

Amiloride inhibition of the proton-translocating NADH-quinone oxidoreductase of mammals and bacteria

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Abstract The proton-translocating NADH-quinone oxidoreductase in mitochondria (complex I) and bacteria (NDH-1) was shown to be inhibited by amiloride derivatives that are known as specific inhibitors for Na^+/H^+ exchangers. In bovine submitochondrial particles, the effective concentrations were about the same as those for the Na^+/H^+ exchangers, whereas in bacterial membranes the inhibitory potencies were lower. These results together with our earlier observation that the amiloride analogues prevent labeling of the ND5 subunit of complex I with a fenpyroximate analogue suggest the involvement of ND5 in H^+ (Na^+) translocation and no direct involvement of electron carriers in H^+ (Na^+) translocation.
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Key words: NADH dehydrogenase; Amiloride derivative

1. Introduction

The proton-translocating NADH-quinone oxidoreductase in mitochondria and bacteria is a component of the oxidative phosphorylation system and catalyzes electron transport from NADH to quinone that is coupled to proton translocation [1–3]. The mitochondrial enzyme, also known as complex I, is located in the inner mitochondrial membrane and composed of 46 different subunits [4]. The bacterial enzyme, NDH-1, is in the cytoplasmic membrane and contains 14 subunits [5]. Despite the difference in the size, complex I and NDH-1 share many properties in terms of structure and function. Both enzymes consist of two major domains [6–8]. One is a peripheral domain that protrudes into the mitochondrial matrix (or the cytoplasm) and bears prosthetic groups such as FMN (flavin mononucleotide) and iron–sulfur centers. The other is a membrane domain bearing hydrophobic subunits. Recent studies suggest that the two domains have distinct functions, the peripheral domain is a cofactor-binding segment and the mem-

brane domain is a proton pumping unit [3,9,10]. It has also been demonstrated that the NDH-1 in *Escherichia coli* and *Klebsiella pneumoniae* can pump Na^+ [11,12]. In fact, some of the subunits in the membrane domain were found to have high sequence similarity to certain Na^+/H^+ antiporters which are considered to house no cofactors [13,14].

We have recently shown that, in bovine complex I, one of the membrane domain subunits, ND5, is labeled by a photo-affinity analogue of a potent inhibitor of complex I, fenpyroximate (TDF), and that this labeling is almost completely prevented by addition of amiloride derivative such as 5-(*N*-methyl-*N*-isobutyl)amiloride (MIA) and 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA) [15]. This is interesting because the amiloride derivatives employed have been known to be specific inhibitors for Na^+/H^+ antiporters [16–18]. In this paper, we report the inhibitory effect of amiloride derivatives on the activities of complex I/NDH-1 from different sources.

2. Materials and methods

Bovine heart mitochondria were generously provided by Dr. C.-A. Yu (Oklahoma State University, OK, USA). Submitochondrial particles (SMP) were prepared as described in [19]. EIPA, MIA and benzamil were from Sigma. All other chemicals were of the highest grade.

An *E. coli* mutant lacking NDH-2 was constructed as follows. The *ndh* gene which encodes NDH-2 was cloned by polymerase chain reaction (PCR) together with a ~900 bp DNA segment upstream and a ~400 bp DNA segment downstream with genomic DNA purified from *E. coli* DH5 α according to [20]. We used a sense primer, CACGAGAAAGGGATCCAATTGCAGTTTATTGACCCGG (underlined bases were altered from *E. coli* DNA and the italicized bases represent the restriction sequence) in order to generate a *Bam*HI site and an anti-sense primer, CCTGCACCGGTGACGATGGGTCACTGTGACG containing the naturally occurred *Sal*I site. The spectinomycin cassette encoding gene from the *Staphylococcus aureus* transposon Tn554 [21] was cloned by using the sense primer, CGGGTCCCATGGTCAGTGGAACGAAACTCACG and the anti-sense primer, AAGGACCATGGTCTTTCTATTTC AATAGT-TAC, both containing a *Nco*I restriction site represented by italicized bases. The DNA fragment containing the *ndh* gene together with the spectinomycin cassette was assembled in pPCR-Script (Stratagene) by using the *Nco*I site naturally occurred in the middle of the *ndh* gene and finally the 2.7 kb *Bam*HI–*Nco*I–*Sal*I fragment was transferred into pKO3, which was kindly provided by Dr. G.M. Church (Harvard Medical School, MA, USA), a gene replacement vector that contains a temperature-sensitive origin of replication and markers for positive and negative selection for chromosomal integration and excision. The resulting construct was designated pKO3(*ndh*-spc). The *E. coli* strain MC4100 was transformed with the pKO3(*ndh*-spc) plasmid, and homologous recombination was carried out as described in [22]. The disruption of the *ndh* gene was verified by PCR and DNA sequencing.

