Biochimica et Biophysica Acta 1807 (2011) 286–292

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Sodium influence on energy transduction by complexes I from *Escherichia coli* and *Paracoccus denitrificans*

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ARTICLE INFO

Article history:
Received 1 September 2010
Received in revised form 30 November 2010
Accepted 11 December 2010
Available online 21 December 2010

Keywords:
NADH dehydrogenase
NADH:quinone oxidoreductase
Respiratory chain
Sodium/proton antiporter

ABSTRACT

The nature of the ions that are translocated by *Escherichia coli* and *Paracoccus denitrificans* complexes I was investigated. We observed that *E. coli* complex I was capable of proton translocation in the same direction to the established $\Delta\Psi$, showing that in the tested conditions, the coupling ion is the H^+. Furthermore, Na^+ transport to the opposite direction was also observed, and, although Na^+ was not necessary for the catalytic or proton transport activities, its presence increased the latter. We also observed H^+ translocation by *P. denitrificans* complex I, but in this case, H^+ transport was not influenced by Na^+ and also Na^+ transport was not observed. We concluded that *E. coli* complex I has two energy coupling sites (one Na^+ independent and the other Na^+ dependent), as previously observed for *Rhodothermus marinus* complex I, whereas the coupling mechanism of *P. denitrificans* enzyme is completely Na^+ independent. This work thus shows that complex I energy transduction by proton pumping and Na^+/H^+ antiporting is not exclusive of the *R. marinus* enzyme. Nevertheless, the Na^+/H^+ antiport activity seems not to be a general property of complex I, which may be correlated with the metabolic characteristics of the organisms.

1. Introduction

Respiratory complex I catalyzes the electron transfer from NADH to quinone in a reaction that is coupled to charge translocation across the membrane, contributing to the establishment of a transmenbrane difference of electrochemical potential. Complex I is a large enzyme that is composed by peripheral and membrane parts in an L-shaped assembly. The crystallographic structure of the peripheral arm was already determined, showing the localization of the respective prosthetic groups (iron–sulfur centers and FMN) [1]. The structures of the membrane part from *Escherichia (E.) coli* complex I and of the entire complexes I from *Thermus (T.) thermophilus* and *Yarrowia (Y.) lipolytica* have recently been solved to 3.9, 4.5, and 6.3 Å resolution, respectively [2,3]. These strongly suggest that conformational changes at the interface of the peripheral and membrane domains may promote the movement of a long amphipathic helix that spans almost the entire membrane domain, which in turn may result in charge translocation [2]. The nature of the ion(s) that is translocated by complex I is still a highly discussed issue. It was for a long time assumed that the ion coupled to the NADH:quinone oxidoreductase activity was the proton [4,5]; however, in recent years, Na^+ has emerged as a new candidate [6,7]. Different energy conservation mechanisms, considering proton or sodium as the coupling ion, have been proposed [6,8–14].

We have recently shown that *Rhodothermus (R.) marinus* complex I is capable of proton and sodium translocation but to opposite directions, being the proton, the coupling ion. A model for the functional mechanism of complex I was then proposed, suggesting the presence of two different energy coupling sites. One coupling site may work as a proton pump and the other as a Na^+/H^+ antiporter [15]. A possible relation of complex I with antiporters has also been suggested based on sequence analyses of the hydrophobic subunits Nqo11, 12, 13, and 14, which are related to subunits of the Mrp type family of Na^+/H^+ antiporters [16,17]. In order to address whether the observed Na^+ transport by complex I was not exclusive of *R. marinus* complex I, we have investigated H^+ and Na^+ translocations by two canonical complexes I, from *E. coli* and *Paracoccus (P.) denitrificans*.

P. denitrificans respiratory enzymes are commonly used as model systems due to their close relation to the mitochondrial respiratory enzymes. Complex I is not an exception, and it has been used in a large number of studies [18–25]. As mitochondrial complex I, *P. denitrificans* enzyme is described as a proton pump and a H^+/e ratio of 1.5 to 2 was estimated [26,27]. The proton transport capacity was observed in

Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; DDM, *n*-dodecyl-β-D-maltoside; DMN, 2, 3-dimethyl-1, 4-naphthoquinone; DUQ, decylubiquinone; $Dy(PPPi)_2^{2-}$, dysprosium (III) tripolyphosphate; *E., Escherichia*; FCCP, carbonylcyanide *p*-trifluoromethoxy-phenylhydrazone; Monen, monensine; NMR, nuclear magnetic resonance; oxonol VI, 1,5-Bis(5-oxo-3-propylisox-azol-4yl)pentamethine oxonol; *P., Paracoccus*; Pier.A, piericidin A; Q, quinone; *R., Rhodothermus*; Rot, rotenone; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxy; $Tm(DOTP)^{5-}$, thulium(III)1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(methylenephosphonate); Val, valinomycin; $\Delta\Psi$, membrane potential; ΔpH, pH difference; ΔpH_{out}, external medium pH difference; ΔNa^+_{out}, external medium sodium concentration changes; pH_{out}, external medium pH

