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Respiratory complex I: A dual relation with H^+ and Na^+?☆

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ABSTRACT

Respiratory complex I couples NADH:quinone oxidoreduction to ion translocation across the membrane, contributing to the buildup of the transmembrane difference of electrochemical potential. H^+ is well recognized to be the coupling ion of this system but some studies suggested that this role could be also performed by Na^+. We have previously observed NADH-driven Na^+ transport opposite to H^+ translocation by menaquinone-reducing complexes I, which indicated a Na^+/H^+ antiporter activity in these systems. Such activity was also observed for the ubiquinone-reducing mitochondrial complex I in its deactive form. The relation of Na^+ with complex I may not be surprising since the enzyme has three subunits structurally homologous to *bona fide* Na^+/H^+ antiporters and translocation of H^+ and Na^+ ions has been described for members of most types of ion pumps and transporters. Moreover, no clearly distinguishable motifs for the binding of H^+ or Na^+ have been recognized yet.
We noticed that in menaquinone-reducing complexes I, less energy is available for ion translocation, compared to ubiquinone-reducing complexes I. Therefore, we hypothesized that menaquinone-reducing complexes I perform Na^+/H^+ antiporter activity in order to achieve the stoichiometry of $4H^+/2e^-$. In agreement, the organisms that use ubiquinone, a high potential quinone, would have kept such Na^+/H^+ antiporter activity, only operative under determined conditions. This would imply a physiological role(s) of complex I besides a simple "coupling" of a redox reaction and ion transport, which could account for the sophistication of this enzyme. This article is part of a Special Issue entitled Respiratory complex I, edited by Volker Zickermann and Ulrich Brandt.

1. Introduction

Energy transduction is a key process for life. In cells, most of the energy is transduced by membrane proteins, present in electron transfer chains, during cellular respiration or photosynthesis. The exergonic electron transfer from electron donors to electron acceptors is coupled to charge translocation across the membrane, establishing a transmembrane difference of electrochemical potential, as proposed by the Chemiosmotic theory [1]. This potential is then used for endergonic processes such as ATP synthesis, active transport or motility.

Complex I (NADH:quinone oxidoreductase, EC. 1.6.5.3) plays a central role in the energy transduction processes of the respiratory chains from mitochondria and many bacteria. It functions as an entry point for electrons into those respiratory chains and catalyzes the two electron oxidation of NADH and the reduction of quinone, coupled to charge translocation from the negatively charged side (N-side, prokaryotic cytoplasm or mitochondrial matrix) to the positively charged side (P-side, prokaryotic periplasm or mitochondrial intermembrane space) of the membrane. This process results in the establishment of a transmembrane difference of electrochemical potential.

Mitochondrial complex I consists of 14 core subunits and a large number of accessory subunits, which account in total for approximately 45 subunits (depending on the species). Those core subunits are responsible for catalysis and ion translocation and are conserved in all species containing complex I. The bacterial complex I (NDH-I) (named Nqo1-14 in *Thermus thermophilus* nomenclature or NuoA-N in *Escherichia coli* nomenclature) is composed of the 14 conserved core subunits and therefore it is considered to be the minimal functional unit of mitochondrial complex I.

The enzyme contains two spatially separated domains; peripheral and membrane domains. The peripheral domain is constituted by 7 subunits (subunits NuoB-G and I) and contains the NADH binding site as well as all prosthetic groups (a FMN and a series of iron-sulfur centers) that conduct electrons from NADH to the quinone, whose catalytic site is located at the base of the peripheral domain, close to the membrane [2–4]. The membrane domain (subunits NuoA, H and J–N) of complex I is involved in charge translocation across the membrane. Subunits NuoL, M and N, located at the distal end of the membrane arm in relation to the base of the peripheral arm, are homologous to each other and to subunits of so-called Mrp (Multi resistance and pH) antiporters [5]. These so-called Na^+/H^+ antiporter-like subunits (NuoL, M and N) are considered to take part in charge translocation. An additional ion translocating pathway was also proposed in subunits NuoH, J and K. In

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this way, complex I was suggested to have four sites for ion translocation [4,6].

The coupling mechanism between electron transfer and charge translocation processes, taking place at two spatially separated domains is a highly debated topic but still poorly understood. Different mechanisms have been proposed, involving either direct [7–8] or indirect [9–11] events or a combination of both [12–13]. The study of complex I and its coupling mechanism was boosted by the publication of the crystal structures of the membrane domain of the *E. coli* enzyme [14] and of the bacterial and mitochondrial complexes I, from *T. thermophilus* [4] and *Yarrowia lipolytica* [6], respectively. Additionally, a structure of complex I from *Bos taurus* heart mitochondria, a close relative of the human enzyme, was determined by single-particle electron cryomicroscopy [15]. The recent structural insights into complex I suggest an indirectly coupling mechanism, indicating the existence of long range conformational changes, connecting the redox events in the peripheral arm to the charge translocation processes in the membrane subunits [6].

The nature of the coupling ion of complex I is also highly discussed. For a long time, it was widely accepted that the coupling ion of all complexes I was the H^+ [16–18]. However, Na^+ was reported as being the coupling ion of complexes I from *E. coli*, *Klebsiella pneumoniae* and *Y. lipolytica* [19–22] and a Na^+/H^+ antiporter activity was reported for complexes I from *Rhodothermus marinus* and *E. coli* [23–25], as well as for the deactive form of the bovine enzyme [26].

In this article, we discuss the ion translocation by complex I, taking into consideration the structural and biochemical data obtained. We observed that most pumps and transporter systems can use H^+ and/or Na^+ as coupling or counter ions, with relevant contribution to the overall cellular bioenergetics.

