Ion translocation by the *Escherichia coli* NADH:ubiquinone oxidoreductase (complex I)

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Abstract

The energy-converting NADH:ubiquinone oxidoreductase, also known as respiratory complex I, couples the transfer of electrons from NADH to ubiquinone with the translocation of ions across the membrane. It was assumed that the complex exclusively works as a proton pump. Recently, it has been proposed that complex I from *Klebsiella pneumoniae* and *Escherichia coli* work as Na⁺ pumps. We have used an *E. coli* complex I preparation to determine the type of ion(s) translocated by means of enzyme activity, generation of a membrane potential and redox-induced Fourier-transform infrared spectroscopy. We did not find any indications for Na⁺ translocation by the *E. coli* complex I.

Introduction

The generation of membrane potential is a basic principle of life. Membrane proteins use cations such as H⁺, Na⁺ and K⁺ to establish the gradient [1,2]. The energy-converting enzymes of the respiratory chain of mitochondria were shown to be proton pumps [3]. However, it is clearly established that some bacterial respiratory enzymes generate an Na⁺ gradient [4]. The first enzyme complex of the respiratory chain, the energy-converting NADH:ubiquinone oxidoreductase, also known as respiratory complex I, couples the transfer of electrons from NADH to ubiquinone with the translocation of ions across the membrane [5–8]. The complex comprises one FMN and up to nine iron–sulphur (Fe–S) clusters as internal redox groups. The operation sequence of these groups as well as the coupling with ion-translocation is not understood due to its complexity. The bovine mitochondrial complex consists of 46 different subunits [5], whereas the bacterial complex consists of 14 subunits [6]. Despite the difference in subunit composition, both the mitochondrial and the bacterial enzymes catalyse the same reaction. There has been some indication that the mitochondrial complex I might be involved in processes other than in energy conversion as well [9–13].

Complex I was believed to work exclusively as a proton pump. However, it has been proposed that complex I from *Klebsiella pneumoniae* and from *Escherichia coli* work as an Na⁺ pump [14,15]. It has been shown for the partially purified enzyme from *K. pneumoniae* that the stoichiometry of Na⁺ to electron is one, thus being half of the stoichiometry of the proton-translocating complex I, and that the complex is not capable of proton translocation [14–16]. The proposal that the *E. coli* complex I also acts as an Na⁺ pump [14,17,18] was derived from experiments with a strain lacking the antiporter genes *nhaA* and *nhaB* [19]. This strain is not capable of growing at elevated Na⁺ concentrations except on glycerol and fumarate [17]. Under these conditions, the expression of the *nuo*-genes coding for the complex is induced 2-fold [20]. Inverted *E. coli* membrane vesicles catalyse NADH-driven Na⁺ uptake, which is not abolished by the addition of the protonophore CCCP but by adding specific complex I inhibitors. It was also shown that the overproduced subunit NuoL mediates Na⁺ uptake after reconstitution in vesicles [21]. This transport was inhibited by the addition of EIPA [5-(N-ethyl)-N-isopropylamiloride], an inhibitor of Na⁺/H⁺ antiporters [22]. An addition of EIPA also protects labelling of the *Paracoccus denitrificans* complex I with fenpyroximate, a specific inhibitor that binds to NuoL [23]. The following summarizes the results of our approach, namely the use of a pure *E. coli* complex I preparation to determine its ability to pump Na⁺ ions by means of enzyme activity, generation of a membrane potential and redox induced FTIR (Fourier-transform infrared) spectroscopy.

Enzyme activity and inhibition

First, we measured the effect of the Na⁺ concentration on the NADH:decyl-ubiquinone reductase activity of the *E. coli* complex I, reconstituted in *E. coli* polar lipids [24]. The Na⁺ concentration varied from 25 μM to 100 mM in a buffer of pH 6.0 and was determined by atomic absorption spectroscopy. The ionic strength on the assay was kept constant by an addition of LiCl. Within the experimental error, the enzyme activity did not change with the Na⁺ concentration (Figure 1A). The activity was 95% inhibited by 10 μM piericidin A and showed the same ν_{max} with either NADH

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**Key words:** complex I, *Escherichia coli*, NADH-dehydrogenase, NADH:ubiquinone oxidoreductase, proton translocation, sodium translocation.