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doi:10.1016/j.bbabio.2010.12.008

comparative studies in which ratios of 8 H^+:O and 3–4 H^+:O were determined in cells, using different electron donors and inhibitors [26–28]. Complex I from *E. coli* is one of the most studied complexes I at the genetic and molecular levels [6,29–35]. It has been proposed that the coupling ion is the H^+ [4,30] and a stoichiometry of 1.5 H^+/e was indirectly determined in cells, using DMSO as an electron donor [4]. However, also Na^+ has been suggested to be the coupling ion of *E. coli* complex I [6]. Additionally, Stolpe and Friedrich observed an increase in proton transport by liposome-incorporated *E. coli* complex I when a sodium gradient was imposed, and a Na^+/H^+ antiport activity coupled to quinone reduction was suggested [30]. It was also shown that the over-expressed C-terminally truncated NuoL (Nqo12) subunit of the *E. coli* complex I mediates sodium uptake when reconstituted into liposomes and this transport was inhibited by the addition of amiloride derivatives, known as Na^+/H^+ antiporter inhibitors [29].

The present work allowed us to further investigate our previous model for energy conservation in *R. marinus* complex I in which we proposed the existence of two energy coupling sites and suggested that this complex I transduces energy by two processes: proton pumping and Na^+/H^+ antiporting [15]. We extended our studies to the canonical complexes I from *E. coli* and *P. denitrificans*, and we observed that the Na^+/H^+ antiport activity is not exclusive of *R. marinus* but is not a general property. We suggest that due to thermodynamic constrains, this property may be correlated with the type of quinone used as substrate. In this way, our findings open new perspectives in the general discussion of energy transduction by complex I.

2. Materials and methods

2.1. Cell growth and membrane vesicles preparation

E. coli was grown anaerobically ($pO_2 = 0\%$) in LB medium at 37 °C until $OD_{600nm} = 0.7$. *P. denitrificans* Pd1222 was grown aerobically in succinate medium at 30 °C, as previously described [36], until $OD_{600nm} = 6$. After harvesting, cells in 2.5 mM HEPES–Tris pH 7.5 with 5 mM K_2SO_4 and 10 mM Na_2SO_4 (buffer A), 25 mM Na_2SO_4 (buffer B) or 50 mM choline chloride (buffer C), were broken in a French Pressure cell at 19,000 psi. The membrane vesicles were obtained by ultracentrifugation of the broken cells at 200,000*g* for 2 h at 4 °C. Integrity of vesicles was checked by K^+/valinomycin assays using 1,5-Bis (5-oxo-3-propylisoxazol-4-yl) pentamethine oxonol (oxonol VI) as a membrane potential ($\Delta\Psi$) indicator (see below Section 2.3.). Protein concentration was determined by the Biuret method modified for membrane proteins [37].

2.2. Internal volume determination

The internal volume of the membrane vesicles was determined by EPR spectroscopy, using 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) [38]. TEMPO (oxidized with $K_3[Fe(CN)_6]$) in the external medium was quenched with 100 mM of potassium chromium(III) oxalate. EPR measurements were performed at room temperature, with a microwave frequency of 9.39 GHz, microwave power 1 mW, and modulation amplitude 0.04 mT.

2.3. $\Delta\Psi$ Detection

$\Delta\Psi$ Generation was detected following oxonol VI absorption (A_{628} *minus* A_{587}) at 18 °C [39]. The vesicles integrity was checked generating K^+ gradients with K^+/valinomycin in an external buffer containing 250 mM K_2SO_4 (internal $[K^+] = 10$ mM). The assays were started by the addition of 2 μM of valinomycin. To detect the NADH-driven $\Delta\Psi$ formation, the assay contained membrane vesicles in buffers B or C. The reaction was started by adding 4 mM of K_2NADH. When referred, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (10 μM), rotenone (10 μM), piericidin A (30 μM), or KCN (5 mM) was added prior to the addition of NADH. Measurements were obtained on an OLIS upgraded Aminco DW2 dual wavelength spectrophotometer.

2.4. Activity measurements

Oxygen consumption was measured with a Clark-type oxygen electrode YSI Model 5300. The assay mixture contained membrane vesicles in buffers B or C. The reaction was started by adding 4 mM NADH. When used, KCN (10 mM), CCCP (10 μM), rotenone (10 μM), and piericidin A (30 μM) were added prior to the addition of the substrate.

NADH:$K_3[Fe(CN)_6]$ oxidoreductase activity was monitored at 420 nm ($\varepsilon = 1020\ M^{-1}\ cm^{-1}$) on an OLIS upgraded Aminco DW2 dual wavelength spectrophotometer. The reaction medium contained membrane vesicles or solubilized membranes in buffers B or C, 250 μM $K_3[Fe(CN)_6]$ and 250 μM NADH. Solubilized membranes were obtained by stirring an aliquot of membrane vesicles with 2 % of *n*-dodecyl-β-D-maltoside (DDM) for 2 h at 4 °C.

NADH oxidation by membrane vesicles was monitored at 330 nm ($\varepsilon = 5930\ M^{-1}\ cm^{-1}$). The reaction medium contained membrane vesicles in the presence of 2.5 mM KCN and 150 μM of quinone analogues. The reaction was started by adding 150 μM of NADH. When used, CCCP (10 μM) was added prior to the addition of the substrate.

