Biochimica et Biophysica Acta 1797 (2010) 509–515

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Energy conservation by *Rhodothermus marinus* respiratory complex I

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ARTICLE INFO

Article history:
Received 27 November 2009
Received in revised form 11 January 2010
Accepted 15 January 2010
Available online 25 January 2010

Keywords:
^{23}Na-NMR
Complex I
Ion transport
NADH dehydrogenase
Proton
Sodium

ABSTRACT

A sodium ion efflux, together with a proton influx and an inside-positive $\Delta\Psi$, was observed during NADH-respiration by *Rhodothermus marinus* membrane vesicles. Proton translocation was monitored by fluorescence spectroscopy and sodium ion transport by ^{23}Na-NMR spectroscopy. Specific inhibitors of complex I (rotenone) and of the dioxygen reductase (KCN) inhibited the proton and the sodium ion transport, but the KCN effect was totally reverted by the addition of menaquinone analogues, indicating that both transports were catalyzed by complex I. We concluded that the coupling ion of the system is the proton and that neither the catalytic reaction nor the establishment of the delta-pH are dependent on sodium, but the presence of sodium increases proton transport. Moreover, studies of NADH oxidation at different sodium concentrations and of proton and sodium transport activities allowed us to propose a model for the mechanism of complex I in which the presence of two different energy coupling sites is suggested.

1. Introduction

Rhodothermus marinus (*R. marinus*) is a halothermophilic bacterium isolated from shallow marine hot springs [1,2] whose optimal growth conditions are 65 °C, 2% NaCl and pH 7. It was phylogenetically placed at the phylum Bacteroidetes [3]. It is considered to be an obligate aerobic organism, with menaquinone-7 being the main quinone in its membranes [4]. Respiratory chain complexes and electron carriers of *R. marinus* have been purified and biochemically characterized in recent years, namely complexes I and II (NADH: menaquinone oxidoreductase and succinate:menaquinone oxidoreductase, respectively) [5–8], an alternative complex III [9,10], three terminal oxygen reductases [11–13] and two soluble electron carriers: a cytochrome *c* and a HiPIP (high-potential iron–sulphur protein) [10,14,15]. Additionally, a so-called type II NADH dehydrogenase coding gene is present in *R. marinus* genome, whereas genes coding for a Nqr Na^+ pumping NADH dehydrogenase are absent [16]. One of the *R. marinus* oxygen reductases was shown to pump protons [17], contributing to the transmembrane difference of electrochemical potential that is the driving force for ATP synthesis.

Complex I of respiratory chains, also called type I NADH dehydrogenase, couples the oxidation of NADH and the reduction of quinone to charge translocation across the membrane, also contributing to the buildup of a transmembrane difference of electrochemical potential. The mitochondrial complex is composed of more than 40 subunits and has a molecular mass of ~1 MDa, while the ~500 kDa bacterial counterpart is typically formed by 13 or 14 subunits, named Nqo1 to 14 or NuoA to N. It consists of a peripheral and a membrane part. The peripheral part contains a series of iron–sulphur centres (binuclear and tetranuclear) and non-covalently bound FMN [18,19]. The mechanism of quinone reduction and its coupling to charge translocation is not known, but it is generally accepted that it occurs in the membranous part of the complex. It was established a long time ago that complex I performs charge translocation by translocating protons. Ratios of 1.5 H^+/e for the *Escherichia coli* [20] and 2 H^+/e for the bovine complexes [21] have been determined. However, it was shown that *Klebsiella pneumoniae* complex I translocates sodium ions [22,23] with a ratio of 1 Na^+/e. In the respective article the proton translocation by this complex was not observed, but that was demonstrated later at pH 6–6.5 [24]. It was also shown that *E. coli* complex I is able to translocate sodium ions [25], in addition to the already established proton translocation. Moreover, Stolpe and Friedrich observed an increase in proton transport by liposome-incorporated *E. coli* complex I when a Na^+ gradient is imposed, and accordingly suggested a sodium/proton antiporter activity coupled to quinone reduction [26]. It remains to be established if these features are specific of the referred organisms or they constitute general

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; Dy(PPPi)$_2^{7-}$, dysprosium (III) tripolyphosphate; HiPIP, high-potential iron–sulphur protein; Menadione, 2-methyl-1,4-naphthoquinone; NQ, 1,4-Naphthoquinone; oxonol V, 1,5-Bis(3-phenyl-5-oxoisoxazol-4-yl) pentamethine oxonol; TEMPO, 2,2,6,6-tetramethyl-piperidine-1-oxyl; Tm(DOTP)$^{5-}$, thulium (III) 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(methylenephosphate); $\Delta\Psi$, transmembrane difference of electric potential

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doi:10.1016/j.bbabio.2010.01.020

properties of complex I. It should be added that the conserved complex I membranous subunits Nqo11, 12, 13 and 14 (Nuo K, L, M and N) resemble subunits from the so-called Mrp type Na^+/H^+ antiporters [27,28].

