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Decoupling of the Catalytic and Transport Activities of Complex I from *Rhodothermus marinus* by Sodium/Proton Antiporter Inhibitor

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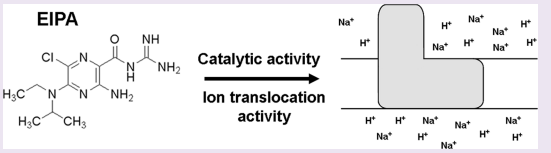
Supporting Information

ABSTRACT: The energy transduction by complex I from *Rhodothermus marinus* was addressed by studying the influence of 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA) on the activities of this enzyme. EIPA is an inhibitor of both Na^+/H^+ antiporter and complex I NADH:quinone oxidoreductase activity. We performed studies of NADH:quinone oxidoreductase and H^+ and Na^+ translocation activities of complex I from *R. marinus* at different concentrations of EIPA, using inside-out membrane vesicles. We observed that the oxidoreductase activity and both H^+ and Na^+ transports are inhibited by EIPA. Most interestingly, the catalytic and the two transport activities showed different inhibition profiles. The transports are inhibited at concentrations of EIPA at which the catalytic activity is not affected. In this way the catalytic and transport activities were decoupled. Moreover, the inhibition of the catalytic activity was not influenced by the presence of Na^+, whereas the transport of H^+ showed different inhibition behaviors in the presence and absence of Na^+. Taken together our observations indicate that complex I from *R. marinus* performs energy transduction by two different processes: proton pumping and Na^+/H^+ antiporting. The decoupling of the catalytic and transport activities suggests the involvement of an indirect coupling mechanism, possibly through conformational changes.



Respiratory complex I couples the transfer of electrons from NADH to quinone with the translocation of ions across the membrane. In this way it contributes to the buildup of a transmembrane difference of electrochemical potential. Dissipation of the latter through the ATP synthase is furthermore used for the synthesis of ATP. Bacterial complex I is generally composed of the so-called minimal functional unit that consists of 14 subunits named Nqo1 to 14 (or NuoA to N). Seven subunits are located in the peripheral arm and seven in the membrane part. The peripheral arm, whose crystallographic structure has already been determined, contains a series of iron−sulfur centers (binuclear and tetranuclear ones) and a noncovalently bound FMN.[1] The α-helical structures of the membrane part of complex I from *Escherichia coli* and of the integral complex I from *Thermus thermophilus* and from the mitochondria of *Yarrowia lipolitica* have recently been determined.[2,3] The structural data 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 over the membrane domain, which in turn may result in proton translocation.[2] Comparisons of amino acid sequences indicated that the hydrophobic subunits Nqo 11, 12, 13, and 14 are related to Na^+/H^+ antiporters of the Mrp family, suggesting that these subunits may participate in charge translocation.[4,5] It is well established that complex I performs proton translocation;[6,7] however, the observation that complexes I from some species translocate Na^+ instead of H^+[8,9] is still under debate.[10−12] We have recently shown that, in fact, complex I from *Rhodothermus marinus* is capable of translocating both H^+ and Na^+, but in opposite directions. The coupling ion of the system is the H^+. The presence of Na^+ is not required for either the catalytic reaction or establishment of the ΔpH, but it increased proton transport.[13] The proposed model for the functional mechanism of complex I suggests the presence of two different energy coupling sites, both coupled to menaquinone reduction. One of the sites is Na^+-independent, while the other requires Na^+ to promote H^+ translocation, possibly working as Na^+/H^+ antiporter.[13] Our model is here further investigated by studying the effect of 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA) on the catalytic activity and ion translocation processes of complex I from *R. marinus*.

EIPA, as other amiloride derivatives, has been known to be an inhibitor of Na^+/H^+ antiporters.[14−16] Since some subunits of complex I are related to Mrp type Na^+/H^+ antiporters,[4,5] amiloride derivatives have also been used in studies of this respiratory complex. Nakamaru-Ogiso and co-workers have shown that the electron transfer of mitochondrial and bacterial complex I is inhibited by amiloride derivatives[17] and that the presence of these compounds prevents labeling of the ND5 (homologue of NuoL/Nqo12) subunit in bovine complex I with a Fenpyroximate analogue, a potent inhibitor of complex I.[18] The inhibitory effect on the catalytic activity was also observed for the *E. coli* enzyme.[12] The expressed C-terminally truncated NuoL subunit of the complex I from *E. coli* was shown to mediate sodium uptake when reconstituted into liposomes, and this process was inhibited by the addition of EIPA.[19] Inhibition of complex I proton pumping by EIPA was also observed in isolated rat liver mitochondria.[20]

The results presented here further support our previous model, in which we proposed the existence of two energy