2.5. Fluorescence spectroscopy

The generation of a proton gradient (ΔpH) was determined by the quenching of the fluorescence of the 9-amino-6-chloro-2-methoxyacridine (ACMA) ($\lambda_{excitation} = 410$ nm, $\lambda_{emission} = 480$ nm). *P. denitrificans* membrane vesicles were incubated aerobically for 5 min at 18 °C in the assay buffers A, B, or C containing 1 μM of ACMA. The reaction was started by adding 50 μM of NADH. When referred, KCN (5 mM), rotenone (10 μM), CCCP (10 μM), or monensine (20 μM) was added prior to the addition of NADH. The external vesicle pH (pH_{out}) was followed using the hydrophilic and membrane-impermeable pH indicator, pyranine ($\lambda_{emission} = 508$ nm). Excitation spectra were obtained before and after NADH addition and data were analyzed based on the differences at $\lambda = 458$ nm [40]. *E. coli* membrane vesicles, when indicated, were incubated in the assay buffers B or C containing 2 μM of pyranine, 5 mM KCN, 200 μM 2,3-dimethyl-1,4-naphthoquinone (DMN), 200 μM decylubiquinone (DUQ) and 2 μM valinomycin. The reaction was started by adding 4 mM of NADH. Fluorescence spectra and kinetics were recorded on a Varian Cary Eclipse spectrofluorimeter.

2.6. ^{23}Na-NMR spectroscopy

NMR spectra were recorded on a Bruker Avance II 500 MHz spectrometer at 18 °C, operating at 132 MHz for ^{23}Na. Experiments were performed as described in Ref. [15]. 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. Since 1 equivalent of this shift reagent contains 5 equivalents of Na^+, the concentration of sodium in the external medium was always higher that inside the vesicles. In average, 400 μL of membrane vesicles containing 27 mg of membrane protein was 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. Spectra were recorded upon addition of 4 mM K_2NADH to membrane vesicles (prepared in buffer B) or to membrane vesicles, which were previously incubated with CCCP (10 or 100 μM), valinomycin (2 μM), rotenone (10 μM), piericidin A (30 μM), KCN (10 mM), DMN (200 μM), and DUQ (200 μM).

2.7. Sequence analysis tools

Searches for amino acid sequences in databases were performed at NCBI using the BLAST network service. Multiple alignments and dendrograms were obtained using CLUSTALX version 2.0. Accession numbers are given in the Supplementary material.

3. Results

3.1. Membrane vesicles characterization

The membrane vesicles preparations of *E. coli* and *P. denitrificans* were mainly composed by tight vesicles, which allowed the formation of a $\Delta\Psi$ using K^+ and valinomycin, stable for at least 30 min (data not shown). The orientation of *E. coli* and *P. denitrificans* membrane vesicles was determined comparing the NADH:$K_3[Fe(CN)_6]$ oxidoreductase activity in membrane vesicles before and after their solubilization with DDM. The activity was approximately the same in both situations and for both organisms, meaning that *E. coli* and *P. denitrificans* membrane vesicles were ~100% inside-out orientated. For both organisms, the internal membrane vesicles volume, determined by EPR spectroscopy and based on the quenching of a paramagnetic probe with a membrane impermeable agent, was found to be ~1 μL mg^{-1} of protein.

3.2. NADH-driven $\Delta\Psi$ generation

E. coli and *P. denitrificans* membrane vesicles were capable of create and maintain a membrane potential after the addition of NADH, stable for at least 20 min (Fig. 1A-a and B-a). The membrane potential was detected by monitoring the change in the absorbance of oxonol VI, which showed the build-up of a $\Delta\Psi$ (positive inside). If the membrane vesicles were preincubated with KCN (inhibitor of heme-copper oxygen reductases), rotenone or piericidin A (complex I inhibitors), the jump in A_{628nm}–A_{587nm} observed upon NADH addition was negligible, indicating that the observed $\Delta\Psi$ was generated by the functioning of the respiratory chain and, as consequence, showing the contribution of complex I to the generated $\Delta\Psi$ (Fig. 1A-b,c and B-b,c). NADH-driven $\Delta\Psi$ was also sensitive to the protonophore CCCP, indicating that $\Delta\Psi$ is also created by H^+ translocation (Fig. 1A-d and B-d).

3.3. Effect of sodium on the NADH:dioxygen oxidoreductase activity

The effect of sodium ions on the oxygen reduction by *E. coli* and *P. denitrificans* respiratory chains was monitored following the NADH:dioxygen oxidoreductase activity. *E. coli* membrane vesicles, prepared in 0 or 50 mM Na^+, were able to consume oxygen with similar rates (33 ± 3 nmol NADH min^{-1} mg^{-1} and 36 ± 3 nmol NADH min^{-1} mg^{-1}, respectively). For *P. denitrificans* membrane vesicles a decrease of approximately 20% on the NADH oxidation was observed when Na_2SO_4 was replaced by choline (54 ± 2 nmol NADH min^{-1} mg^{-1} *versus* 44 ± 2 nmol NADH min^{-1} mg^{-1}, respectively). In all the four cases, NADH oxidation was almost completely inhibited by KCN and by piericidin A or rotenone. An increase of up to 30% on the dioxygen consumption was observed in the presence of the protonophore, CCCP. Focusing on the NADH:quinone segment of the *E. coli* respiratory chain, a preincubation of membrane vesicles with KCN and quinone analogues was performed and the NADH consumption was spectrophotometrically monitored. An increase of up to 20% on the NADH:quinone oxidoreduction was observed in the presence of CCCP, indicating that this activity was limited by ΔpH.