R. marinus complex I seems to be a canonical type I NADH dehydrogenase [5]: it was isolated with one non-covalently bound FMN, ~3 menaquinone molecules per complex and six to eight iron–sulphur centres of the $[2Fe–2S]^{2+/1+}$ and $[4Fe–4S]^{2+/1+}$ types [8]. Moreover, it is sensitive to the complex I specific inhibitor rotenone. Upon reconstitution in liposomes, it presented CCCP (carbonyl cyanide m-chlorophenyl hydrazone) stimulated NADH:quinone oxidoreductase activity, which is an indication for proton transport coupled to that activity. Furthermore, among *R. marinus* complex I genes, and co-transcribed with them, an additional gene encoding for a putative sodium/proton antiporter was identified. Its sequence exhibits higher similarity to Nha type sodium/proton antiporters than to Mrp type antiporters or to other complex I subunits [29,30].

Here, we report a study in which the nature of the charge(s) transported by *R. marinus* complex I was investigated. The ACMA (9-amino-6-chloro-2-methoxyacridine) fluorescence quenching method was used to monitor proton transport by this complex. Furthermore, due to the possible relation between complex I and sodium ions, the Na^+ transport by this complex was also investigated. We used ^{23}Na-NMR spectroscopy, which has the advantage of directly monitoring changes in sodium concentration. To our knowledge, this is the first report on the use of the ^{23}Na-NMR to monitor substrate-driven Na^+ transport by membrane vesicles. The obtained findings have a significant impact on general understanding of the coupling mechanisms of complex I.

2. Materials and methods

2.1. Materials

Na_2SO_4 was potassium free ($[K^+]<10$ μM) and K_2SO_4 and choline chloride were sodium free ($[Na^+]<10$ μM). Stock solutions of oxonol V (1,5-Bis(3-phenyl-5-oxoisoxazol-4-yl) pentamethine oxonol), CCCP, rotenone and monensin were prepared in ethanol. 500 mM KCN was prepared in 400 mM HEPES–Tris pH 7.5. $Dy(PPPi)_2^{7-}$ (dysprosium (III) tripolyphosphate) was prepared from sodium triphosphate and dysprosium chloride [31].

2.2. Cell growth and membrane vesicles preparation

Rhodothermus marinus PRQ 62B was grown as described previously [10]. After harvesting, cells in 10 mM HEPES–Tris buffer pH 7.5 with 10, 25 or 50 mM Na_2SO_4, were broken in a French Pressure cell at 6000 psi. For control experiments, Na_2SO_4 was replaced with 10 mM choline chloride or 50 mM K_2SO_4. The membrane vesicles were obtained by ultracentrifugation of the broken cells at 205 000 × *g*, 2 h, 4 °C followed by re-suspension in the same buffer. Integrity of vesicles was checked by the K^+/valinomycin assay using oxonol as a ΔΨ (transmembrane difference of electric potential) sensitive dye (see Section 2.3). Lipids were extracted from membrane preparations [32]. Protein concentration was determined by the Biuret method modified for membrane proteins [33].

2.3. ΔΨ detection

ΔΨ generation was detected following oxonol V absorption (A_{640} minus A_{612}) by an OLIS upgraded Aminco DW2 dual wavelength spectrophotometer, at 27 °C [34]. Membrane vesicles (in HEPES–Tris buffer pH 7.5 and 50 mM Na_2SO_4) were added to 1.5 μM oxonol V in 10 mM HEPES–Tris pH 7.5 buffer with the appropriate salt. To demonstrate the membrane vesicles integrity, K^+ gradients were generated with K^+/valinomycin in an external buffer containing 50 mM K_2SO_4 (internal K^+ concentration was <10 μM). The assay was started adding 2 μM valinomycin, creating a positive-inside ΔΨ due to the influx of K^+. To detect the NADH-driven ΔΨ formation, the assay contained membrane vesicles in 10 mM HEPES–Tris buffer pH 7.5 with 50 mM Na_2SO_4. The reaction was started by adding 4 mM of NADH. When referred, 40 μM rotenone, 10 or 100 μM CCCP, 2.5 mM KCN and 100 μM DMN (2,3-dimethyl-1,4-naphthoquinone) were added prior to the addition of NADH.

2.4. Determination of the internal volume of membrane vesicles

The internal volume of the membrane vesicles was determined by EPR spectroscopy, using TEMPO (2,2,6,6-Tetramethyl-1-piperidinyloxy) [35]. The samples contained approximately 40 mg protein mL^{-1} and 100 μM TEMPO oxidized with $K_3[Fe(CN)_6]$. TEMPO in the external medium was quenched with 100 mM of potassium chromium(III) oxalate trihydrate. Relative decrease of TEMPO concentration upon addition of the quencher was 96% indicating that the internal volume is 4% of the total volume. 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.