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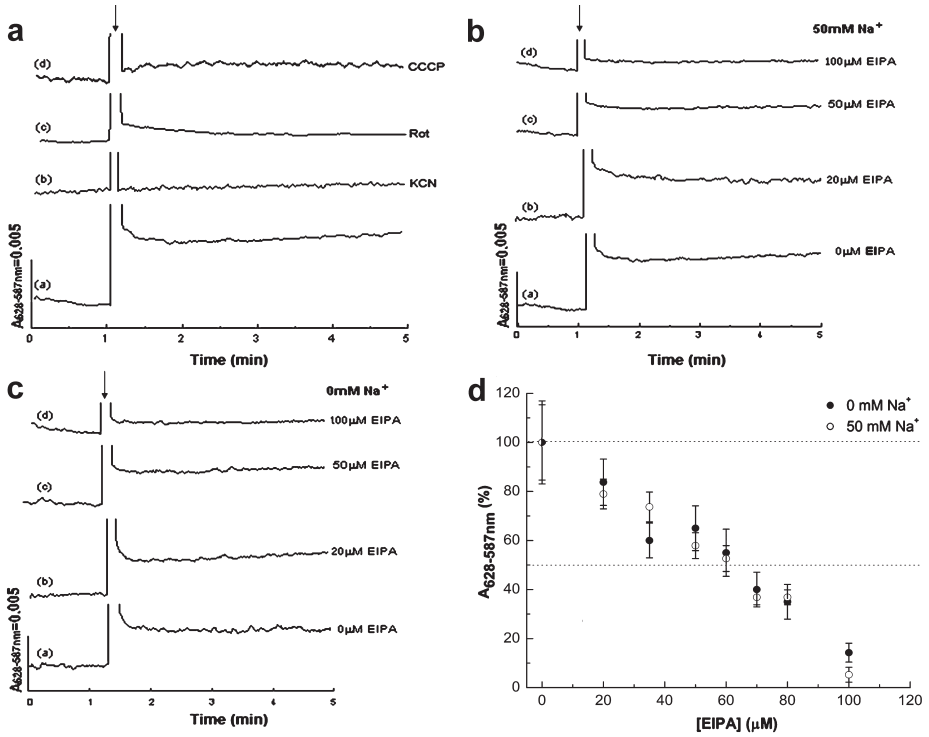

Figure 1. Generation of a membrane potential ($\Delta\Psi$) by the functioning of the *R. marinus* respiratory chain, monitored by the absorbance difference ($A_{628\,nm} - A_{587\,nm}$) of oxonol VI. Membrane vesicles were prepared in 2.5 mM HEPES-Tris pH 7.5, 5 mM K_2SO_4, 25 mM Na_2SO_4 or 2.5 mM HEPES-Tris pH 7.5, 5 mM K_2SO_4, 50 mM choline chloride ($[Na^+] < 10\ \mu M$). The reaction was started by addition of 4 mM NADH (indicated by an arrow). Panel a: Absorbance difference of oxonol VI, in the presence of Na^+, upon the addition of NADH to vesicles (a) and preincubated vesicles with 5 mM KCN (b), 10 μM rotenone (Rot) (c), or 10 μM CCCP (d). Panels b−d: Influence of EIPA on the NADH-driven $\Delta\Psi$ in the presence (panel b, ○ panel d) or the absence (panel c, ● panel d) of Na^+. The represented data are the average ± SD of at least three independent assays (panel d). NADH-driven $\Delta\Psi$ formation in the absence of EIPA was used as reference (100%).

coupling sites and suggest that *R. marinus* complex I transduces energy by two processes; proton pumping and Na^+/H^+ antiporting. Moreover, our findings suggest the involvement of an indirect mechanism for energy transduction, possibly through conformational changes.

RESULTS AND DISCUSSION

We have previously shown that complex I from *R. marinus* performs three activities: (i) the catalytic activity NADH:quinone (Q) oxidoreductase, (ii) H^+ translocation in the same direction of the established $\Delta\Psi$, and (iii) Na^+ transport to the opposite direction of (ii).[13] In this work we studied the influence of a Na^+/H^+ antiporter inhibitor, EIPA, on these activities. We use membrane vesicles from *R. marinus*, with an internal volume of 1 μL mg^{-1} of protein. These vesicles allowed the formation of a membrane potential using K^+ and valinomycin, which was stable for at least 20 min. This observation indicated that the *R. marinus* vesicles were tight. The orientation of the vesicles was determined comparing the NADH:$K_3[Fe(CN)_6]$ oxidoreductase activity of vesicles before (0.54 ± 0.12 μmol $K_3[Fe(CN)_6]$ min^{-1} mg^{-1}) and after (0.55 ± 0.14 μmol $K_3[Fe(CN)_6]$ min^{-1} mg^{-1}) their solubilization with *n*-dodecyl-β-D-maltoside (DDM). The activity was approximately the same in both situations, showing that the membrane vesicles obtained had an inside-out orientation.