3.4. NADH-driven ΔpH generation

Upon NADH addition to *P. denitrificans* membrane vesicles, a quenching in ACMA fluorescence intensity (used as a ΔpH indicator) was observed, indicating the establishment of a ΔpH (Fig. 2A-a). If the vesicles had been previously incubated with rotenone, KCN, the protonophore CCCP or the Na^+/H^+ exchanger monensine, the ACMA fluorescence quenching was negligible upon addition of the electron donor (Fig. 2A-b to e). It was previously shown that the NADH-driven ΔpH generation by complex I from *R. marinus* was not dependent on sodium, but that the presence of this ion increased proton transport

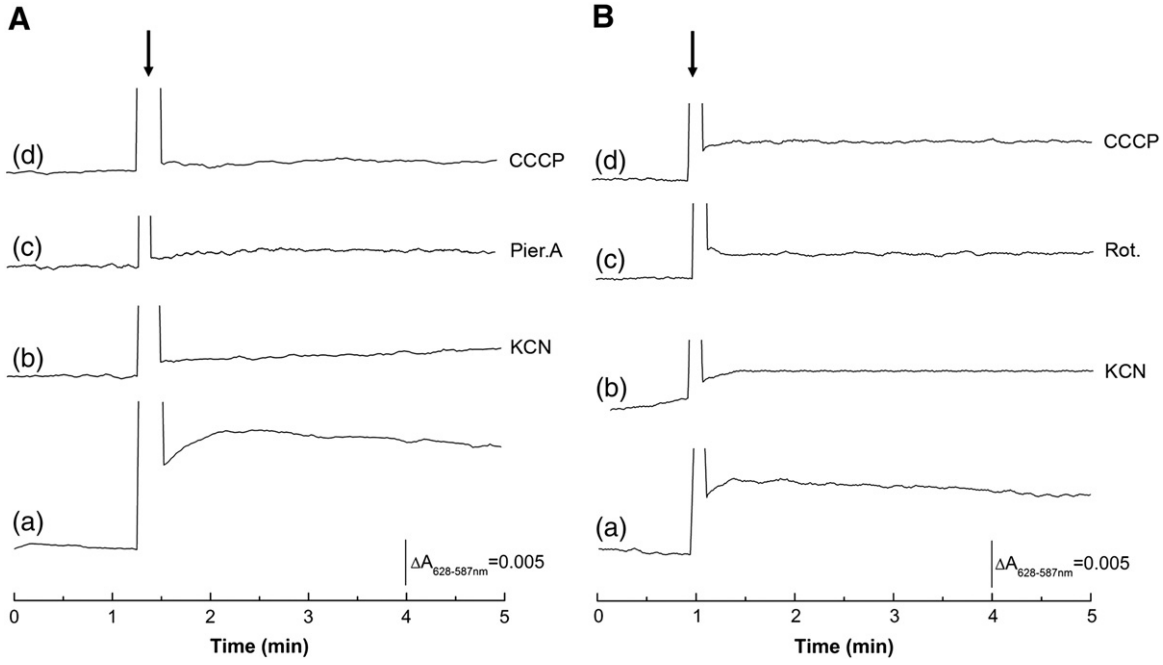

Fig. 1. Generation of a $\Delta\Psi$ by the functioning of *E. coli* and *P. denitrificans* respiratory chains, monitored by the absorbance difference of oxonol VI. The reaction was started by the addition of 4 mM of NADH (indicated by an arrow). (A) Absorbance difference of oxonol VI upon addition of NADH to *E. coli* membrane vesicles (a) and preincubated vesicles with 5 mM KCN (b), 30 μM piericidin A (Pier.A) (c), or 10 μM CCCP (d). (B) Absorbance difference of oxonol VI upon addition of NADH to *P. denitrificans* membrane vesicles (a) and preincubated vesicles with 5 mM KCN (b), 10 μM rotenone (Rot.) (c), or 10 μM CCCP (d).