**Abbreviations used:** ACMA, 9-amino-6-chloro-2-methoxyacridin; ANS, 8-anilinonaphthalene-1-sulphon acid; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; DCCD, dicyclohexylcarbodi-imide; DPA, 5,5'-diethyl-N-isopropylamiloride; FTIR, fourier-transform infrared; NQR, NADH:ubiquinone reductase.

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**Figure 1** | (A) Steady state NADH:decyl-ubiquinone reductase activity of *E. coli* complex I reconstituted in *E. coli* polar lipids at various Na\(^+\) concentrations and (B) inhibition of this activity by DCCD.

The decrease in the NADH concentration was measured at 340–400 nm. The assay (A) contained 20 µg of complex I in 50 mM Mes/KOH, 100 mM KCl and 5 mM MgCl\(_2\) (pH 6.0). The Na\(^+\) concentration was varied by the addition of 50 mM Mes/NaOH, 100 mM NaCl and 5 mM MgCl\(_2\) (pH 6.0). The assay (B) contained 30 µg of complex I in 50 mM Mes/NaOH, 100 mM NaCl and 5 mM MgCl\(_2\) (pH 6.0). The wavelength used for detection were: excitation wavelengths, 380 nm (ANS) and 410 nm (ACMA), and emission wavelength, 480 nm (ANS and ACMA). The additions are indicated at the individual traces. Pic A, piericidin A.

**Figure 2** | Ion translocation induced by the redox reaction of *E. coli* complex I reconstituted in proteoliposomes

(A) Generation of a membrane gradient monitored by ANS fluorescence and (B) generation of a pH gradient monitored by ACMA fluorescence. The assay contained 20 µg of purified complex I in 5 mM Mes/KOH, 80 mM NaCl and 50 µM decyl-ubiquinone (pH 6.0). The reaction was started by the addition of 50 µM NADH indicated by the arrow. The wavelengths used for detection were: excitation wavelengths, 380 nm (ANS) and 410 nm (ACMA), and emission wavelength, 480 nm (ANS and ACMA). The additions are indicated at the individual traces. Pic A, piericidin A.

In contrast, primary sodium pumps seem to show no turnover in the absence of Na\(^+\) [25,26]. The activity of the Na\(^+\) pumping complex I from *K. pneumoniae* decreases to 50% of the \(v_{\text{max}}\) at 332 µM Na\(^+\) [15]. The enzymatic activity of the Na\(^+\)-translocating NQR (NADH:quinone reductase), an enzyme complex analogous to complex I (see below), from *Vibrio cholerae* is lowered by 50% at approx. 10 mM Na\(^+\) [26]. The independence of the NADH:decyl-ubiquinone reductase activity of the isolated *E. coli* complex I from the Na\(^+\) concentration indicates that this enzyme is not involved in primary Na\(^+\) translocation.

DCCD (dicyclohexylcarbodi-imide) is well known as an inhibitor of electron transfer and proton translocation of complex I [24,27,28]. It is thought to modify a protonable acidic amino acid located in a hydrophobic environment and to be essential for the proton pathway. If the *E. coli* complex is able to translocate Na\(^+\), it would be reasonable to assume that the affinity of DCCD depends on the Na\(^+\) concentration. In a buffer of pH 6.0, DCCD inhibited 90% of the enzyme activity with an apparent IC\(_{50}\) of 0.25 mM, independent of the Na\(^+\) concentration (Figure 1B). Amiloride derivatives like EIPA and benzamil, which have originally been described as inhibitors of Na\(^+\)/H\(^+\) antiporters [22], inhibited electron transfer of complex I [23,29]. For the isolated and reconstituted *E. coli* complex I, we determined an IC\(_{50}\) of 100 and 70 µM for EIPA and benzamil respectively [24], regardless of whether the assay contained 25 µM or 100 mM Na\(^+\). Thus these inhibitors do not act on a hypothetical Na\(^+\)-binding site in the *E. coli* complex I.