Effect of EIPA on NADH-Driven Membrane Potential ($\Delta\Psi$) Generation. The addition of the substrate NADH to the vesicles originated in a jump in the absorbance difference, $A_{628\ nm} - A_{587\ nm}$, of 1,5-bis(5-oxo-3-propylisoxazol-4-yl) pentamethine oxonol (oxonol VI), indicating the establishment of a $\Delta\Psi$ (positive inside) (Figure 1 and Supporting Figure 1). When the vesicles were preincubated with the terminal dioxygen reductases inhibitor, KCN, the jump in the absorbance difference observed upon substrate addition was negligible. This finding indicates that the observed $\Delta\Psi$ was created by the functioning of the respiratory chain (trace b, Figure 1a). A similar behavior was observed in the presence of rotenone (inhibitor of complex I) or in the presence of the protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) (traces c and d, Figure 1a and Supporting Figure 1). These observations are in agreement with the results obtained before.[13] Approximately the same trend of absorbance difference was detected in the presence of 25 mM Na_2SO_4 or 50 mM choline chloride ($[Na^+] < 10\ \mu M$) (traces a, Figure 1, panels b−d) indicating that sodium ions do not influence the establishment of $\Delta\Psi$.

The effect of EIPA on the $\Delta\Psi$ was tested in the presence and absence of Na^+ (Figure 1, panels b−d). Different concentrations of EIPA were added to the reaction mixture prior to the addition of NADH. For EIPA concentrations up to 20 μM, no clear effect was observed, independently of the presence of Na^+ (traces a and b, Figures 1, panels b−d). Approximately 50% inhibition of $\Delta\Psi$ formation was observed at 60 μM EIPA for both conditions (Figure 1d).

Effect of EIPA on the NADH:Dioxygen Oxidoreductase Activity. *R. marinus* vesicles were capable of consuming dioxygen upon addition of NADH, with an approximate rate of 23 nmol O_2 mg^{-1} min^{-1} at 27 °C. As previously observed,[13] this

activity was not dependent or stimulated by the presence of Na^+. In the presence of valinomycin and CCCP the consumption of dioxygen increased (~38 nmol O_2 mg^{-1} min^{-1}), corroborating the above results that oxidation of NADH contributed to the generation of a membrane potential in tight vesicles. The NADH:dioxygen oxidoreductase activity was affected by EIPA, revealing a 10−20% inhibition in the presence of 50 μM EIPA (Figure 2). The inhibition increased with the increase of the concentration of EIPA. The effect of EIPA on the NADH:dioxygen oxidoreductase activity was not affected by the presence of Na^+ and approximately 600 μM EIPA for half maximal inhibition (IC_{50}) was estimated (Figure 2).

Effect of EIPA on the NADH:DMN Oxidoreductase Activity. NADH:Q oxidoreductase activity of purified complex I from *R. marinus* was determined using the menaquinone analogue, 2,3-dimethyl-1,4-naphthoquinone (DMN), at different concentrations of Na^+ (0−200 mM). The specific activity was 0.8 μmol of NADH min^{-1} mg^{-1} in the absence of Na^+, a value in the range of those determined for other isolated bacterial enzymes in the presence of detergent, without the addition of lipids.[12] NADH:DMN oxidoreductase activity was not stimulated by the presence of Na^+; on the contrary, a slight decrease in activity was observed upon increasing Na^+ concentration (Figure 3a). It is worth mentioning that such a decrease was not observed in the case of vesicles.

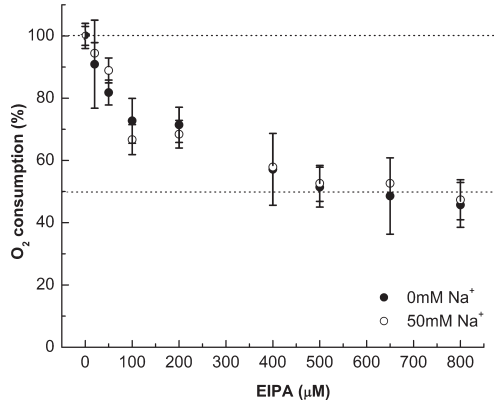

Figure 2. Effect of EIPA on the NADH:O_2 oxidoreductase activity of *R. marinus* membrane vesicles. The activity was measured in the presence (○) or absence (●) of Na^+. The reaction was started by addition of 4 mM NADH. The represented data are the average ± SD of at least three independent assays. NADH:O_2 oxidoreduction in the presence of Na^+ and in the absence of EIPA was used as reference (100%).

It is well established in the literature that EIPA inhibits catalytic activity of complex I, most probably by blocking the quinone binding site. It has been observed that 100 μM EIPA can prevent labeling of bovine complex I with a Fenpyroximate analogue, a specific inhibitor of complex I which is assumed to bind at or close to a quinone binding site.[18] In the case of complex I from *R. marinus* no inhibition of NADH:Q oxidoreductase activity was observed up to at least 20 μM EIPA and an IC_{50} for EIPA of approximately 230 μM was estimated, irrespective of the presence of Na^+ (Figure 3b and Table 1). This IC_{50} value is in the range of those determined for reconstituted complex I from *E. coli* (IC_{50} = 100 μM)[12] for complex I in bacterial membrane vesicles (*E. coli*, *Paracoccus denitrificans*) and submitochondrial particles.[17]