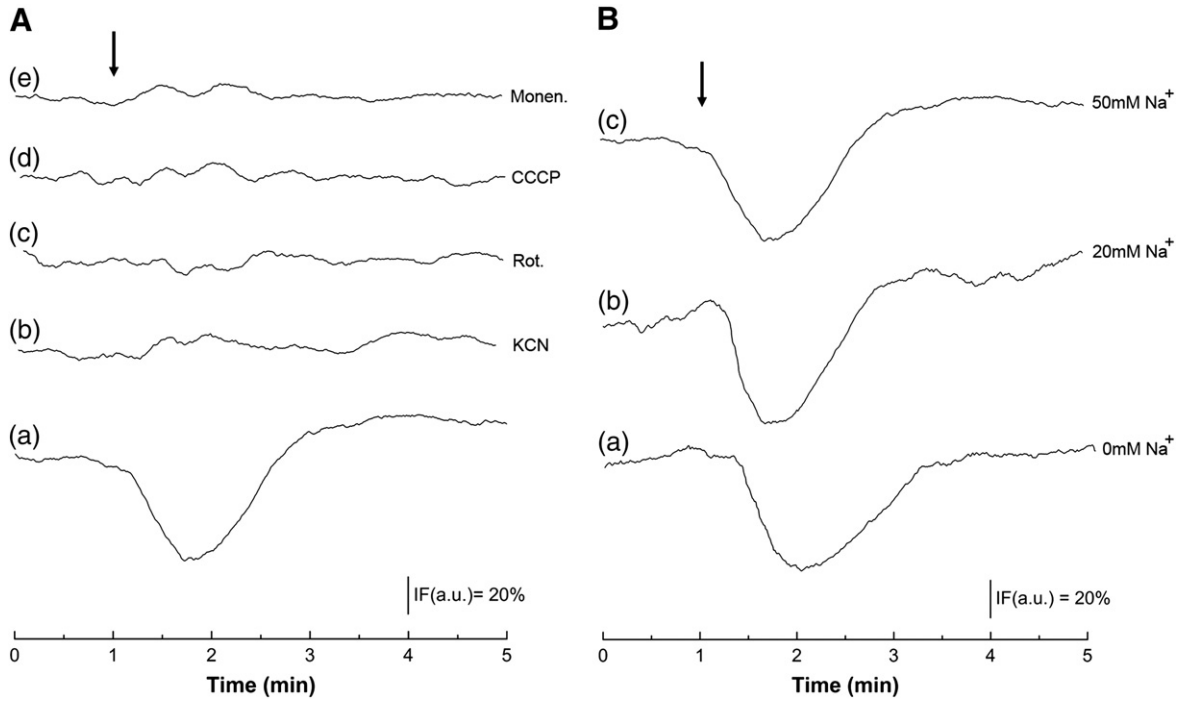

Fig. 2. Generation of a ΔpH by the respiratory chain of *P. denitrificans* monitored by ACMA fluorescence. The ACMA fluorescence was detected using an excitation wavelength of 410 nm and an emission wavelength of 480 nm. (A) The assay contained membrane vesicles in 2.5 mM HEPES–Tris pH 7.5, 5 mM K_2SO_4, 25 mM Na_2SO_4. The reaction was started by the addition of 50 μM NADH (indicated by an arrow) without preincubating the membrane vesicles (a), after preincubation with 5 mM KCN (b), 10 μM rotenone (c), 10 μM CCCP (d), 20 μM monensin (e). (B) The assay contained membrane vesicles in 2.5 mM HEPES–Tris pH 7.5 with 50 mM choline chloride (a), with 10 mM Na_2SO_4 (b), and with 25 mM Na_2SO_4 (c). The reaction was started by the addition of 50 μM NADH (indicated by an arrow).

[15]. A similar behavior was not observed for *P. denitrificans* complex I. Membrane vesicles prepared in the presence of 0, 20 or 50 mM Na^+ showed the same ΔpH amplitude upon NADH addition (Fig. 2B-a to c). Thus, in the case of *P. denitrificans* vesicles, the presence of sodium was not necessary for the establishment of a ΔpH and did not influence it.

3.5. NADH-driven external-vesicle pH (pH_{out}) variation

The use of the ACMA as a ΔpH indicator is not a straightforward procedure in the case of *E. coli* membrane vesicles [6]. Thus, in this case, we opted to monitor the change in pH outside the membrane vesicles upon NADH addition. This was performed by following the changes on pyranine fluorescence intensity. To focus on the NADH: quinone segment of the *E. coli* respiratory chain, a preincubation of membrane vesicles with KCN, DMN, and DUQ was performed. Addition of NADH (in the presence and absence of sodium) promoted an increase in the pyranine fluorescence intensity at 458 nm, indicating an increase in the pH_{out}. The pH difference was due to the proton translocation, from the outside to the inside of the vesicles, in addition to the proton uptake upon quinone reduction. The change in the pH_{out} was higher for membrane vesicles prepared with 50 mM Na^+ (Fig. 3). Approximately three- and four-fold increases of the pH_{out} change were observed in the presence of valinomycin for the membranes prepared in the absence and presence of sodium, respectively, indicating that in both cases, the NADH:quinone oxidoreductase activity was limited by $\Delta\Psi$ (Fig. 3). This observation excluded the possibility that the observed increase in H^+ uptake in the presence of Na^+ was due to a simple charge compensation effect.

3.6. NADH-driven ΔNa^+_{out} generation

^{23}Na-NMR spectroscopy was chosen to directly monitor changes in Na^+ concentration. In the case of *E. coli* membrane vesicles (prepared with 50 mM Na^+), an increase in the external [Na^+] of approximately 30 nmol of Na^+ per milligram of protein was observed upon NADH addition, indicating that Na^+ was transported from the inside to the outside of the membrane vesicles (Fig. 4A). Identical directionality of Na^+ transport was observed for *P. denitrificans*, but the amount of translocated Na^+ was smaller (10 nmol Na^+ per milligram of protein) (Fig. 4B). If membrane vesicles had been previously incubated with KCN or with a complex I inhibitor (piericidin A or rotenone), no Na^+ transport from the inside to the outside was observed (Fig. 4A and B).