**Ion translocation and inhibition**

The ability of the isolated complex to translocate ions was measured in proteoliposomes, where the enzymes were orientated in such a way that two-thirds of the NADH-binding sites were accessible from the outside [24]. The membrane potential was measured with the fluorescent probe ANS (8-anilinonaphthalene-1-sulphonic acid). An addition of NADH to the proteoliposomes, incubated with decyl-ubiquinone and ANS, showed an enhanced fluorescence signal (Figure 2A), indicating that the reconstituted complex generated a membrane potential. This signal was sensitive to the addition of piericidin A, a specific complex I inhibitor, and to the addition of the protonophore CCCP (carbonyl cyanide m-chlorophenylhydrazone) (Figure 2A). Thus a proton gradient was measured that derived from the redox reaction of complex I. In contrast, the Na\(^+\)-specific ionophore ETH-157 had no effect on the fluorescence signal, indicating that there was no contribution to the membrane potential from a primary NADH-induced Na\(^+\) gradient (Figure 2A). Using the fluorescent probe ACMA (9-amino-6-chloro-2-methoxyacridin) in the same system, we directly measured...
proton pumping by the *E. coli* complex. Addition of NADH to proteoliposomes, incubated with decyl-ubiquinone and ACMA, showed a quenched fluorescence signal (Figure 2B). This fluorescence signal was sensitive to the addition of piericidin A and CCCP (Figure 2B). The addition of the Na+/H⁺ exchanger monensin led to a loss of the ACMA signal, indicating the absence of an Na⁺ gradient.

These experiments were conducted at pH 6.0 due to the poor stability of the preparation at more basic pH values [30–32]. However, it was questioned whether Na⁺ pumping by the *E. coli* complex I had been overlooked due to the high proton concentration. To clarify this point, the experiments were repeated in a Tris/HCl buffer at pH 7.5 (Figure 2B). Due to the instability of the isolated complex, the enzyme was prepared at pH 6.0 and the pH was shifted during reconstitution in proteoliposomes. The ACMA signal induced by the redox reaction of complex I was also detected at pH 7.5 (Figure 2B), indicating that the complex also functions as a proton pump at a lower proton concentration. As described for the measurements at pH 6.0, the signal was sensitive to piericidin A, CCCP and monensin. The amplitude of the ACMA signal at pH 7.5 was approximately four times smaller compared with the signal obtained at pH 6.0, which is partly due to the property of the dye and partly due to the decreased stability of the complex.

It has been reported that the single subunit NuoL is capable of a passive H⁺/Na⁺ antiport when reconstituted in proteoliposomes [21]. To determine whether this subunit can act as an antiporter when it is part of the fully assembled complex I, we measured the effect of Na⁺ concentration on the magnitude of the proton gradient [24]. Proteoliposomes containing complex I were loaded with 80 mM NaCl and added to an assay buffer with the same osmolarity containing 1 mM NaCl. In the absence of NADH and decyl-ubiquinone, no ACMA signal was detectable as would have been expected in the case of a passive Na⁺/H⁺ antiport mediated by complex I. Proteoliposomes that were loaded with 1 mM NaCl and added to a buffer with 80 mM NaCl also showed no effect [24]. However, subsequent addition of decyl-ubiquinone and NADH led to the rise of an ACMA signal in both assays. Remarkably, the amplitude of the ACMA signal induced by the redox reaction of the complex was unambiguously enhanced when the proteoliposomes were loaded with an Na⁺ concentration higher than the assay buffer. This effect was eliminated when the proteoliposomes were incubated with the Na⁺ ionophore ETH-157 [24]. These findings indicate that the redox reaction of the *E. coli* complex I is coupled with some type of secondary Na⁺/H⁺ antiporter.