Effect of EIPA on NADH-Driven External-Vesicle pH (pH_{out}) Change. Considering the EIPA inhibitory profile of the catalytic reaction, concentrations between 0 to 20 μM (a range in which neither the catalytic reaction nor the integrity of the membrane vesicles are affected) were chosen for the ion translocations studies. Changes on the pH_{out}, due to the activity of complex I, were measured using the pyranine fluorescence probe. Pyranine is a hydrophilic and membrane-impermeable pH

Table 1. Inhibition of Activities of Complex I from *R. marinus*: NADH:quinone Oxidoreductase Activity (NADH:Q OR), Proton Transport (ΔpH_{out}), and Sodium Transport (ΔpNa^+_{out}), by EIPA

	% of inhibition				
	0 mM Na^+		50 mM Na^+		
[EIPA] (μM)	NADH:Q OR	ΔpH_{out}	NADH:Q OR	ΔpH_{out}	ΔpNa^+_{out}
10	0	0	0	32	50
20	0	46	0	70−80	70−80
230	50	*a*	50	*a*	*a*

[a] At 230 μM EIPA, NADH:quinone oxidoredutase activity is already 50% inhibited and thus ΔpH_{out} and ΔpNa^+_{out} are also directly affected by that inhibition.

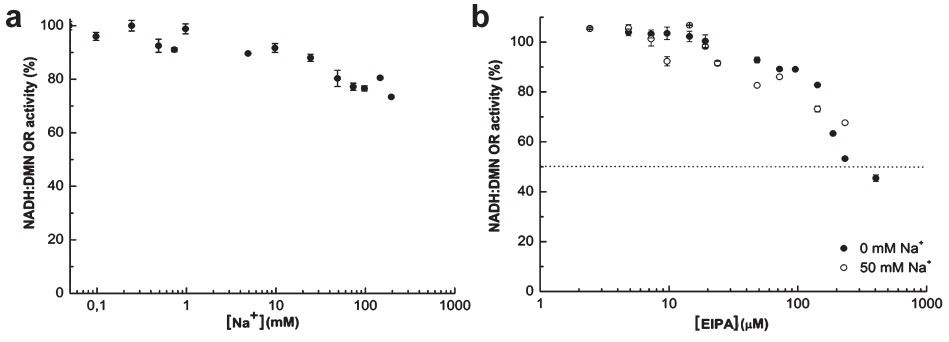

Figure 3. NADH:2,3-dimethyl-1,4-naphthoquinone (DMN) oxidoreductase activity profiles of purified complex I from *R. marinus*. The assay mixture contained 24 μg of purified complex I in 200 mM KPi pH 7.5 with 0.1% *n*-dodecyl-β-D-maltoside, 50 μM NADH, and 50 μM DMN. The reaction was started by addition of complex I and was monitored at 330 nm. The represented data are the average ± SD of at least three independent assays. Panel a: Influence of the Na^+ concentration on the catalytic activity. NADH:DMN oxidoreduction in the absence of Na^+ ([Na^+] < 10 μM) was used as reference (100%). Panel b: Effect of EIPA on the catalytic activity in the presence (○) and absence (●) of Na^+. NADH:DMN oxidoreduction in the absence of EIPA was used as reference (100%).

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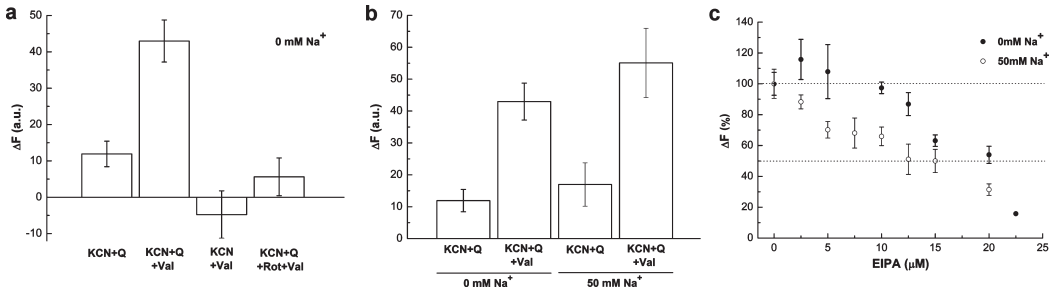

Figure 4. Effect of membrane potential ($\Delta\Psi$), inhibitors of respiratory chain complexes, and EIPA on NADH-driven pH_{out} variation by *R. marinus* complex I. The pyranine fluorescence was measured using $\lambda_{excitation}$ = 458 nm and $\lambda_{emission}$ = 508 nm. The reaction was started by addition of 4 mM NADH. The represented data are the average ± SD of at least three independent assays. Panel a: Influence of $\Delta\Psi$ and of inhibitors on NADH-driven pH_{out} variation. The assay mixture contained vesicles, prepared in the absence of Na^+, preincubated with KCN (5 mM) and 2,3-dimethyl-1,4-naphthoquinone (DMN) (200 μM) (KCN + Q); KCN (5 mM), DMN (200 μM) and valinomycin (2 μM) (KCN + Q + Val); KCN (5 mM) and valinomycin (2 μM) (KCN + Val); and KCN (5 mM), DMN (200 μM), rotenone (10 μM) and valinomycin (2 μM) (KCN + Q + Rot + Val). Panel b: Influence of $\Delta\Psi$ on NADH-driven pH_{out} variation in the presence and absence of Na^+. The assay mixture contained vesicles, prepared in the absence or presence of Na^+ (50 mM Na^+), preincubated with KCN (5 mM) and DMN (200 μM) with or without valinomycin (Val) (2 μM). Panel c: Influence of EIPA on NADH-driven pH_{out} variation. The assay mixture contained vesicles, prepared in the absence (●) or presence of Na^+ (○), preincubated with KCN (5 mM), DMN (200 μM), and valinomycin (2 μM). NADH-driven pH_{out} variation in the presence of 0 μM EIPA was used as reference (100%). See Supporting Figure 2 for examples of kinetic traces.