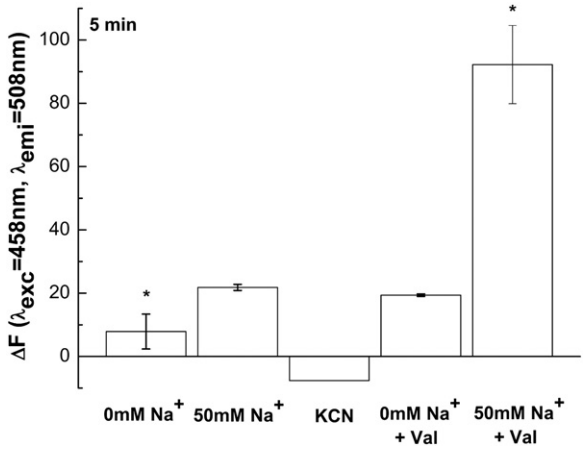

Fig. 3. Effect of sodium ions and $\Delta\Psi$ on NADH-driven pH_{out} variation by *E. coli* complex I. The pyranine fluorescence was detected using an excitation wavelength of 458 nm and an emission wavelength of 508 nm. The vesicles were preincubated with KCN (5 mM), DMN (200 μM), and DQU (200 μM). The assays contained membrane vesicles in 2.5 mM HEPES–Tris pH 7.5, 5 mM K_2SO_4 with 25 mM Na_2SO_4 (50 mM Na^+) or 50 mM choline chloride (0 mM Na^+). When referred the assays contained membrane vesicles in the presence valinomycin (Val) (2 μM). The reaction was started by the addition of 4 mM NADH. The represented data are the average of at least three independent assays (*$p<0.05$, *versus* membranes prepared with 25 mM Na_2SO_4 and preincubated with KCN *plus* DMN *plus* DUQ).

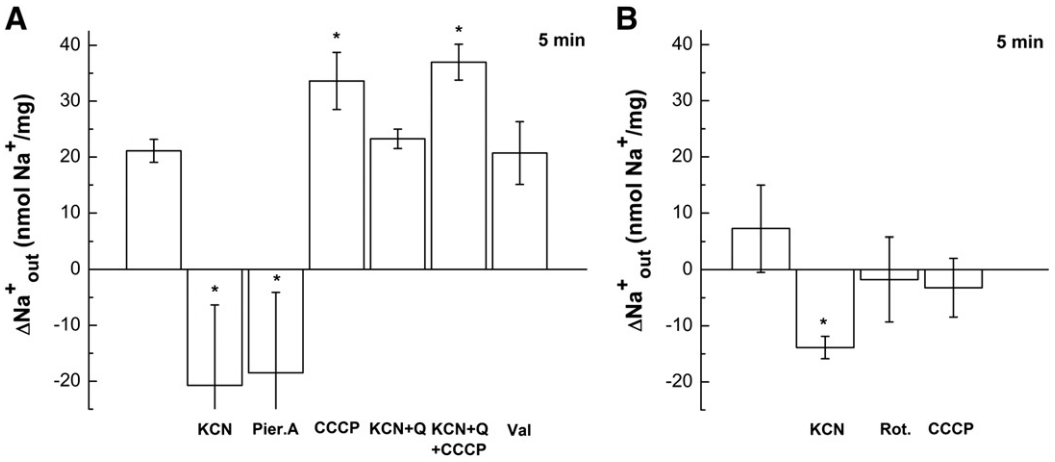

Fig. 4. Sodium ion transport by *E. coli* and *P. denitrificans* NADH-respiring membrane vesicles. (A) Sodium ion transport by *E. coli* membrane vesicles. Effect observed 5 min after the addition of 4 mM K_2NADH on external Na^+ concentration of membrane vesicles containing 50 mM Na^+ without preincubations or after preincubation with 30 μM piericidin A (Pier. A), 10 mM KCN (KCN), 10 or 100 μM CCCP (CCCP), 10 mM KCN plus 200 μM of menaquinone analogue DMN and ubiquinone analogue, DUQ (KCN + Q), 10 mM KCN plus 200 μM of DMN and DUQ plus 10 μM CCCP (KCN + Q + CCCP) or 2 μM valinomycin (Val). The represented data are the average of at least three independent assays (*$p<0.05$ *versus E. coli* membranes vesicles without preincubations).(B) Sodium ion transport by *P. denitrificans* membrane vesicles. Effect of 4 mM K_2NADH on external Na^+ concentration of membrane vesicles containing 50 mM Na^+ without preincubations or after preincubation with 10 μM rotenone (Rot), 10 mM KCN (KCN) or 10 μM CCCP (CCCP). The represented data are the average of at least three independent assays (*$p<0.05$ *versus P. denitrificans* membranes vesicles without preincubations).

Thus, Na^+ transport through the membrane vesicles occurred during NADH consumption by the *E. coli* and *P. denitrificans* respiratory chains. To distinguish between an active Na^+ transport by some respiratory enzyme and a possible secondary effect due to the proton gradient formed by respiration, the ionophores valinomycin and CCCP (10 and 100 μM) were added to the vesicles prior to the addition of NADH. Dissipation of the $\Delta\Psi$ and of the proton gradient with valinomycin and CCCP did not decrease Na^+ transport by *E. coli* membrane vesicles, showing that Na^+ transport to the outside of the membrane vesicles was actively performed by the *E. coli* respiratory chain (Fig. 4A). This active Na^+ transport was not observed in *P. denitrificans*. In this case, the NADH-driven ΔpNa was completely dissipated by the presence of CCCP (Fig. 4B), indicating that most probably, the observed Na^+ transport was a secondary event established by the action of the respiratory chain. Hypothesizing that the respiratory enzyme responsible for the *E. coli* active Na^+ transport was complex I, as in the case of *R. marinus* [15], the membrane vesicles from *E. coli* were preincubated with KCN and with ubiquinone and menaquinone analogues, DUQ and DMN. In these conditions, Na^+ transport was found to be similar to the one observed with non-inhibited vesicles in the absence of added quinones (Fig. 4A). *E. coli* complex I Na^+ transport activity was further supported by performing the same type of assays in the presence of KCN, external quinones and CCCP. Representative NMR spectra of the data represented in Fig. 4 are given in Supplementary Figure S1 in Supplementary material.