2. The role of H^+ and Na^+ in bioenergetics

The Chemiosmotic theory, formulated in 1961, only considered the establishment of a proton electrochemical potential ($\Delta\mu H^+$ in $kJ \cdot mol^{-1}$ units, corresponding to proton motive force, *pmf* or Δp, in mV units), generated by the proton translocation activity of the respiratory complexes [1]. However, few years later, Mitchell proposed that ΔpH, built by the respiratory chain, could be used by Na^+/H^+ antiporters in order to create a difference in Na^+ concentration (ΔpNa) [27–29]. Moreover, the transport of Na^+ across the membrane was shown to help to maintain $\Delta\mu H^+$ and pH homeostasis in the case of limitation of substrates or growth in alkaline environments [30–31]. Since then, Na^+/H^+ antiporters have been widely described in prokaryotes and eukaryotes, establishing the foundations for the study of the bioenergetic role of Na^+ electrochemical potential [32–34].

The first evidence of the role of Na^+ as coupling ion emerged when a primary Na^+ pump activity was determined for oxaloacetate decarboxylase of *Klebsiella aerogenes* (*K. pneumoniae*) [35]. Additionally, it was observed that in alkaline conditions, NADH:quinone oxidoreductase activity of the respiratory chain of the marine bacterium *Vibrio alginolyticus* is coupled to Na^+ translocation across the membrane [36–37]. Furthermore, it was recognized that some extremophilic (hyperthermophilic, alkaliphilic and/or halophilic) organisms and bacterial pathogens seem to depend on Na^+ to survive and grow [38–40]. In fact, it was demonstrated that, in some circumstances, Na^+ can substitute H^+ as coupling ion and the resulting $\Delta\mu Na^+$ can be used for ATP synthesis, motility and/or solute import [35,37,41–42]. This is possible because, from a thermodynamic point of view, the $\Delta\mu H^+$ and $\Delta\mu Na^+$ are equivalent, and composed of both chemical, ΔpH or ΔpNa, and electrical components, $\Delta\Psi$ [38].

2.1. Coexistence of H^+ and Na^+ cycles

Most organisms use both H^+ and Na^+ cycles, either alternatively or concomitantly, and several enzymes seem to participate in the two cycles, being able to use either Na^+ and/or H^+ ions [43–52]. The interconversion between $\Delta\mu Na^+$ and $\Delta\mu H^+$ may increase the robustness and adaptation of organisms to different environments and stress conditions, as observed in bacterial pathogens, for example. These organisms need to survive in their natural environment and once inside the host a rapid adaptation to the new conditions is required. In fact, the coexistence of H^+ and Na^+ cycles in most bacterial pathogens is guaranteed by Na^+ and H^+ transporters in respiratory chains and multiple Na^+/H^+ antiporters [40].

While in prokaryotes both Na^+ and H^+ cycles seem to coexist in the same membrane, in animal cells, H^+ and Na^+ cycles occur simultaneously, but in different cell locations. In one hand, the establishment of an exclusive proton electrochemical potential across the inner mitochondrial membrane, by the electron transport chain, is essential for respiration. On the other hand, in the cytoplasmic membranes of most animal cells, a Na^+ cycle is established. The electrogenic Na^+/K^+-ATPase, which is present in the cytoplasmic membrane of all animals, uses the energy released by the hydrolysis of ATP to exchange 3 Na^+ ions with 2 K^+ ions, both against their concentration gradients [53–54]. The established Na^+ electrochemical potential provides the driving force for many antiporters, such as Na^+/H^+ antiporter and Na^+/Ca^{2+} antiporter [55–56]. The ionic fluxes promoted by the Na^+ cycle are involved in several cellular processes, such as, the regulation of cell volume, the maintenance of the cell membrane resting potential, the generation of action potential in neuronal cells, the regulation of signal transduction mechanisms and the import of glucose, amino acids and other nutrients into the cell by processes that require a ΔpNa [57–59].

3. Complex I contributes directly to the establishment and maintenance of the transmembrane difference of electrochemical potential

3.1. H^+ as the coupling ion of complex I

Complex I is generally accepted to contribute to the transmembrane difference of electrochemical potential by translocating H^+ ions across the membrane, in a process that is coupled to its redox activity. However, the determination of the number of H^+ ions or charges translocated *per* catalytic turnover (corresponding to $2e^-$ transfer between NADH and quinone) has not been a trivial task.

Early observations by Lawford and Garland (1972) and Ragan and Hinkle (1975) indicated a maximal $H^+/2e^-$ stoichiometry of 2 for rat liver and bovine heart mitochondrial complex I [60–61]. Later, studies performed by the groups of Lehninger [62] and Azzone [63] indicated a stoichiometry of approximately $4H^+/2e^-$ in rat liver and heart mitochondria, which is now generally accepted [17,64–67]. The same value was obtained for complex I from the yeast *Y. lipolytica*, either using intact mitochondria or purified enzyme reconstituted into liposomes [68]. However, Wikström and Hummer using the values of the H^+/ATP ratio of the F1FO-ATP synthase of animal mitochondria and the measured $ATP/2e^-$ ratios for different segments of the mitochondrial respiratory chain, calculated a value of $3H^+/2e^-$ for mitochondrial complex I [69]. Indeed, such stoichiometry was observed by Jonge and Westerhoff [70] using submitochondrial particles. Only two studies addressed the $H^+/2e^-$ stoichiometry by bacterial complexes I; a stoichiometry of 3 to $4H^+/2e^-$ was calculated for complex I of aerobically grown *Paracoccus denitrificans* [71], while a value of 3 was indirectly determined for dimethyl-sulfoxide respiring *E. coli* cells [18]. $H^+/2e^-$ stoichiometry of 3 for complex I does not agree with the existence of 4 translocating sites if all of these are considered to be active.

3.2. Na^+ as the coupling ion

The established role of H^+ as coupling ion in complex I was challenged by the studies of Steuber and coworkers using two closely

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related organisms, *K. pneumoniae* and *E. coli* [21]. By atomic absorption spectroscopy and radioactive $^{22}Na^+$ techniques, the authors observed NADH-driven Na^+ uptake in inverted membrane vesicles of *K. pneumoniae*. This Na^+ transport was abolished by the complex I inhibitor, rotenone, but not in the presence of the protonophore CCCP. These observations were indicative of a primary Na^+ pump activity by complex I and not a combined action of a proton pump and Na^+/H^+ antiporters [19]. Similar conclusions were obtained using a Na^+/H^+ antiporter deficient *E. coli* strain. In this study, the authors observed an increase of Na^+ tolerance of the studied strain when the expression of complex I was higher. Na^+ translocation by complex I was confirmed by using the complex I inhibitor rotenone and a mutant devoid of complex I [21]. Interestingly, it was observed that the C-terminally truncated subunit NuoL of *E. coli* complex I (reconstituted into liposomes [72] or expressed in *Saccharomyces cerevisiae* [73]) performed passive Na^+ uptake.