indicator that responds rapidly to changes in pH in the physiological range.[21] The vesicles were preincubated with KCN and DMN, the inhibitor of the oxygen reductases and a menaquinone analogue, respectively, which allowed us to focus on the NADH-Q segment of the respiratory chain from *R. marinus*. An increase in the pyranine fluorescence intensity is observed, upon addition of NADH, indicating an increase in the pH_{out} due to the functioning of complex I (Figure 4, panels a and b). In the presence of valinomycin more than a 2-fold increase of the pH_{out} change was observed revealing that NADH:DMN oxidoreductase activity was previously limited by $\Delta\Psi$ (Figure 4, panels a and b). When the vesicles were preincubated with KCN (without DMN) or rotenone, the alkalinization upon NADH addition was considerably impaired (Figure 4a). The observed increase by the presence of valinomycin was the same in the presence and absence of Na^+. However, the change in pH_{out} is more pronounced when Na^+ is present (Figure 4b).

The influence of EIPA on the NADH-driven pH_{out} change was furthermore investigated using different concentrations of EIPA, in the presence or absence of Na^+ (Figure 4c and Supporting Figure 2). These assays were performed in the presence of valinomycin in order to overcome the limitation of activity by $\Delta\Psi$. In the absence of Na^+, ~46% of inhibition was reached at 20 μM EIPA and no inhibition was detected at 10 μM (● in Figure 4c; Table 1 and Supporting Figure 2a). In the presence of Na^+, at 10 μM EIPA a decrease in the change of the pyranine fluorescence intensity of ~32% was observed, and at 20 μM EIPA the decrease in that change was ~70% (○ in Figure 4c; Table 1 and Supporting Figure 2b). An IC_{50} value of 21 ± 1 μM EIPA was estimated for H^+ transport in the absence of Na^+ and 13.5 ± 1.5 μM in the presence of this ion.

These results show different inhibitory behaviors for complex I proton transport by EIPA in the presence and absence of Na^+. In its absence, 20 μM EIPA was responsible for a decrease of ~46% in ΔpH_{out}. Since at this concentration of EIPA, the NADH:Q oxidoreduction was not affected (Figure 3b and Table 1), the observed decrease in ΔpH_{out} was not due to a decrease in proton consumption for quinone reduction but originated from a decrease in proton uptake for translocation. Therefore we conclude that in those conditions EIPA inhibited H^+ translocation and promoted decoupling of the oxidoreductase and H^+ translocating activities. The decoupling of H^+ transport from the catalytic reaction was further supported by the assays performed in the presence of Na^+ in which 5 μM EIPA was sufficient to inhibit ~30% of the H^+ transport.

Effect of EIPA on ΔpH Generation. The decoupling of H^+ transport from the catalytic activity was further corroborated by the studies performed with the integral respiratory chain from *R. marinus*, in which an inhibition of the proton transport was observed at EIPA concentrations at which the catalytic activity was not affected. It was previously shown that the functioning of complex I from *R. marinus* allowed the establishment of a ΔpH, which was completely sensitive to the presence of the protonophore CCCP and to the Na^+/H^+ exchanger monensin. Although the presence of Na^+ was not a requisite for the establishment of ΔpH, it increased the ΔpH.[13] In the current study, the effect of EIPA on the NADH-driven ΔpH was monitored using 9-amino-6-chloro-2-methoxyacridine (ACMA) in the presence and absence of Na^+ (Figure 5). Since we have observed an NADH-driven ΔpH increase of 20−30% in the absence of $\Delta\Psi$, these assays were performed in the presence of valinomycin and K^+, in order to guarantee that the establishment of ΔpH was not limited by $\Delta\Psi$. In the presence of Na^+, ~30% and ~45% decrease of the ΔpH amplitude was observed at 10 and 20 μM EIPA, respectively. A corresponding IC_{50} value of 20.5 ± 0.5 μM was determined (Figure 5a and Supporting Figure 3). When Na_2SO_4 was replaced by choline chloride, only ~20% inhibition of ΔpH amplitude was observed at 20 μM EIPA. In the absence of Na^+, 10 μM EIPA had no effect on the NADH-driven ΔpH (Figure 5b and Supporting Figure 3).

