polyacrylamide Gel Electrophoresis, using a gradient gel (5-13%), of solubilized membranes of *E. coli* K12 (WT) and *E. coli* ΔNuoL strains and in gel activity (NADH:Nitrobluetetrazolium (NBT) oxidoreductase activity). WT and ΔNuoL presents bands compatible to the expected molecular mass of complex I (~540±50kDa and ~474±50kDa, respectively). NADH:NBToxidoreductase activity was observed for both strains.



Supplementary Figure 2 – EPR spectrum of reduced solubilized membranes from *E. coli* expressing Complex I devoid of NuoL subunit I. The solubilized membranes were reduced with 5 mM K_2-NADH in the presence of 5 mM KCN. The spectrum was obtained at 10 K, microwave frequency: 9.38 GHz and microwave power 2.4 mW.