All complex I inhibitors used in our assays simultaneously prevented proton translocation and electron transfer. This was found with piericidin A, an inhibitor binding at or close to the quinone-binding site, with DCCD, which is believed to bind covalently to acidic amino acids, and with amiloride derivatives, the action of which on complex I is unknown. These findings indicate strong coupling between the electron transport and the proton translocation in complex I. They are also an indication of indirect coupling of electron transfer and proton translocation because comparable properties were reported for the ATP synthase but not for the *bc₁* complex and cytochrome *c* oxidase [33].

**FTIR spectroscopy**

The Na⁺-pumping NQR from *V. cholerae* catalyses the same redox reaction as complex I, but the genes of these two enzymes show no detectable sequence homology [26,34]. The enzyme is made up of six subunits and contains four flavins, a [2Fe–2S] cluster and most likely a tightly bound quinone as cofactor. It has been demonstrated that its redox reaction is strictly coupled with Na⁺ translocation [35,36]. Samples of the NQR were prepared either in the presence of Na⁺ or K⁺. Both samples showed similar electron transfer activity but the K⁺ sample exhibited reduced ion translocation activity. The absence of the substrate Na⁺ affected the enzyme, which was monitored using electrochemically induced FTIR difference spectroscopy. Oxidized–minus-reduced FTIR difference spectra were obtained (Figure 3A). The difference spectra are dominated by vibrational modes of the flavins and also include reorganizations of the backbone. The spectra of the Na⁺ samples revealed Na⁺-dependent signals. These shifts mainly occur in the amide I range, between 1690 and 1620 cm⁻¹. Shifts in the vibrational modes of carboxylate groups might represent CO₂⁻/Na⁺ vibrations [37]. The same experiment was performed with the *E. coli* complex I. The enzyme was prepared in the presence of Na⁺ ions and a part of the preparation was transferred to a K⁺ buffer by repeated dilution and concentration. The residual Na⁺ concentration of the sample was <100 µM. Redox-induced FTIR spectra were obtained from both aliquots (Figure 3B). The comparison revealed only minor variations between both the
difference spectra, mainly in the range between 1500 and 1400 cm$^{-1}$ where CO$_2$-Na$^+$ signals were expected. The small effect of the variation of the Na$^+$ concentration on the difference spectra and the comparison with the difference spectra of NQR, a primary Na$^+$ pump, revealed that there are no clear perturbations of complex I induced by Na$^+$ ions, although a general interaction of complex I with Na$^+$ ions cannot be excluded.

**Conclusion**

It was proposed that the redox reaction of the respiratory complex I from *K. pneumoniae* and *E. coli* is coupled with the translocation of Na$^+$ across the membrane [38]. However, earlier measurements with whole cells indicated that the *E. coli* complex I is a proton pump with a stoichiometry of at least 1.5 H$^+$/e$^-$ [39–41]. These results are supported by our experiments, namely by the independence of the enzymatic activity and the IC$_{50}$ of DCCD from the Na$^+$ concentration, the direct measurement of a proton gradient with the complex reconstituted in protoliposomes and the lack of any indication of primary Na$^+$ translocation in these experiments. Finally, we found no spectroscopic evidence of Na$^+$ binding due to the redox reaction of complex I, which was detected with the Na$^+$-translocating NQR.

The homomeric of the membranous complex I subunits NuoL, NuoM and NuoN to cation/proton antiporter [42–44] suggests that the complex might either be involved in primary proton translocation or capable of secondary Na$^+$/H$^+$ antiport. While we found no indication for the first assumption, the increase in the amplitude of the ACMA signal in the presence of a higher Na$^+$ concentration inside the pro-membrane, revealed that there are no clear perturbations of complex I induced by Na$^+$ ions, but is not capable of translocating protons [16].

The proposal that complex I from *K. pneumoniae* pumps Na$^+$, but is not capable of translocating protons [16], has been recently questioned [45]. The genome of *K. pneumoniae* does not only code for complex I and the non-energy converting NADH dehydrogenase but also for a Na$^+$-translocating NQR-type enzyme described above. By means of gene disruption, it was shown that complex I contributes to a proton gradient, while the reaction of the NQR is coupled with the generation of an Na$^+$ gradient [45]. The reason for this discrepancy is not yet understood.

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**References**


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