Complex I of *K. pneumoniae* was reconstituted into liposomes and a $Na^+/2e^-$ ratio of 2 was determined [20]. When complex I from *K. pneumoniae* and the Na^+-dependent ATP synthase from *Ilyobacter tartaricus* were reconstituted together into liposomes, it was observed that the $\Delta\mu Na^+$ generated by NADH-driven Na^+ translocation by complex I could serve as driving force for the ATP synthesis and that the $\Delta\mu Na^+$ generated by ATP hydrolysis could drive NADH formation by reversed electron transfer in complex I [74].

Na^+ pumping activity was also detected in complex I of *Y. lipolytica* and again, NADH-driven Na^+ translocation from the negatively charged side to the positively charged side of the membrane of submitochondrial particles was observed [22].

All these finding were highly debated because in *K. pneumoniae* [75], *E. coli* [16] and *Y. lipolytica* [68,76], H^+ translocation from the negative to the positive side of the membrane was observed. Moreover, the results obtained in studies involving complex I from *Y. lipolytica* reconstituted into liposomes and Na^+/H^+ antiporter monensin seem to rule out the hypothesis of Na^+ translocation by this enzyme [76].

Na^+ translocation was attributed to Na^+-translocating NADH:quinone oxidoreductase (Na^+-NQR) in *K. pneumoniae* [75] and to secondary Na^+/H^+ antiporter activity in *E. coli* [16]. Nevertheless, growth and experimental conditions were different among the different studies, which might strongly influence the expression of respiratory enzymes and the nature of the coupling ion.

3.3. Na^+/H^+ antiporter activity of complex I

The first suggestion of a Na^+/H^+ antiporter activity by complex I was made by Stolpe and Friedrich using isolated *E. coli* complex I reconstituted into liposomes [16]. The results showed NADH-driven H^+ translocation, which increased in the presence of Na^+. The authors suggested that a putative translocation of Na^+ by complex I drives further the translocation of H^+ in the opposite direction, increasing ΔpH. These results indicated the role of H^+ as the coupling ion of complex I and a possible Na^+/H^+ antiporter activity by complex I in *E. coli*.

NADH-driven Na^+/H^+ antiporter activity of complex I was directly observed using a ^{23}Na-NMR spectroscopy method developed by our group [23–25]. This technique allowed the direct monitoring of the changes in Na^+ concentration inside and outside of inverted membrane vesicles of *R. marinus* and *E. coli* [23–25]. The results showed proton translocation in the same direction of the established $\Delta\Psi$ and for the first time, translocation of Na^+ by complex I in the direction opposite to that of the established $\Delta\Psi$. In these systems, Na^+ translocation increased in the presence of the protonopohore CCCP, showing that Na^+ transport is not dependent on ΔpH and thus is a primary event. The presence of Na^+ was also observed to increase the ΔpH, although it was not necessary for the catalytic or proton transport activities, which was in agreement with the observations by Stolpe and Friedrich for *E. coli* [16].

The Na^+/H^+ antiporter activity of complex I of *R. marinus* was further corroborated by investigations with the Na^+/H^+ antiporter inhibitor EIPA. This inhibitor promoted the decoupling of the catalytic and transport activities of complex I from *R. marinus* and two different inhibition profiles of H^+ translocation, in the presence and absence of Na^+ ($[Na^+] < 10$ μM), were observed [77]. We hypothesized that complexes I from *E. coli* and *R. marinus* have two types of energy coupling sites, one Na^+ independent, working as proton pump and the other Na^+ dependent, working as Na^+/H^+ antiporter. Recently, the ion translocation activity by complex I from *E. coli* devoid of subunit NuoL was investigated and we observed that the mutated complex I does not translocate Na^+ and is less effective in proton pumping [78]. These studies corroborated the idea that H^+ is the coupling ion in complex I from *E. coli* and that subunit NuoL may be involved in the translocation of H^+ and Na^+ ions, possibly acting as a Na^+/H^+ antiporter or influencing the activity of other subunits of complex I. In addition, these observations are in agreement with those by Friedrich and coworkers, who previously reconstituted into liposomes an *E. coli* ΔNuoL mutant of complex I and observed a reduced rate of proton pumping relatively to the wild-type complex I [79].

We also investigated Na^+ translocation by complex I from *P. denitrificans*. In this case we observed translocation of H^+ but not of Na^+. Knowing that the two complexes I for which we observed Na^+ translocation use menaquinone as substrate, and the one from *P. denitrificans* uses ubiquinone, we hypothesized a correlation between the type of quinone used as substrate and the Na^+/H^+ antiporter activity [24] (see Section 4.2). Hirst and coworkers reconstituted complex I from *B. taurus* heart mitochondria (which reduces ubiquinone) into liposomes and observed H^+ translocation by complex I in its "deactive" form (i.e. no redox activity due to lack of substrates), when a Na^+ electrochemical potential was imposed [26]. This suggested that mitochondrial complex I may perform Na^+/H^+ antiporter activity under certain conditions. The conversion between an "active" and a "deactive" form of complex I is a characteristic of some eukaryotes, never observed in bacteria [80]. Hirst and coworkers suggested that the redox and translocation modules of complex I become disconnected in the "deactive" form, possibly allowing the antiporter-like subunits of complex I to perform their native function (i.e. Na^+/H^+ antiporter activity) [26].