Our observations are in agreement with the results obtained by Dlasková and co-workers, who observed inhibition of H^+ transport by EIPA using glutamate and malate as substrates and rat liver mitochondria without affecting respiration.[20] An apparent IC_{50} value for EIPA inhibition of that transport, when added before the substrates, was estimated to be 27 μM. The decoupling effect was assigned to decoupling of complex I. The authors also observed no inhibition of mitochondrial respiration of the mentioned substrates until EIPA concentration reached 250−500 μM.[20]

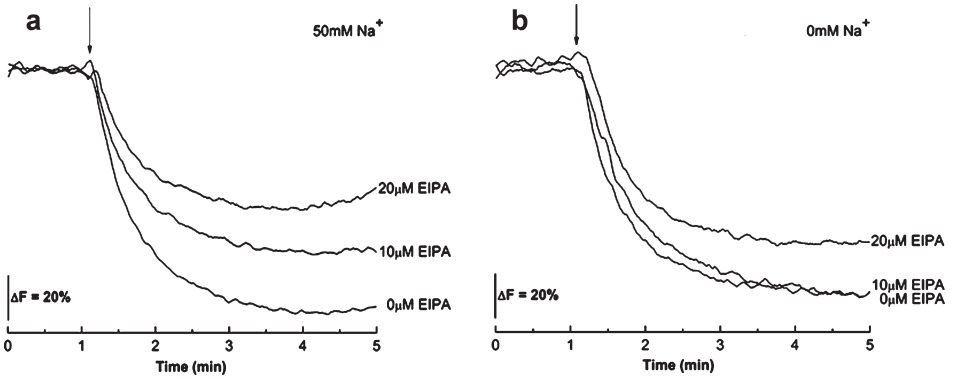

Figure 5. Effect of EIPA on NADH-driven ΔpH generation by the respiratory chain of *R. marinus*. The 9-amino-6-chloro-2-methoxyacridine (ACMA) fluorescence was measured using $\lambda_{excitation}$ = 410 nm and $\lambda_{emission}$ = 480 nm. The reaction was started by the addition of 100 μM NADH (indicated by an arrow). Influence of EIPA (0, 10, and 20 μM) on NADH-driven ΔpH generation by vesicles prepared in the presence (panel a) or absence (panel b) of Na^+.

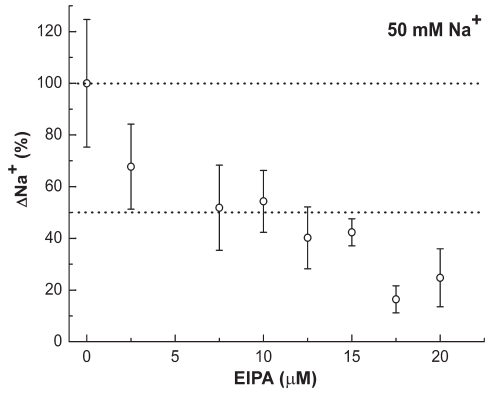

Figure 6. Effect of EIPA (0 to 20 μM) on NADH-driven $\Delta[Na^+]_{out}$ by complex I from *R. marinus* monitored by ^{23}Na NMR. The reaction was started by the addition of 4 mM NADH. The assay mixture contained vesicles preincubated with KCN (5 mM), DMN (200 μM), and valinomycin (2 μM). The represented data are the average ± SD of at least three independent assays. NADH-driven $\Delta[Na^+]_{out}$ in the presence of 0 μM EIPA was used as reference (100%).

Effect of EIPA on Na^+ Transport. It was previously shown using ^{23}Na NMR spectroscopy that NADH consumption was accompanied by Na^+ transport from the inside to the outside of *R. marinus* vesicles, which was completely sensitive to KCN, monensin, and rotenone. This transport was actively performed since it was stimulated by the protonophore CCCP, excluding a transport by a secondary event dependent on a proton gradient or simply by a charge compensation event.[13] The effect of EIPA on Na^+ transport by complex I from *R. marinus* was also investigated using vesicles preincubated with KCN and DMN. After NADH addition, an increase in the external medium of 17 ± 1.3 nmol of Na^+ mg^{-1} of protein was observed (Supporting Figure 4), and an increase of ~42% was observed in the presence of valinomycin (Supporting Figure 4), showing that Na^+ transport was limited by ΔΨ. In the presence of valinomycin, EIPA inhibits the external increase of Na^+ concentration with an estimate IC_{50} of 9 ± 1 μM (Figure 6).

Taken together, the data from the H^+ and Na^+ transport studies reveal that in the presence of Na^+, 10 μM EIPA was sufficient to inhibit both H^+ and Na^+ transports, but to different extensions (Table 1). In these conditions, decreases of ~32% and ~50% were observed for NADH-driven pH_{out} and NADH-driven $[Na^+]_{out}$ changes, respectively. At 20 μM EIPA, both the pH_{out} and $[Na^+]_{out}$ changes decreased to ~70−80%. Since NADH:Q oxidoreductase activity was not affected at these concentrations of EIPA, we conclude that H^+ and Na^+ transports were both decoupled from the oxidoreductase activity.