2.5. Activity measurements

Oxygen consumption was measured with a Clark-type oxygen electrode YSI Model 5300 at 27 °C and 45 °C. The assay mixture contained membrane vesicles and 10 mM HEPES–Tris pH 7.5 with 10, 25 or 50 mM Na_2SO_4, or 10 mM choline chloride or 50 mM K_2SO_4. The reaction was started by adding 4 mM NADH. When used, ionophores or inhibitors were added prior to the addition of substrates.

NADH oxidation by membrane vesicles was monitored at 330 nm ($\varepsilon = 5930\ M^{-1}\ cm^{-1}$) with a Shimadzu UV-1603 spectrophotometer. The reaction medium contained membrane vesicles in the presence or absence of 2.5 mM KCN and 100 μM DMN. The reaction was started by adding 100 μM NADH.

NADH:$K_3[Fe(CN)_6]$ oxidoreductase activity was monitored at 340 nm ($\varepsilon = 6220\ M^{-1}\ cm^{-1}$). The reaction medium contained membrane vesicles or solubilized membranes (added to start the reaction) in 10 mM HEPES–Tris pH 7.5, 25 or 50 mM Na_2SO_4, 1 mM $K_3[Fe(CN)_6]$ and 200 μM NADH. Solubilized membranes were obtained by stirring an aliquot of membrane vesicles with 9% of DDM (n-dodecyl-β-D-maltoside) for 1 h at 4 °C.

2.6. Fluorescence spectroscopy

The generation of pH difference (ΔpH) was determined by the quenching of the fluorescence of ACMA, recorded on a Varian Cary Eclipse spectrofluorimeter (excitation λ = 410 nm, emission λ = 480 nm). Membrane vesicles were incubated aerobically for 5 min at 27 °C in the assay buffer (10 mM HEPES–Tris pH 7.5, 10 or 25 mM Na_2SO_4 or 10 mM choline chloride) containing 1 μM of ACMA. The reaction was started by adding 50 μM of NADH. When referred, CCCP (10 μM), rotenone (40 μM), monensin (20 μM), KCN (5 mM) and DMN (50 μM) were added prior to the addition of NADH.

2.7. ^{23}Na-NMR spectroscopy

NMR spectra were recorded on a Bruker Avance II 500 MHz spectrometer at 27 °C, operating at 132 MHz for ^{23}Na, equipped with a 10 mm sodium selective probe and a vertical bore magnet. Experiments were performed using a 13.5 μs 90° pulse and 8 k data points were recorded over a spectral width of 40 kHz. 1051 scans were accumulated for each experiment. The vesicles were prepared in 10 mM HEPES–Tris, pH 7.5 with 10, 25 or 50 mM Na_2SO_4 or 10 mM choline chloride without shift reagents and the intra-vesicle sodium signal was not shifted. $Tm(DOTP)^{5-}$ (thulium (III) 1,4,7,10-

tetraazacyclododecane-1,4,7,10-tetrakis(methylenephosphate)) at a concentration of 3, 4.5 or 6 mM (depending on the Na_2SO_4 concentration in the buffer) was added to prepared membrane vesicles and used as a shift reagent for the sodium signal of the suspension medium. Since 1 equivalent of $Tm(DOTP)^{5-}$ contains 5 equivalents of Na^+, the concentration of sodium in the external medium was always higher than inside the vesicles. In average, 400 μL of membrane vesicles containing 20 mg of membrane protein were used in each NMR experiment in a 5 mm diameter tube. A concentric capillary tube with 2 mm diameter, containing 50 μL of the shift reagent $Dy(PPPi)_2^{7-}$ at a concentration of 22 mM, placed inside the NMR tube, was used in all experiments as external reference. The integral of the resonance frequency of Na^+ in the presence of $Dy(PPPi)_2^{7-}$ (this reagent contains 10 equivalents of Na^+) was used to calibrate the concentration of sodium in the suspension medium [31]. The sodium concentration of the suspension medium was measured by integration of the sodium shifted signal. The intra-vesicle concentration of sodium was not monitored due to its small signal to noise ratio. Spectra were recorded under the same conditions as above described, upon addition of 4 mM NADH (in the form of potassium salt) to membrane vesicles which were previously incubated with or without inhibitors, quinones or ionophores.

3. Results

3.1. Membrane vesicles preparation

The membrane preparations obtained by the procedure described above (Section 2.2) were found to be composed of tight vesicles which allowed formation of a ΔΨ using K^+ and valinomycin (data not shown), stable for at least 30 min, and detected by monitoring the change in the absorbance of oxonol V. If external potassium was replaced by sodium, no oxonol V response was observed (data not shown). The positive oxonol V response could only be observed at a certain oxonol V to membrane ratio, typically 0.75 μmol oxonol per 1 μg of lipid, which was optimized by changing the membrane concentration at a fixed oxonol V concentration of 1.5 μM. Closed membrane vesicles were stable at temperatures up to 45 °C.