4. The physiological relevance of Na^+ translocation by complex I

Sodium translocation by complex I is still not fully accepted by some members in the community. Besides the experimental evidences, many facts support, or at least do not exclude Na^+ translocation. Complex I has three subunits homologous to *bona fide* Na^+/H^+ antiporters (NuoL, M and N) and other subunits (NuoK, J and H) that also share key structural features with the same transporters (see Section 4.1.1) and therefore suggests a similar activity. Furthermore, the existence of Na^+ binding site(s) in complex I cannot be excluded, since these, as exemplified by several proteins involved in H^+ and Na^+ translocation, have not been clearly established, yet (see Section 4.1.2). Complex I is suggested to have 4 functional ion translocation pathways, which would allow the translocation of 4 ions. In the case of menaquinone reducing complexes I there is not enough energy available by the oxidoreduction reaction to supply the energy needed for the translocation of 4 charges per catalytic turnover (see Section 4.2). Thus an additional energy source has to be involved in order to achieve this number. We suggested this energy could be the dissipation of the Na^+ gradient potential.

4.1. Structural considerations

4.1.1. Complex I contains subunits homologous to Na^+/H^+ antiporters

Subunits NuoL, M and N of complex I are homologous to subunits of so-called Mrp antiporters [5]. The X-ray crystal structures of complex I showed that the three antiporter-like subunits are homologous to

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each other and contain a highly conserved core of two sets of transmembrane helices (TM4-8 and TM9-13), related to each other by a pseudo-2-fold symmetry, being so-called inverted structural repeats. Each repeat contains charged or polar amino acid residues in the middle of the membrane, near to discontinuous or partially unwound transmembrane helices. These structural characteristics are observed in *bona fide* antiporters or transporters [14,81], such as NhaA. However, the repeats present in the subunits of complex I have a face-to-back arrangement, not observed before [14,82]. This arrangement is also expected to occur in the case of Mrp antiporters, as suggested by homology structural models [83].

The members of Mrp family perform electrogenic secondary Na^+/H^+ antiporter activity and play a key role in the cytoplasmic pH homeostasis under alkaline growth conditions and in Na^+ resistance [5,84–85]. Mrp antiporters form hetero-oligomeric complexes containing 6 or 7 subunits, MrpA-G, which are all required for the proper function of the complex, but MrpA and MrpD have been shown to play a key role in the Na^+/H^+ antiporter activity [86–91]. Subunits NuoL and NuoM/N of complex I possess high sequence similarity to MrpA and MrpD subunits, respectively. Moreover, subunits NuoK and NuoJ of complex I are homologous to subunit MrpC and part of C-terminal domain of MrpA, respectively.

Using *Bacillus subtilis* ΔMrpA and ΔMrpD deletion strains, Hägerhäll and coworkers suggested that each one of MrpA and MrpD subunits is responsible for the translocation of only one type of ion. One of the subunits may be responsible for the translocation of H^+ and the other for the translocation of Na^+ [91]. Complementation studies using *B. subtilis* ΔMrpA and ΔMrpD deletion strains demonstrated that subunits NuoL and NuoN from complex I could replace MrpA and MrpD subunits, respectively [88,91]. This suggested a functional specialization of NuoL and NuoN and the authors hypothesized the existence of a Na^+ channel in NuoL subunit and a H^+ channel in NuoN subunit [88,91].

Besides these three subunits, complex I has a fourth subunit closely related with Na^+/H^+ antiporters. Based on structural homology studies, a relation between NuoH, which is involved in quinone binding, and the antiporter-like subunits (NuoL, NuoM and NuoN) was put forward [92], which was corroborated by the crystal structure of *T. thermophilus* complex I [4]. NuoH presents a set of helices with the same fold as the half-channels of the antiporter-like subunits. Therefore, a proton pathway composed of two half-channels; one corresponding to NuoH and another to subunits NuoJ and NuoK, linked to each other by acidic amino acid residues, was suggested [4].

4.1.2. Does complex I structure present Na^+ binding sites?

Transport of both H^+ and Na^+ ions is common among several membrane transporters and enzymes. While some enzymes are able to switch between Na^+ or H^+ coupling ions, depending on environmental conditions, others appear to use both Na^+ and H^+ gradients simultaneously [43–52]. However, the structural requirements that modulate ion specificity in most of these processes are still poorly understood.

While strongly H^+ selective ATP synthases are capable of using only proton as the coupling ion, weakly H^+-selective ATP synthases are capable of using both Na^+ and H^+ ions [44]. Leone and coworkers proposed that H^+ selectivity in ATP synthases is conferred by two factors, the availability of the coupling ion and the balance between hydrophobic and polar groups in vicinity of the side chains of conserved glutamate/aspartate residues in rotor rings [44]. In ATP synthase from *Propionigenium modestum*, hydrophobic substitutions (S66A and Q32I) in c-ring contribute to a greater selectivity towards H^+ [93]. In ATPases from *I. tartaricus* and *Enterococcus hirae*, serine, valine and leucine residues appear to be involved the Na^+-binding sites [94–95].

Membrane-bound pyrophosphates (mPPases) are key energetic players during periods of stress such as starvation or exposure to salinity or toxins, performing transmembrane transport of H^+ or Na^+ ions [45,96–97]; Whereas H^+-mPPases are unable to transport Na^+ ions, Na^+-mPPases are capable of translocating both Na^+ and H^+ ions at Na^+ concentration below 5 mM [45–46]. Interestingly, a third subfamily of mPPases, Na^+, H^+-mPPases, mainly found in bacteria of the human gastrointestinal tract, mediate transport of both Na^+ and H^+ ions, even at high Na^+ concentrations [45]. Subtle sequence changes differentiate mPPases coupled to H^+ translocation from the ones coupled to Na^+ transport [97]. According to Luoto and coworkers, a K681N substitution in Na^+-mPPase abolished H^+ but not Na^+ transport, which indicates the involvement of K681 in H^+ selectivity at low Na^+ concentration [46]. Moreover, the relocation of a conserved residue (E246) along the same transmembrane helix (TM) or to another TM seems to be involved in the shift from Na^+ to H^+ specificity [98]. Replacement of E246 with an aspartate residue in Na^+-PPase leads to a decrease in Na^+ binding [43,96,98].