Complex I from *R. marinus* Has Two Energy Transducing Sites. We have previously proposed a model for energy transduction by complex I from *R. marinus*, in which we suggested the presence of two energy transducing sites, one involving Na^+-independent H^+ translocation and the other processing Na^+-dependent H^+ translocation.[13] This model was based on the observations that complex I from *R. marinus* performs NADH:Q oxidoreduction and H^+ and Na^+ transports and that Na^+ neither influences the catalytic activity nor is necessary for H^+ transport. However the presence of Na^+ increases H^+ transport. The studies here described not only corroborate the presence of two proton translocations sites but also show that the two sites have different inhibition profiles for EIPA. In the absence of EIPA, the two H^+ translocations sites (Na^+-dependent and -independent) are perfectly operational; NADH-driven H^+ transport occurs, as well as an opposite NADH-driven Na^+ transport is performed. In the presence of 10 μM EIPA, the antiporter site is partially inhibited (50%), whereas the sodium-independent H^+ translocating site is not affected. These findings explain why, in these conditions, the $\Delta[Na^+]_{out}$ decreases in a higher amplitude when compared to ΔpH (30% for ΔpH_{out} and 50% for ΔpNa^+; Table 1). In the presence of Na^+, 20 μM EIPA causes 70−80% of inhibition of H^+ and Na^+ transports. However, in the absence of Na^+, the H^+ transport is only ~50% inhibited at the same concentration of EIPA, reflecting the inhibition profile of the Na^+-independent H^+ translocating site. The two different inhibition profiles for H^+ translocation, in a concentration range in which the oxidoreduction activity is not affected, reinforce the hypothesis that two H^+ translocating sites are present in complex I from *R. marinus*.

It was reported that in some situations EIPA could also act as a protonophore, even stronger than CCCP.[14,22,23] This effect is completely absent in our studies. EIPA did not influence NADH-driven formation of ΔΨ in the concentration range used for the translocation studies (0−20 μM). This conclusion was further supported by the ^{23}Na NMR experiments in the presence of EIPA, in which an increase of Na^+ translocation was not observed, as expected for a protonophore;[13] on the contrary it worked as a Na^+ transport inhibitor.

Complex I Transduces Energy by an Indirect Coupling Mechanism. Strong indications for the coupling mechanism for energy transduction in complex I have recently been provided by the structural data of the membrane domain of complex I from *E. coli* and of integral complex I from *T. thermophilus* and mitochondria of *Y. lipolitica*. The structures of the bacterial enzymes suggest that 3 protons may be translocated by an indirect coupling mechanism, involving conformational changes from near the quinone binding site to the antiporter subunits, with the participation of a long amphipatic helix, which spans almost the entire membrane domain and may work as a transmission element.[2] In order to achieve the stoichiometry of 4 translocated H^+ per NADH molecule, proposed for complex I,[7] an additional H^+ translocating site was considered. It would operate through a redox-driven mechanism, representing a direct coupling mechanism in which H^+ uptake would occur upon reduction of cluster N2.[24] This hypothesis further suggested that the same process for H^+ uptake would be operational for H^+ translocation and for quinone reduction. Such a possibility seems unlikely in the case of complex I from *R. marinus* since the catalytic reaction and H^+ translocation, irrespective of the dependence of Na^+, can be decoupled, *i.e.*, catalytic activity was not affected at certain conditions that highly inhibited H^+ translocation. The recent structural data[2,3] do not exclude the possibility that the translocation of the fourth proton may be driven by conformational change. Moreover, on the basis of our results and the available structures, we may speculate that EIPA could interfere with the transmission element hampering the required conformational changes, which follows the redox reaction, to propagate to the pumping site(s). In this case the energy released by the catalytic reaction would not be transduced into a transmembrane difference of electrochemical potential but instead would be lost as heat. The possible decoupling of the activities of complex I could be physiologically relevant in a situation in which the cell would need to regenerate NADH but had already reached its maxima of the transmembrane difference of electrochemical potential.

CONCLUSIONS

We propose that complex I from *R. marinus* has two types of energy transducing sites, both dependent on menaquinone reduction and both promoting H^+ translocation. However, one of the sites requires Na^+ for its operation. Complex I from *R. marinus* also promotes Na^+ transport in the opposite direction to that of H^+ transport. Furthermore, different EIPA inhibition profiles were observed for the two coupling sites. Taking together all data, we can hypothesize that one type of coupling site may work as a proton pump, while the other may operate by a Na^+/H^+ antiporter mechanism. In both cases energy transduction occurs through indirect coupling, most probably by conformational changes. These conclusions are the agreement with the recently obtained complex I structural data and deepen the clarification of the coupling mechanism for energy transduction by complex I.