The internal membrane vesicles volume, determined by EPR spectroscopy, was found to be 1 μL per mg of protein.

Since NADH is unable to cross lipid membranes, one way of determining the percentage of inside-out membrane vesicles is to compare NADH:$K_3[Fe(CN)_6]$ oxidoreductase activity in membrane vesicles before and after their solubilization by detergent. After solubilization of the membrane vesicles with the detergent DDM, the activity was increased at most 10%. Thus, it can be concluded that the membranes obtained by French press cell rupture are composed preferentially of inside-out vesicles.

3.2. NADH and dioxygen consumption by R. marinus respiratory chain

R. marinus membrane vesicles were able to reduce dioxygen to water in 10 mM HEPES–Tris buffer pH 7.5 with 50 mM Na_2SO_4, upon addition of NADH, with an approximate rate of 12 nmol O_2 min^{-1} mg^{-1} at 45 °C. The same rate was observed in the presence of 10 mM Na_2SO_4, 25 mM Na_2SO_4 and also if Na_2SO_4 in the membrane preparation and in the assay buffers was replaced by K_2SO_4 or choline chloride ($[Na^+] < 10$ μM). Maximal NADH oxidase inhibition by rotenone was observed to be ~70% at concentrations ≥10 μM of the inhibitor. KCN could inhibit the same activity at 95% when present at ≥10 mM concentration.

When the NADH consumption was spectrophotometrically monitored, it was observed that KCN also completely inhibited NADH oxidation. This inhibition was totally overcome by the presence of 100 μM menaquinone analogue DMN (data not shown).

3.3. NADH-driven ΔΨ generation by R. marinus membrane vesicles

To analyze the generation of a ΔΨ during NADH oxidation by the vesicles, the optimal oxonol V to membrane ratio (determined in control experiments performed with the K^+/valinomycin assay — Section 3.1) was chosen. A jump in absorbance (A_{640} minus A_{612}) of oxonol V was observed upon addition of NADH to membrane vesicles prepared in buffer containing 50 mM Na_2SO_4 (Fig. 1A-a), demonstrating the buildup of a ΔΨ (positive inside) across the membrane vesicles. If the membrane vesicles were pre-incubated with the respiratory chain inhibitors rotenone or KCN, the jump in A_{640} minus A_{612} observed upon NADH addition was much smaller (Fig. 1A-b and c), indicating that the observed ΔΨ was generated by the functioning of the respiratory chain. The ΔΨ was dissipated by the addition of the protonophore CCCP (Fig. 1A-d). To focus on the NADH–menaquinone segment of this respiratory chain, a preincubation of the membrane vesicles with KCN and the menaquinone analogue DMN was performed. After NADH addition, changes in the oxonol absorbance were observed, showing the formation of a ΔΨ (Fig. 1B-a). This ΔΨ was assigned to complex I, since in the presence of rotenone no absorbance differences were detected (Fig. 1B-b). Average and standard deviation of the results represented in Fig. 1 are given in Supplementary Fig. S1 in Supplementary material.

3.4. Investigation of NADH-driven proton transport

NADH-driven proton transport by the membrane vesicles was monitored by fluorescence spectroscopy using ACMA as a ΔpH indicator (Fig. 2). After NADH addition, a quenching in the ACMA fluorescence intensity was observed, indicating proton transport to the inside of the vesicles (Fig. 2A-a). Prior incubation of the membrane vesicles with the complex I specific inhibitor rotenone led to a lower ACMA quenching (Fig. 2A-b). If the vesicles had been previously incubated with KCN, the protonophore CCCP, or the sodium/proton exchanger monensin, the ACMA fluorescence quenching was negligible upon addition of NADH (Fig. 2A-c to e). These observations reflect the NADH-driven proton transport by the whole *R. marinus* respiratory chain. To focus on the NADH–menaquinone segment as for ΔΨ generation (Section 3.3) the membrane vesicles were pre-incubated with KCN and the menaquinone analogue DMN. The absence of quenching of ACMA fluorescence upon addition of NADH in the presence of KCN was, similarly to NADH oxidation and ΔΨ generation, totally reversed by the addition of DMN (Fig. 2A-f). The influence of the sodium concentration on ACMA fluorescence quenching was also investigated (Fig. 2B). Being maximal at 50 mM Na^+ (inside the vesicles and in the outside buffer) (Fig. 2B-c), the extent of ACMA fluorescence quenching was found to decrease to 43% in the presence of 20 mM Na^+ (Fig. 2B-b) and to 30% when Na_2SO_4 is substituted by choline chloride (Fig. 2B-a).