Flagellar motors are also powered by an electrochemical potential of H^+ or Na^+ ions across bacterial cytoplasmic membrane [43,99–101]. Some bacteria, like *E. coli* or *Salmonella typhimurium* have proton-driven motors. In contrast, the flagella of *Vibrio* spp. and alkalophilic *Bacillus* species use Na^+ electrochemical potential for motility [102]. Dual flagellar systems are also used in nature, contributing to swimming under different physical and chemical conditions [43,102–104]. The MotAB-like system from *Bacillus clausii* is an example of a single stator that can use both ions to power motility, using preferentially H^+ ions at pH 7, Na^+ ions at pH 11 and both ions at pH values ranging from 8 to 10, in a variety of Na^+ concentrations [43]. Terahara and coworkers identified triple mutations that can convert a bifunctional flagellar motor to a specific H^+ or Na^+-coupled profile. In order to produce a H^+-motor candidate, Q43S mutation and either G42S or Q46A alterations were necessary to accomplish loss of Na^+ coupling. In order to change a H^+-coupled motor to a Na^+-coupled profile at low pH, V37L mutation and either A40S or G42S mutation were required [43].

Some transport systems are coupled to Na^+ and/or H^+ ions, such as melibiose transporter (MelB) from *E. coli* [104], alanine carrier protein (Acp) from thermophilic bacterium PS3 [105], glutamate carrier (GltT) from *Bacillus stearothermophilus* [106] and citrate transporter (CitS) from *K. pneumoniae* [107]. Whereas *E. coli* melibiose transporter uses Na^+ (most effective), H^+ and Li^+ for the co-transport of melibiose, the enzyme from *K. pneumoniae* uses H^+ or Li^+ but not Na^+ [48,104]. It has been determined that the N-terminal region of the enzyme plays a key role in the ion specificity [108]. In this region, only five residues are not conserved between the *K. pneumoniae* and *E. coli* enzymes. Interestingly, a single mutation, A58N, allowed the melibiose transporter from *K. pneumoniae* to use Na^+ as coupling ion and the reverse mutation, N58A, led to a severe reduction of Na^+ transport by *E. coli* melibiose transporter [109–110]. Moreover, residue K337 seems to play a key role in ion specificity by interacting with the nearby aspartate residues (D55, D59, D124) [109–111]. Some studies suggest the involvement of this residue in spatial organization of Na^+ binding site and in substrate binding [111].

In recent years, the increasing knowledge on ion pumping systems led to the discovery of Na^+-pumping counterparts of well-established H^+-pumping enzymes. One example is the case of heme–copper oxidases (HCOs), which were believed to conserve energy by H^+ pumping across cell membrane. However, the existence of some Na^+-pumping HCOs has been reported [47,112–114], including the recent report on the cbb_3 oxidase from *Thioalkalivibrio versutus* [114]. Other recent discovery relates to light-driven ion-pumping rhodopsins, which are widely distributed in many microorganisms. These enzymes convert the energy of sunlight into electrochemical ion gradients. H^+ was described to be the pumped ion, but in 2013, the first light-driven Na^+ pump, KR2, was discovered in the marine flavobacterium *Krokinobacter eikastus* [115]. While in the absence of Na^+ and Li^+, KR2 transports H^+, at physiological conditions it only pumps Na^+ ions [116].This rhodopsin contains a unique NDQ (N112, D116 and Q123 residues) motif, which seems to be involved in Na^+ binding and translocation.

As far as we know, H^+ and Na^+ ions have been described to be translocated by members of all types of ion pumping enzymes. The

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Na^+/H^+ antiporter activity by complex I makes this complex not an exception to the rule.

We explored possible structural determinants that would allow us to recognize H^+ and Na^+ binding sites in complex I. However, this was a challenging task since no obvious conserved motifs for H^+ or Na^+ binding site have been established yet. Several factors may contribute to the specificity of ion binding sites such as the number of ligands, atomic composition of the binding site, size of the binding site and ion coordination geometry [117–118]. While H^+ binding requires the presence of hydrophilic groups, Na^+ coordination requires several ligands with an appropriate spatial geometry [44,93,119]. In some enzymes, subtle differences in just one specific amino acid residue seem to define the enzyme specificity for Na^+ or H^+ ions [44,96,108–110,120]. Negatively charged residues (aspartates and glutamates) positioned in the transmembrane portions of proteins are common constituents of Na^+ binding sites and good candidates for Na^+ translocation pathways [121], as has been observed for Na^+-NQR, HCO from *T. versutus* [114], ATPases from *P. modestum* and *Pyrococccus furiosus* [122], Na^+-mPPases [98], aspartate transporter GltPh [123] and Na^+/H^+ antiporters [124–129].

Nevertheless, the presence of net negative charges is not a strict requirement for Na^+ transport, as has been described for leucine/Na^+ co-transporter (LeuT) [118]. Other amino acid residues, such as serines, asparagines, histidines and lysines and the backbone carbonyl group of valine and leucine residues may be involved in Na^+ coordination in several transporters [43,94–95,108–111,126].

Subunits NuoL, M and N of complex I present structural features similar to the ones observed in *bona fide* antiporters or transporters [14,81]. In fact, those subunits contain conserved charged or polar amino acid residues in the middle of the membrane, near to discontinuous or partially unwound transmembrane helices (Fig. 1). Conserved glutamate, lysine and histidine residues were suggested to play a key role in ion translocation in complex I [14]. The existence of structural determinants for Na^+ binding in complex I is not clear, as also observed in the case of the multiple examples pointed here (Fig. 1). Also taking into account these examples, at the present stage, the existence of a Na^+ binding in (some) complex I cannot be excluded.