3.7. Amino acid sequence analyses of subunit Nqo11, 12, 13, and 14

Subunits Nqo11, 12, 13, and 14, due to their relation to the Mrp Na^+/H^+ antiporter subunits are the strongest candidates to be involved in Na^+ transport. Taking in consideration that complexes I from *E. coli* and *R. marinus* are able to translocate Na^+, whereas *P. denitrificans* complex I is not, we performed amino acid sequence alignments of those subunits in order to identify possible features that could be correlated to Na^+ transport (data not shown). From the obtained dendrograms, we observed that the sequences of Nqo12 and Nqo14 subunits from *E. coli* and *R. marinus* are closer to each other than to the sequences from *P. denitrificans* (data not shown). Inspection of the sequence alignments did not reveal any particular difference between the groups of sequences including those from *E. coli*, *R. marinus* and closest ones and the groups containing *P. denitrificans* sequences and its closest ones.

4. Discussion

In order to investigate whether the presence of two coupling sites was a specific characteristic of *R. marinus* complex I or if the proposed mechanism could be extended to other complexes I, H^+ and Na^+ transports were studied using *E. coli* and *P. denitrificans* complexes I. Inside-out membrane vesicles from these two organisms were prepared and were active towards NADH oxidation. These vesicles were capable of create and maintain a $\Delta\Psi$, positive inside. The abolishment of the $\Delta\Psi$ in the presence of the protonophore CCCP indicated the existence of a proton gradient generated by NADH oxidation. This effect was observed in membrane vesicles from the two organisms, showing that the functioning of each respiratory chain was responsible for the formation of a ΔpH.

The nature of the charge that is translocated by *E. coli* complex I has been determined to be H^+ [4,11] or Na^+ [6]. To focus on the NADH-quinone segment of the *E. coli* respiratory chain, the membrane vesicles were preincubated with KCN and quinone analogues, and H^+ and Na^+ translocations studies were performed. The change on the pH_{out} was followed using the pH indicator, pyranine. Upon NADH addition, an increase on the fluorescence intensity was observed being this change more pronounced when Na^+ was present. Since NADH oxidase activity is not stimulated by Na^+, we concluded that the ΔpH_{out} observed in these conditions was due to an increase in H^+ translocation and not to a higher H^+ consumption by quinone reduction. Thus, our results showed that *E. coli* complex I promoted H^+ translocation and corroborated the previous results obtained by Stolpe and Friedrich, who observed an increase in proton transport by liposome-incorporated *E. coli* complex I when a sodium gradient was imposed [30]. By ^{23}Na-NMR spectroscopy, Na^+ translocation from the inside to the outside of *E. coli* membrane vesicles was observed. This NADH-driven Na^+ transport was inhibited by KCN and piericidin A, specific inhibitors of heme-copper oxygen reductases and complex I, respectively, showing that Na^+ transport was performed by the functioning of the respiratory chain. The Na^+ transport was actively performed by a respiratory complex since it was not impaired by the presence of the protonophore CCCP. The site of Na^+ transport could be identified at the NADH-quinone segment because the Na^+ transport was recovered in membrane vesicles preincubated with KCN and DMN + DUQ. The possible contribution of the alternative NADH

dehydrogenase to the observed results was excluded since this enzyme is not electrogenic neither it is inhibited by complex I-specific inhibitors at the concentrations ranges used in the present work [41,42]. Also, the participation of a Na^+-dependent NADH dehydrogenase was not considered because the observed NADH:quinone oxidoreductase activity was not dependent on the presence of Na^+. Furthermore *E. coli* does not contain genes coding for a Na^+-dependent NADH dehydrogenase (www.genome.jp). In this way, the observed Na^+ transport was assigned to the functioning of *E. coli* complex I. Although kinetic resolution for sodium transport should be obtained, our results constitute a first step in the characterization of such transport by *E. coli* complex I.

The addition of NADH to *P. denitrificans* membrane vesicles resulted in the generation of a ΔpH, as indicated by the quenching of ACMA. The ACMA fluorescence did not change when the membrane vesicles were incubated with rotenone, KCN, CCCP, or monensin, showing a NADH-driven ΔpH formation by the functioning of the respiratory chain. These observations suggested that in *P. denitrificans*, the coupling ion between NADH oxidation and the establishment of the $\Delta\Psi$ is the proton. This result is in perfect agreement with previous studies performed in *P. denitrificans* cells in which a H^+/e stoichiometry of 1.5 to 2 was estimated [26]. It was also observed that the rate of NADH oxidation by *P. denitrificans* membrane vesicles increased in the presence of the protonophore carbonylcyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP), which corroborates the existence of a proton gradient [28]. We also performed Na^+ transport experiments with *P. denitrificans* membrane vesicles, using ^{23}Na-NMR spectroscopy and a slight increase in Na^+ concentration in the outside of the membrane vesicles upon NADH addition was observed. However, this increase upon NADH oxidation collapsed in the presence of the protonophore CCCP, indicating that the Na^+ transport was a secondary event. Thus, we concluded that *P. denitrificans* complex I was not able to perform Na^+ translocation, at least under the tested conditions. This is in agreement with the absence of any effect of Na^+ on NADH-driven ΔpH.