3.5. Investigation of NADH-driven sodium ion transport

The involvement of Na^+ in respiration was studied by following NADH-driven Na^+ transport by membrane vesicles. ^{23}Na-NMR spectroscopy was chosen to directly monitor changes of sodium ions concentration. By adding the shift reagent $Tm(DOTP)^{5-}$ to the external medium of the membrane vesicles, it is possible to distinguish between internal and external Na^+, since the resonance of the latter is shifted to higher frequency (Fig. 3). After NADH addition, the external Na^+ concentration increased, *i.e.*, sodium ion was transported from the inside to the outside of the membrane vesicles (Fig. 4-filled circles). As described in Materials and methods, four different internal sodium concentrations were used: approximately 0 (Na_2SO_4 is substituted by choline chloride), 20, 50 and 100 mM. Knowing that the membrane vesicles internal volume is 1 μL mg^{-1} of protein, approximately 0, 20, 50 or 100 nmol of Na^+ per mg of protein,

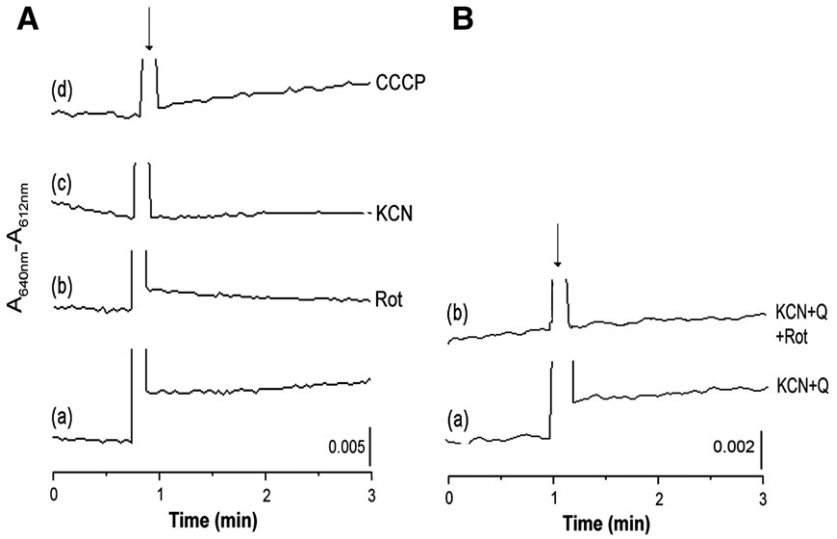

Fig. 1. Generation of a $\Delta\Psi$ by *R. marinus* membrane vesicles. Absorbance difference A_{640} minus A_{612} of 1.5 μM oxonol V in membrane vesicles with 10 mM HEPES–Tris buffer pH 7.5, 50 mM Na_2SO_4 upon addition of 4 mM NADH (indicated by an arrow). Panel A) without preincubating the membrane vesicles (a), after preincubation with 40 μM rotenone (b), in the presence of 2.5 mM KCN (c), or 100 μM CCCP (d). Panel B) preincubating the membrane vesicles with 2.5 mM KCN plus 100 μM DMN (a), or with 2.5 mM KCN plus 100 μM DMN plus 40 μM rotenone (b). See Supplementary material Fig. S1 for statistical information.

respectively, were available to be transported to the outside. No change was observed in the external sodium concentration when choline was present inside the vesicles. In the case of 20 mM Na^+ inside ($[Na_i^+]$), an increase of 20 nmol of Na^+ per mg of protein was observed in the external medium, while an increase of 25 nmol of Na^+ per mg of protein was observed for the conditions of 50 or 100 mM Na^+. Taken together, these observations suggest that the system is operating under $[Na_i^+]$-limiting conditions for Na^+ transport in the presence of choline or 20 mM Na^+, but that it is under non-$[Na_i^+]$-limiting conditions for 50 or 100 mM Na^+, even considering that 10% of the vesicles are inside-in oriented and thus unable to react with NADH.

If membrane vesicles had been previously incubated with rotenone, the increase in external sodium ion concentration was much smaller (Fig. 4—open diamonds). Furthermore, when incubated with KCN, no sodium ion transport from the inside to the outside was observed (Fig. 4—filled diamonds). Thus, sodium ion transport through the membrane vesicles occurred during NADH consumption by the respiratory chain. As expected, a decrease in the ion transport was observed when the sodium/proton exchanger monensin was added (Fig. 4—filled triangles). To distinguish between an active transport by some respiratory complex and a possible secondary sodium transport due to the proton gradient formed by respiration, the uncoupler and protonophore CCCP was added to the vesicles prior to the addition of NADH. Dissipation of the proton gradient with CCCP increased sodium transport (Fig. 4—open triangles). The CCCP experiment shows that Na^+ transport to the outside of the membrane vesicles is not a secondary event that arises from a