4.2. Thermodynamic considerations

Since complexes I from *E. coli*, *R. marinus* and *P. denitrificans* are very similar, the differences observed in terms of Na^+ translocation may be related to the metabolic characteristics of each organism, namely the quinones used by complex I. Both *E. coli* (grown under anaerobic or microaerophylic conditions) and *R. marinus* complexes I reduce menaquinone ($Em,7 \approx -80$ mV), whereas complex I from *P. denitrificans* uses ubiquinone ($Em,7 \approx +100$ mV) as substrate [130]. The difference in the reduction potentials of the two types of quinones has strong thermodynamic implications. Considering the reduction potential of the NAD^+/NADH pair ($Em,7 \approx -320$ mV), the redox potential difference (ΔE) between NADH and menaquinone is 240 mV and between NADH and ubiquinone is 420 mV [131–132] (Fig. 2). As can be observed, the energy available for proton translocation is very different in these two situations. Considering that in both cases the $H^+/2e^-$ stoichiometry would be the same, an immediate raised question was whether Δp would be the same in organisms that produce menaquinone and in those that synthesize ubiquinone. Unfortunately, the information on the determination of Δp for different organisms or growth conditions is scarce. As far as we know, the only study that addresses this question is the work by Tran and Unden [131], which determined that in *E. coli*, Δp is 160 mV and 140 mV in aerobic and anaerobic respiration, respectively. *E. coli* uses ubiquinone in aerobic conditions and menaquinone in anaerobic conditions. Thus, these results suggest that Δp of menaquinone reducing organisms would not be much different from that of ubiquinone reducing organisms.

For a two electron redox reaction, ΔE is related to Δp by the following expression, $n\Delta p = 2\Delta E$, where n is the number of protons that can be pumped [132].

If we consider an average Δp value of 150 mV, the oxidation of menaquinone and ubiquinone allows a maximal $H^+/2e^-$ stoichiometry of 3 and 5, respectively (Fig. 2). However, it is important to note that these calculations were performed based on the midpoint potentials of the substrates, considering that the pools of substrates are 50% oxidized and 50% reduced. In physiological conditions, the NADH/NAD^+ ratio is smaller than 1 (either in aerobic or anaerobic conditions) and then the actual reduction potential for the NADH/NAD^+ couple would be higher, possibly leading to slightly smaller $H^+/2e^-$ stoichiometries than those we present here [133].

In the organisms that use ubiquinone as the electron acceptor (e.g. *P. denitrificans* and eukaryotes), the energy obtained by the reduction of ubiquinone seems to fulfill the 'universally accepted' stoichiometry of $4H^+/2e^-$. In the case of organisms that use menaquinone (e.g. *E. coli* and *R. marinus*), the energy obtained by the menaquinone reduction is only sufficient for a stoichiometry of $3H^+/2e^-$, which is in accordance with the value suggested by Bogachev et al. when using DMSO respiring *E. coli* cells [18]. Nevertheless, the crystal structure of complex I from *T. thermophilus* (a menaquinone reducing organism) suggests the

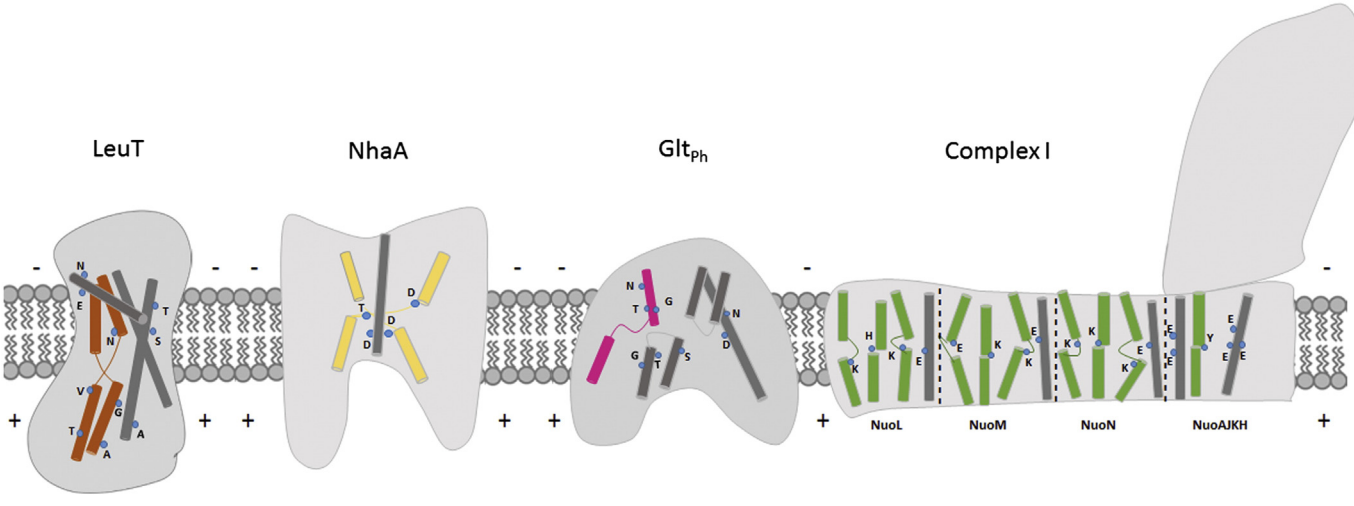

Fig. 1. Complex I presents common structural features to Na^+ transporters. Schematic representation of complex I and some transporters involved in Na^+ and H^+ transport, leucine transporter (LeuT), Na^+/H^+ antiporter NhaA and aspartate transporter (Gltph). Charged and polar residues present near discontinues helices are supposed to participate in ion translocation, being commonly associated with proteins that transport Na^+ and/or H^+. Transmembrane helices (rods) and crucial amino acid residues (blue circles) involved in cation transport are presented. Orange, yellow, pink and green rods indicate the presence of discontinuous helices. In case of oligomers, only monomers are represented.