The observations that *R. marinus* and *E. coli* complexes I are able to transport Na^+, whereas *P. denitrificans* complex I is not raise one essential question: What is/are the main difference(s) between these complexes? All the three enzymes have the typical features of canonical complexes I [30,35,43–45]. Assuming that the responsible for Na^+ transport are the Mrp-like subunits (Nqo11 to Nqo14), the difference(s) between *E. coli* and *R. marinus* complexes I and that from *P. denitrificans* should reside on those subunits and thus we addressed this issue performing amino acid sequence analysis. These showed that Nqo12 and Nqo14 subunits from *P. denitrificans* complex I are more related to those of the mitochondrial complex I, than to those of complex I from *E. coli* and *R. marinus*. However, no relevant difference in the amino acid sequences could be observed between the cluster including *P. denitrificans* and the mitochondrial sequences and the cluster containing the sequences from *R. marinus* and *E. coli.*

A common aspect to *E. coli* and *R. marinus* complexes I is that these reduce menaquinone, whereas complex I from *P. denitrificans* uses ubiquinone as substrate. The difference in the reduction potentials of the two different types of quinones ($E_{m,7} \approx -80$ mV for menaquinone *versus* $E_{m,7} \approx +120$ mV for ubiquinone) [46] has strong thermodynamic implications. Considering the reduction potential of NADH, −320 mV, the free energy obtained by its oxidation by menaquinone can be ~240 mV, whereas in the case of oxidation by ubiquinone, the free energy obtained can be ~440 mV. Thus, considering a membrane potential usually of ~120 mV, in the first situation, the H^+/2e cannot exceed 2 while in the latter it can be up to 4. Although completely speculative, these observations may suggest that complexes I using ubiquinone as the electron acceptor transduce energy just by a proton pumping process (as in the case of *P. denitrificans*) while those that use menaquinone (as in the case of *E. coli* and *R. marinus*) transduce energy by proton pumping *plus* sodium/proton antiporting. In this case, the additional Na^+ transport may occur in order to guaranty the high efficiency of complex I in the overall energy transduction process. In evolutionary terms, it was suggested that the menaquinone represents the ancestral type of quinone in bioenergetics systems and thus, the only type of quinone in early branching archaeal and bacterial phyla [47]. Therefore, menaquinone reducing complexes may be considered ancestor enzymes. Thus, the ubiquinone reducing enzymes may have lost the need to use ΔpNa to promote additional proton translocation, transducing energy only by a proton pumping process. The possibility that some complexes I may perform an H^+/Na^+ antiporter activity is also sustained by the recently obtained structural data for complexes I from *T. thermophilus* and *Y. lipolytica*, in which structural features typical of those types of antiporters, such as the presence of discontinous transmembrane helices [48], are observed [2,3]. The situation created by the operation of complexes I from *E. coli* and *R. marinus* could result in sodium accumulation inside the cells, which in turn should be removed by the action of Na^+/H^+ antiporters. Such a scenario could create a sodium cycle. In fact such sodium cycle has been proposed before for *E. coli* cells, based on the observation of global H^+ and Na^+ transports [49,50]. It cannot be anticipated whether this cycle will be energetically unfavorable. The overall process will depend on the different stoichiometries of the individual transporting processes, thus the overall energy yielding is a combination of multiple factors.

In summary, our study indicates that complex I from *E. coli* transduces energy by proton pumping and by Na^+/H^+ antiporting, showing that these properties are not exclusive of *R. marinus* complex I. However, the Na^+/H^+ antiporting seems not to be a general property of complexes I since it was not observed for *P. denitrificans* complex I. Whether complex I from *P. denitrificans* does not contain a second coupling site or the second coupling site lost its Na^+ dependency is still an open question. The findings here reported open new perspectives in the studies of the process of energy transduction by complex I.

Acknowledgments

We thank Miguel Teixeira for the critical reading of the manuscript, to João Carita and Bruno C. Marreiros for cell growth, and to Dr. Rita Ventura and Filipa Sousa for performing the synthesis of DMN in Prof. Chris Maycock laboratory at ITQB. We acknowledge CERMAX at ITQB and Rede Nacional de RMN for access to the facilities, supported with funds from FCT, Projecto de Re-Equipamento Científico (REDE/1517/RMN/2005). The EPR equipment was funded by FCT (REEQ/336/BIO/2005 to ITQB). A.P.B. is recipient of a grant from Fundação para a Ciência e a Tecnologia (SFRH/BD/25288/2005). This project was funded by Fundação para a Ciência e a Tecnologia (POCI/QUI-BIQ/100302/2008 to M.P.).

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbabio.2010.12.008.

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