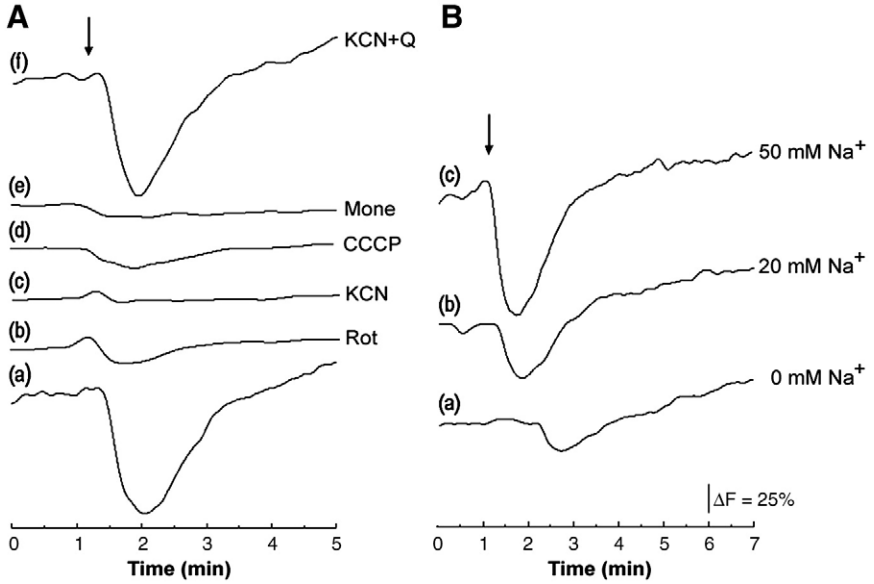

Fig. 2. Generation of a pH difference by the respiratory chain of *R. marinus* monitored by ACMA fluorescence. The ACMA fluorescence was detected using an excitation wavelength of 410 nm and an emission wavelength of 480 nm. Panel A) The assay contained membrane vesicles in 10 mM HEPES–Tris buffer pH 7.5 with 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 40 μM rotenone (b), 5 mM KCN (c), 10 μM CCCP (d), 20 μM monensin (e) or 5 mM KCN plus 50 μM DMN (f). Panel B) The assay contained membrane vesicles in 10 mM HEPES–Tris buffer pH 7.5 with 10 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).

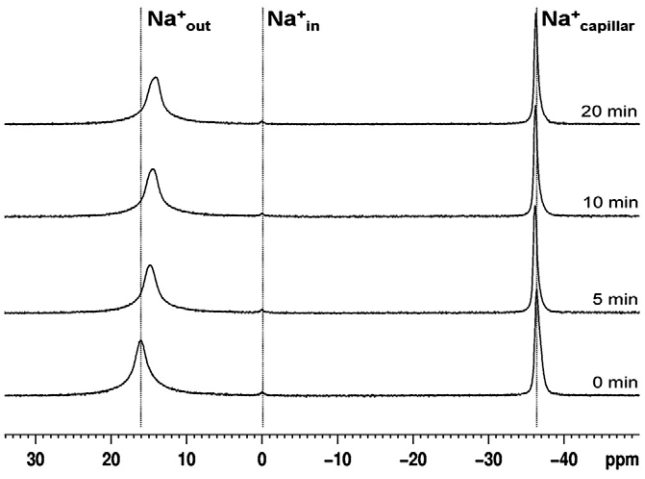

Fig. 3. ^{23}Na-NMR spectra of membrane vesicles in 10 mM HEPES–Tris buffer pH 7.5 with 10 mM Na_2SO_4 and containing 3 mM $Tm(DOTP)^{5-}$ in the suspension medium, obtained before addition of NADH and 5, 10 and 20 min after the addition. The signal at −36.17 ppm is due to the external reference present in the concentric capillary tube inside the NMR tube, which contained 22 mM $Dy(PPPi)_2^{7-}$. The weak signal at 0 ppm originates from the intra-vesicle Na^+ and the resonance at ~15 ppm is the signal from the Na^+ present in the suspension medium. This signal changes its resonance frequency because Na^+ magnetic resonance system for the equilibrium exchange of Na^+ ions between free and shift reagent bound states is in the fast-exchange-limit, and the concentration ratio of Na^+ and the shift reagent is increasing in the outside medium, together with other factors.

proton gradient formation, and thus it is actively performed by a component of the respiratory chain. Hypothesizing that this component is located at the NADH–menaquinone segment of the respiratory chain, and using the same rationale applied to NADH oxidation, $\Delta\Psi$ generation (Section 3.3) and proton transport (Section 3.4), the addition of quinones to vesicles pre-incubated with KCN should restore sodium ion transport activity. Indeed, when adding NADH to KCN-treated membrane vesicles in the presence of the menaquinone analogues DMN, NQ (1,4-Naphthoquinone) and menadione (2-methyl-1,4-naphthoquinone), sodium ion transport was found to be similar to the one observed with non-inhibited vesicles in the absence of added quinones (Fig. 4—open circles).