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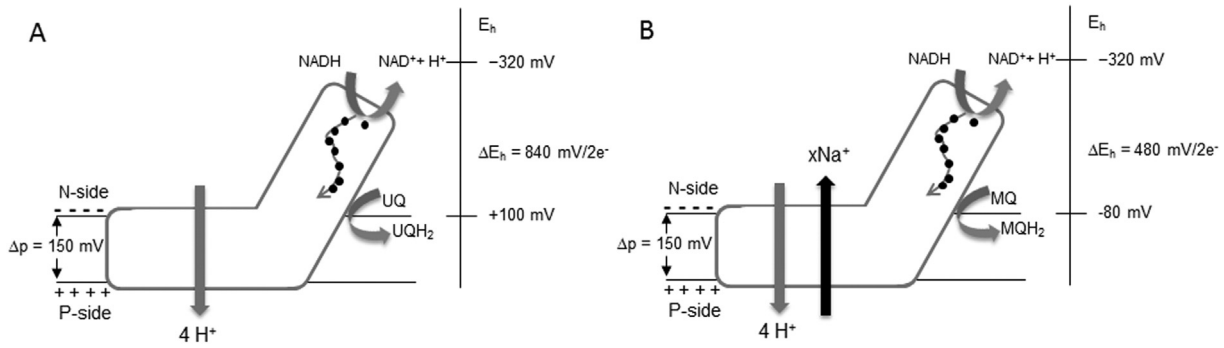

Fig. 2. Thermodynamic contributions for complex I using menaquinone (MQ) or ubiquinone (UQ) as substrate. Schematic representation of ubiquinone-reducing (A) and menaquinone-reducing (B) complexes I. The free energy obtained from the redox reaction drives ion translocation across the membrane, against an average Δp of 150 mV. Considering the reduction potential of the NAD^+/NADH pair (Em,7 ≈ −320 mV), the redox potential difference (ΔE) between NADH and menaquinone is 240 mV and between NADH and ubiquinone is 420 mV. For a two electron redox reaction, ΔE is related to Δp by the following expression, $n\Delta p = 2\Delta E$, where n is the number of protons that can be pumped. The menaquinone-reducing complexes I would not have enough available energy to reach the accepted stoichiometry of $4H^+/2e^-$. We hypothesize that the dissipation of Na^+ chemical gradient (Δp) would provide the required energy. In ubiquinone-reducing complexes I enough ΔE is already available by the redox reaction and only protons are translocated.

presence of four ion channels, which may allow the translocation of 4 protons *per* catalytic turnover [4].

We hypothesized that in the menaquinone reducing complex I, at least one of the coupling sites could function as a Na^+/H^+ antiporter [24]. The energy released by dissipation of Na^+ chemical gradient (Δp), $[Na^+]_{out} > [Na^+]_{in}$, would allow the translocation of additional protons in order to achieve the stoichiometry of $4H^+/2e^-$ (Fig. 2). A simple exchange of 1 H^+ per 1 Na^+ would not contribute to an increase of the electrical component of the electrochemical potential but would contribute to increase ΔpH. If the Na^+/H^+ antiporter activity of complex I would be electrogenic i.e., Na^+/H^+ stoichiometry different from 1, the Na^+/H^+ antiporter activity could also contribute to ΔΨ.

Menaquinone reducing complexes are present in early branches of prokaryotic phyla and can be considered ancestor enzymes, relative to ubiquinone reducing complexes [134]. When the substrate changed from menaquinone to ubiquinone, the NADH–quinone redox reaction provided more energy for ion translocation, and the complex I would have eventually lost the need to use ΔpNa to promote additional H^+ translocation (Fig. 2).

Na^+/H^+ antiporters were reported to change its directionality and stoichiometry in response to the environmental conditions. In *E. coli*, an electroneutral (1:1) H^+/Na^+ antiporter operates under acidic and neutral conditions and an electrogenic activity ($H^+/Na^+ = 2$) with export of Na^+ is detected under alkaline conditions. Taking this into consideration, the possibility of Na^+ translocation to the positive side of the membrane may also be put forward. Whether this would contribute to the establishment of a transmembrane difference of electrochemical potential depends on the stoichiometry of the transport [135]. The

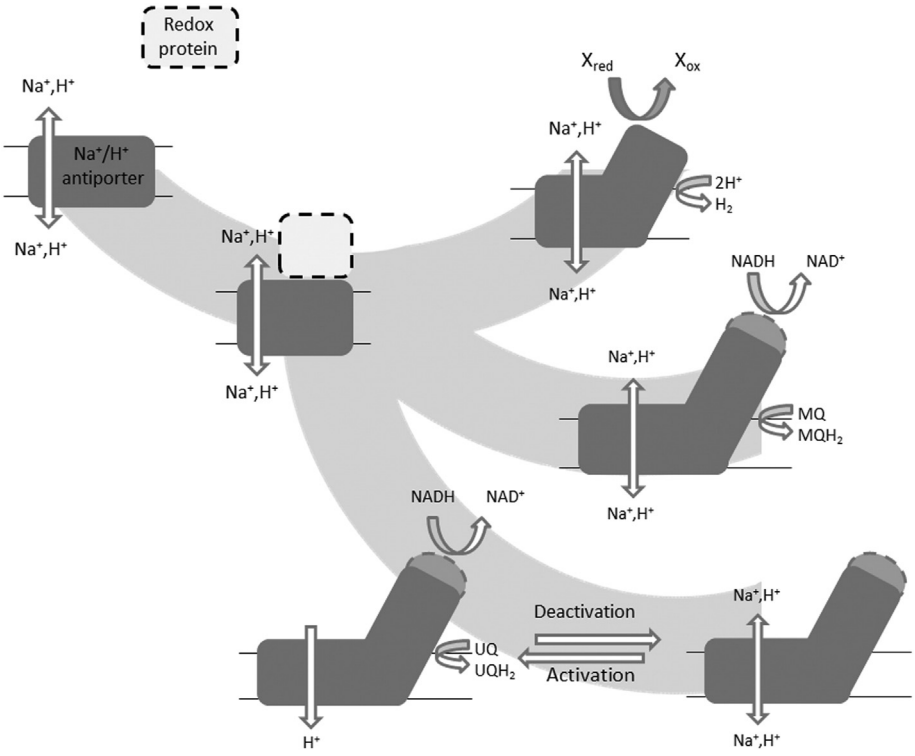

Fig. 3. Proposal of an evolutionary scenario for complex I and type 4 membrane-bound [NiFe] hydrogenases. A soluble redox enzyme performing electron donor: H^+ oxidoreductase activity would have associated with a Na^+/H^+ antiporter in order to allow energy coupling and generation of transmembrane difference of electrochemical potential (1 and 2). This complex would give rise to type 4 membrane-bound [NiFe] hydrogenases (3) and complex I (4 and 5). Menaquinone-reducing complexes I (4) need to perform Na^+/H^+ antiporter activity in order to achieve the $4H^+/e^-$ stoichiometry. Ubiquinone-reducing complexes I transport solely protons when coupled to the catalytic reaction (5) but could have kept Na^+/H^+ antiporter activity independently of catalytic activity, in its deactive form, for example (6).