METHODS

Cell Growth and Membrane Vesicles Preparation. *Rhodothermus marinus* PRQ 62B was grown as described previously,[25] in growth medium containing 100 mM glutamate. After harvesting, cells were suspended in 2.5 mM HEPES-Tris pH 7.5, 5 mM K_2SO_4, 25 mM Na_2SO_4 (buffer A) or 2.5 mM HEPES-Tris pH 7.5, 5 mM K_2SO_4, 50 mM choline chloride ($[Na^+] < 10\ \mu M$) (buffer B) and 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*, 2 h at 4 °C followed by resuspension in buffer A or buffer B. Integrity of vesicles was checked by the K^+/valinomycin assay using oxonol VI as a $\Delta\Psi$ sensitive dye (see below). Protein concentration was determined by the Biuret method modified for membrane proteins.[26]

Protein Purification. Protein was purified as described before,[27] introducing an additional chromatographic step using a Mono Q column and 20 mM Tris-HCl pH 8, 1 mM phenylmethanesulfonyl fluoride, 0.1% DDM (w/v) as buffer. The complex was eluted in a linear gradient of 0 to 100% NaCl (1 M). Protein concentration was determined by the bicinchoninic acid method as described before.[28]

$\Delta\Psi$ Detection. $\Delta\Psi$ generation was detected following oxonol VI absorption ($A_{628} - A_{587}$) at 27 °C, on an OLIS upgraded Aminco DW2 dual wavelength spectrophotometer.[29] The integrity of the vesicles was verified by generating K^+ gradients with K^+/valinomycin in an external buffer containing 50 mM K_2SO_4 (internal $[K^+] = 10$ mM). The assay was started by adding 2 μM valinomycin. To detect the NADH-driven formation of $\Delta\Psi$, 4 mM K_2-NADH was added to vesicles in buffer A or buffer B. When referred, EIPA (0 to 100 μM), CCCP (10 and 100 μM), rotenone (10 μM), and KCN (5 mM) were added prior to the addition of NADH.

Determination of the Internal Volume of Membrane Vesicles. The internal volume of the vesicles was determined by EPR spectroscopy, using 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) oxidized with $K_3[Fe(CN)_6]$.[30] TEMPO was quenched with 100 mM of potassium chromium(III) oxalate, in the external medium. EPR measurements were performed at RT, with a microwave frequency of 9.39 GHz, microwave power 1 mW, and modulation amplitude 0.04 mT.

Activity Measurements. Oxygen consumption was measured with a Clark-type oxygen electrode YSI model 5300. The assay mixture contained vesicles in buffer A or buffer B. The reaction was started by adding K_2-NADH. When used, EIPA (0 to 800 μM), valinomycin (2 μM), or CCCP (10 μM) were added prior to the addition of substrate. NADH:Q oxidoreductase activity was monitored on an OLIS upgraded Aminco DW2 dual wavelength spectrophotometer, at 55 °C, following the NADH oxidation at 330 nm (ε = 5930 M^{-1} cm^{-1}). The reaction mixture contained 200 mM KPi pH 7.5, 0.1% DDM (w/v) and 0 ($[Na^+]$<10 μM) or 25 mM Na_2SO_4, 50 μM DMN, and 50 μM NADH. The reaction was started by the addition of complex I. When used, NaCl (0 to 200 mM) or EIPA (0 to 250 μM) was added prior to the addition of substrate. NADH:$K_3[Fe(CN)_6]$ oxidoreductase activity was monitored at 420 nm (ε = 1020 M^{-1} cm^{-1}). The reaction medium contained membrane vesicles or solubilized membranes in buffer A or buffer B, 250 μM $K_3[Fe(CN)_6]$, and 250 μM NADH. Solubilized membranes were obtained by stirring an aliquot of membrane vesicles with 10% of DDM (w/v) for 4 h at 4 °C.

Fluorescence Spectroscopy. Fluorescence spectroscopy was performed on a Varian Cary Eclipse spectrofluorimeter. Generation of ΔpH was determined by the quenching of the fluorescence of ACMA ($\lambda_{excitation}$ = 410 nm, $\lambda_{emission}$ = 480 nm). Vesicles were incubated aerobically for 5 min at 27 °C in buffer A or buffer B containing 1 μM ACMA. The reaction was started by adding 100 μM NADH. When referred, valimonycin (2 μM) and EIPA (0 to 25 μM) were added prior to the addition of NADH.

The extra-vesicle pH (pH_{out}) was monitored using the hydrophilic and membrane-impermeable pH indicator pyranine ($\lambda_{excitation}$ = 458 nm, $\lambda_{emission}$ = 508 nm). Vesicles were incubated in buffer A or buffer B containing 2 μM pyranine. The reaction was started by adding 4 mM NADH. When referred, valimonycin (2 μM), rotenone (10 μM), KCN (5 mM), DMN (200 μM), or EIPA (0 to 22.5 μM) was added prior to the addition of NADH.

^{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.[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. A capillary tube containing the shift reagent dysprosium(III) tripolyphosphate (22 mM) was used in all experiments as external reference.[31] Spectra were recorded upon addition of 4 mM K_2-NADH to vesicles that were previously incubated with KCN (10 mM), DMN (200 μM), valinomycin (2 μM), or EIPA (0 to 20 μM).

■ ASSOCIATED CONTENT

Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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