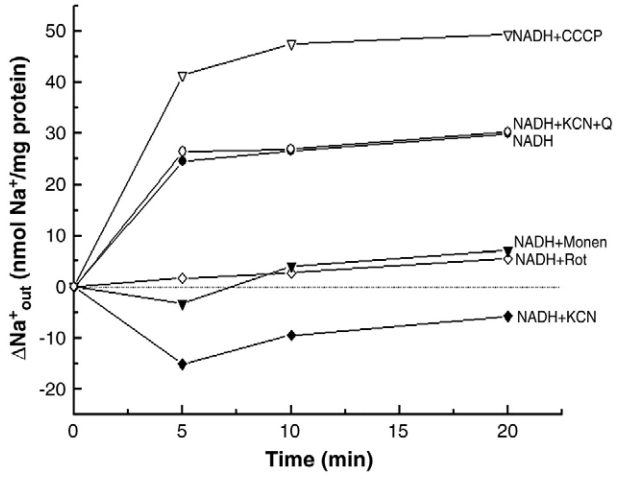

Fig. 4. Sodium ion transport by *R. marinus* NADH-respiring membrane vesicles. Effect of 4 mM K_2-NADH on external Na^+ concentration at time zero to membrane vesicles containing 50 or 100 mM Na^+ without pre-incubations (filled circles) or after preincubation with 40 μM rotenone (open diamonds), 10 mM KCN (filled diamonds), 10 μM CCCP (open triangles), 20 μM monensin (filled triangles) or 10 mM KCN plus 200 μM of each menaquinone analogue DMN, NQ and menadione (open circles). The represented data are the average of at least three independent assays. See Supplementary material Fig. S2 for standard deviation.

Supplementary Fig. S2 shows average and standard deviation of the results from Fig. 4.

3.6. Sodium ion transport and NADH consumption ratio

Measuring the amount of dioxygen consumed in the first 5 min after NADH addition to membrane vesicles under the same conditions used in the NMR experiments, allowed the calculation of the amount of NADH oxidized during this period. By comparing this result with the sodium ion transported by the membrane vesicles, under non-$[Na_i^+]$-limiting conditions, during the first 5 min after NADH addition, we estimated that 0.3 to 1 Na^+ was transported upon consumption of 1 NADH molecule. This nearly 1 to 1 stoichiometry supports the hypothesis that the observed sodium ion transport is a primary event caused by NADH consumption. It should be noted that if the Na^+ transport observed after the first 5 min had occurred in a shorter period of time, and/or if the Na^+ flux observed in the reverse direction when the respiratory chain is inhibited by KCN (Fig. 4) can be taken as a baseline, the indicated Na^+/NADH values appear underestimated.

4. Discussion

The membrane vesicles prepared as described, were active with respect to NADH oxidation and dioxygen reduction, and could create and maintain an NADH-driven $\Delta\Psi$ positive inside. This $\Delta\Psi$ was fully sensitive to the protonophore CCCP, indicating a ΔpH contribution to the $\Delta\Psi$, as confirmed by monitoring the ΔpH using the indicator ACMA. The ACMA fluorescence quenched after the addition of NADH, and did not change when the membrane vesicles were incubated with rotenone, CCCP, monensin or KCN. A proton translocation site in the respiratory chain of *R. marinus* could be attributed to the NADH: menaquinone electron transfer segment, since proton transport was observed upon short cutting KCN inhibited membrane vesicles with the menaquinone analogue DMN. Due to the fact that only the inside-out membrane vesicles contribute to the observed results, it can be concluded that H^+ transport into the vesicles corresponds to the proton transport from the cytoplasm to the periplasm, in agreement with the $\Delta\Psi$ (positive inside) detected with oxonol V. These results show that the coupling ion between NADH:quinone oxidoreductase activity and the establishment of the $\Delta\Psi$ is H^+.

^{23}Na-NMR spectroscopy showed that NADH consumption was accompanied by a net sodium ion transport from the inside to the outside of the membrane vesicles. The long time scale of this process (Fig. 4) is due to the temperature of the assays, 27 °C, which is much lower than the physiological *R. marinus* growth temperature (65 °C). It was shown that the sodium ion transport is actively performed by a respiratory chain component since it was stimulated by the protonophore CCCP, excluding a transport by a secondary event dependent on a proton gradient. Na^+ transport was also prevented by KCN, the inhibitor of terminal dioxygen reductases which hampers the electron transfer from NADH to dioxygen by 95%. The site of sodium ion transport could undoubtedly be identified at the NADH to menaquinone electron transfer segment, since the sodium ion transport activity was recovered upon providing a shortcut for electrons by the presence of menaquinone analogues to KCN inhibited membranes. Moreover, because the specific complex I inhibitor rotenone also inhibited sodium ion transport, we assign the Na^+ translocation activity of the respiratory chain of *R. marinus* to complex I. So far, neither an experimental evidence for the presence of a Na^+-NQR type NADH dehydrogenase (e.g. [36]) in this respiratory chain exists, nor genes coding for such a complex were found in *R. marinus* genome [16]. Furthermore, Na^+-translocating NADH dehydrogenases (Na^+-NQRs) are not inhibited by rotenone, excluding a contribution of this enzyme to the observed activity. It was also observed that in the conditions in which Na^+ transport is

limited ($\leq$20 mM-Na_i^+), the NADH consumption by the respiratory chain is the same as the one observed in non-[Na_i^+]-limiting conditions, implying that Na^+ does not influence the NADH oxidase activity of *R. marinus* complex I.