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idea of a reversible Na^+/H^+ antiporter could partially solve the apparent controversy of the nature of the coupling ion.

5. Evolution considerations

We have proposed that Na^+/H^+ antiporter activity reflects a possible evolutionary origin of complex I [92]. It is well established that complex I has subunits homologous to subunits of Mrp Na^+/H^+ antiporters, soluble NAD^+ reducing hydrogenases and type 4 membrane-bound [NiFe] hydrogenases [83,136–137]. We hypothesized that the precursor of complex I and membrane bound hydrogenases was the result of the association of a soluble enzyme performing thermodynamic favorable redox reaction with a transmembrane protein performing passive or secondary charge transport [92]. In this way, we put forward the idea that a soluble redox enzyme performing electron donor: H^+ oxidoreductase activity, which gave rise to the precursor of the peripheral arm of type 4 membrane-bound [NiFe] hydrogenases, would have associated with a Na^+/H^+ antiporter to allow energy coupling. This antiporter in the new established complex would work in the reverse direction of that performed when isolated and in this way would contribute to the establishment of the transmembrane difference of electrochemical potential. Specifically, we suggested that this complex was the precursor of type 4 membrane-bound [NiFe] hydrogenases and later of complex I by integration of a NADH interacting subunit(s) and loss of the [NiFe] center. The binding site for this center seems to have been converted into a quinone binding site (Fig. 3) [92].

In menaquinone-reducing complexes I, the antiporter activity is still observed in order to promote the translocation of four protons across the membranes. When menaquinone was substituted by ubiquinone, with higher redox potential, complex I lost the need to use Na^+ membrane potential for the translocation of the four protons and thus Na^+ transport is not observable during catalysis. The results obtained for the bovine enzyme suggest that Na^+/H^+ antiporter activity was maintained in ubiquinone-reducing complexes I, but only functions under certain circumstances. This may reflect an evolutionary step forward in the regulation and operative modes of the complex (Fig. 3).

6. Conclusion

Complex I is one of the most complex respiratory enzymes present in the mitochondria of Eukarya and in the respiratory chains of Bacteria. An 11 subunit version with a different input model is observed in some archaea.

Several reports suggested that complex I is involved in proton and sodium bioenergetics, either by using proton or sodium as coupling ion, or by performing Na^+/H^+ antiporter activity. In fact complex I has several subunits homologous to *bona fide* Na^+/H^+ antiporters. The structural determinants of Na^+ and H^+ binding sites are not yet clearly and unambiguously distinguishable yet, as exemplified by several proteins involving H^+ and/or Na^+ translocation. Thus, the existence of Na^+ binding site(s) in complex I cannot be excluded.

Na^+/H^+ antiporter activity has been shown in Bacteria and in Eukarya complexes I. However, the physiological relevance of Na^+ transport in these organisms may be different; in the first case it may be a requirement in order to couple the redox reaction to the translocation of 4 ions, and in the second case, the antiporter activity may be under tight regulation and only operative in certain conditions, as a reflection of the sophistication of eukaryotic complex I. So far, two bacterial complexes I were shown to perform Na^+/H^+ antiporter activity during catalytic turnover. We hypothesized that the need for Na^+ transport by complex I is related to the available ΔE, defined by the type of quinone used by the organism. The two bacteria whose complexes I were observed to have Na^+/H^+ antiporter activity use a low potential quinone, menaquinone, as electron acceptor. The menaquinone dependent complexes I would need an extra source of energy to reach the accepted stoichiometry of $4H^+/2e^-$. This extra energy can be achieved by dissipation of ΔpNa. In ubiquinone dependent complexes I, enough ΔE is already available by the redox reaction itself to transport 4 protons across the membrane. Therefore, we suggested that both Bacterial and Eukarya complexes I that use ubiquinone would be specialized to transport solely protons when coupled to the catalytic reaction. Moreover, in ubiquinone dependent complexes I, Na^+ transport activity could have been kept independently of catalytic activity and could be activated under specific circumstances. As suggested by Hirst and coworkers [26], who observed Na^+/H^+ antiporter activity by the deactive form of complex I from *B. taurus* mitochondria, such a specific circumstance could be an ischemia-reperfusion event. The authors discussed that complex I driven transmembrane fluxes of Na^+ and H^+ may influence intra-mitochondrial Ca^{2+} homeostasis, which is related to important cell functions, including apoptosis [26,138].

Complex I seems to have a very sophisticated structure for its function. Specifically when compared for example to the last enzyme of aerobic respiratory chains, HCO, which couples O_2 reduction to proton pumping. The free energy available from O_2 reduction to water drives the pumping of 4 protons across the mitochondrial inner membrane (H^+/e^- stoichiometry of 1, half of the stoichiometry of complex I) [139]. O_2 reduction and proton translocation takes place in the same subunit, which has 50 kDa. This is in sharp contrast to complex I that contains 550 kDa in bacteria or 1 MDa in eukaryotes and in which the catalytic reaction involves seven subunits, while ion translocation seems to take place in several subunits. In fact, as mentioned before, 4 proton translocation sites were hypothesized in complex I, 3 of them present in the antiporter like subunits with 50 kDa each. In the two systems, complex I and HCOs, proton translocation is coupled to redox reactions and thus we find it most intriguing the high sophistication of complex I when comparing to HCOs. Why would cells invest so much energy building up such a complex machine when simpler solutions exist? We believe that complex I has a more complex operative mechanism than a simply “coupling” of a redox reaction and ion transport.

Transparency document

The Transparency document associated with this article can be found, in online version.

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