The observed Na^+ efflux corresponds to a transport from the periplasm to the cytoplasm in the living organism, whereas H^+ influx corresponds to a transport from the cytoplasm to the periplasm. Consequently, the transport of H^+ is higher than that of Na^+, since it accompanies the positive establishment of the $\Delta\Psi$. How these transport activities are processed is still not known. Na^+ is not required for NADH oxidation nor for the establishment of ΔpH. Considering these observations, we propose a possible mechanism of *R. marinus* complex I schematized in Fig. 5. NADH oxidation drives electrogenic proton translocation (represented by 1) to the periplasm and sodium import (represented by 2). It was observed that sodium is not necessary for proton transport but its presence increases the NADH-driven proton transport, possibly indicating the presence of another site for proton transport. Thus, we suggest that there is an export of H^+, associated with sodium import (represented by 2 in Fig. 5), possibly performed by the action of an antiporter. The involvement of a Na^+/H^+ antiporter is the most attractive scenario since complexes I, including the one from *R. marinus*, possess the so-called Mrp like subunits, related to these types of antiporters. In the case of complex I from *R. marinus*, there is a second candidate, a Nha like sodium/proton antiporter. Namely, its gene was found among *R. marinus* complex I genes, and is co-transcribed with the canonical ones [29]. Whether this Na^+/H^+ exchange contributes to the increase of the transmembrane difference of electrochemical potential created by the respiratory chain depends on the sodium/proton stoichiometry of this module: a simple exchange of 1 H^+ per 1 Na^+ would not contribute to an increase of the transmembrane difference of electrochemical potential, but could benefit ATP synthesis by ATP synthase if it is H^+-dependent.

The mechanism that we propose here can be further applied to other prokaryote complexes I. All known complexes I have the so-called Mrp like subunits. Moreover, in the case of *E. coli* an increase in the proton transport was observed upon a Na^+ gradient [26]. Thus, we suggest that the complex I may have two coupling sites, both coupled to menaquinone reduction. One of the sites is independent of the presence of sodium ions (site 1), while the other (site 2) needs sodium ions to promote proton translocation.

In summary, our study indicates that complex I from *R. marinus* is electrogenic involving the transport of protons and sodium ions. It has been already demonstrated that complex I from different organisms contribute, by proton translocation to the periplasm, to the establishment of a transmembrane difference of electrochemical potential. In addition, *R. marinus* complex I translocates Na^+ in the opposite direction, i.e., to the cytoplasm. This finding opens new perspectives for the investigation of the electron transfer/charge transport mechanism in complexes I in general, which may be more versatile than one could anticipate.

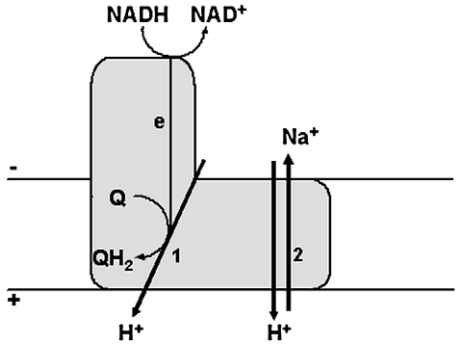

Fig. 5. Schematic representation of a proposed mechanism for *R. marinus* complex I, considering the H^+ and Na^+ transport observed in this study. 1— Represents the coupling event between NADH oxidation and the translocation of electrogenic charge, H^+. 2— Represents the coupling between NADH oxidation and the gating mechanism that allows the observed Na^+ transport.

Acknowledgements

We are indebted to Prof. Carlos Geraldes for numerous suggestions and advices, and for critically reading the manuscript. We greatly appreciated the discussions with Alexander Konstantinov Miguel Teixeira, the critical reading by Smilja Todorovic, as well as scientific and technical advice from Po-Chi Lin from the University of Zürich on the experiments with oxonol dye. We would like to give our thanks to João Carita for cell growth and to Dr. Rita Ventura and Filipa Sousa for performing the synthesis of DMN in Prof. Chris Maycock laboratory in 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). A. S. Fernandes was recipient of a grant from Fundação para a Ciência e a Tecnologia (SFRH/BPD/34493/2006). A. P. Batista 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/BIA-PRO/58374/2004 and POCI/QUI-BIQ/100302/2008 to M.P., FCT-REEQ/336/BIO/2005 to ITQB) and by the Parkinson Schweiz (to J. S.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2010.01.020.

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