Cholate-treated *Paracoccus denitrificans* membranes, *E. coli* and

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Abbreviations: Q₁, ubiquinone-1; NDH-1, bacterial proton-translocating NADH-quinone oxidoreductase; NDH-2, NADH-quinone oxidoreductase lacking energy coupling; complex I, mitochondrial proton-translocating NADH-quinone oxidoreductase; SMP, submitochondrial particles; EIPA, 5-(*N*-ethyl-*N*-isopropyl) amiloride; MIA, 5-(*N*-methyl-*N*-isobutyl)amiloride; TDF, (trifluoromethyl)phenyldiaziriny fenpyroximate; PCR, polymerase chain reaction

Bacillus subtilis membranes were prepared according to the method reported previously [7].

SMP (10–30 $\mu\text{g ml}^{-1}$) or bacterial membranes (20–140 $\mu\text{g ml}^{-1}$) were incubated in the presence of an amiloride derivative for 5 min at 30°C in a buffer containing 50 mM sodium phosphate (pH 7.4) and 1 mM EDTA. Activities were measured at 30°C in the same buffer as a decrease of absorbance at 340 nm ($\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) with 150 μM NADH (NADH oxidase) or, with 150 μM NADH and 100 μM ubiquinone-1 (Q_1) in the presence of 2 mM KCN and 0.2 μM antimycin A (NADH-quinone reductase). The NADH- $\text{K}_3\text{Fe}(\text{CN})_6$ reductase activity was determined from an absorbance decrease at 420 nm ($\epsilon_{420} = 1.0 \text{ mM}^{-1} \text{ cm}^{-1}$) with 150 μM NADH, 1 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 2 mM KCN and 0.2 μM antimycin A.

Protein concentration was estimated by the method of Lowry et al. [23]. Any variations from the procedures and details are described in the figure legends.

3. Results

As mentioned above, the ND5 subunit of complex I in bovine heart SMP can be labeled by a photoaffinity analogue of TDF and the labeling was almost completely prevented by the presence of amiloride analogues as well as other complex I inhibitors [15]. Since amiloride derivatives have been known as specific inhibitors for Na^+ -related transporters such as Na^+/H^+ exchangers and Na^+/K^+ ATPase [16,18,24], we investigated the effect of these inhibitors on complex I activities. Incubation of SMP with an amiloride derivative, MIA, resulted in inhibition of the NADH oxidase activity as shown in Fig. 1. The concentration of half maximal inhibition (IC_{50}) for MIA was approximately 5 μM . The inhibition profile remained the same regardless of the SMP concentration used. Addition of NaCl at 50 mM did not affect the degree of inhibition. Two other amiloride derivatives tested, benzamil and EIPA, both inhibited the NADH oxidation in a similar fashion. Benzamil and EIPA were slightly less effective than MIA both with an IC_{50} value of 17 μM (Fig. 1 and Table 1). In the case of the Na^+/H^+ exchangers, inhibitory concentrations reported for MIA, benzamil and EIPA vary from low (less than micromolar) to high (hundreds of micromolar) depending on the types and sources [17,25,26]. The effective concentrations of the amiloride inhibition of complex I are well within the reported range for inhibition of the Na^+/H^+ exchangers. The greater potency of MIA over EIPA agrees with our previous result of suppression of the TDF labeling of the ND5 subunit in which MIA was shown to be more effective than EIPA [15]. The amiloride derivatives also inhibited NADH-quinone reductase activity but did not affect NADH- $\text{K}_3\text{Fe}(\text{CN})_6$ reductase activity or succinate oxidase activity in bovine SMP at up

Table 1
 IC_{50} values of amiloride analogues for NADH oxidase activity

Membrane	IC_{50} for NADH oxidase activity (μM)		
	MIA	Benzamil	EIPA
Bovine SMP ^a	5	17	17
<i>P. denitrificans</i>	20	42	63
<i>E. coli</i> (NDH-1) ^b	45	45	> 100
<i>E. coli</i> (NDH-2) ^c	$\geq 100^d$	$\geq 100^d$	no inhibition
<i>B. subtilis</i> (NDH-2)	~ 100	~ 100	$\geq 100^d$

^aThere were slight variations in the IC_{50} values among preparations. However, bovine SMP reproducibly showed lower values than bacterial membranes.

^bAn *E. coli* mutant lacking NDH-2 was used.

^cActivity was measured with wild-type *E. coli* under the conditions in which >95% of the activity of NDH-1 is inhibited in the presence of 2.5 μM piericidin A.

^dInhibition was 30% or less at 100 μM of the inhibitor.

to 100 μM (data not shown). Therefore, it is likely that the inhibition site is located in the membrane domain of complex I, consistent with the result of suppression of ND5 labeling with TDF [15].

Complex I inhibitors that are known to act downstream of iron-sulfur centers may be grouped into two types on the basis of kinetic behavior against quinone. One group of inhibitors including piericidin A displays competitive inhibition with respect to quinone and most likely share a common binding site with quinone. The other group shows non-competitive inhibition and their binding site may not overlap with the quinone binding site. We have examined the competition mode of MIA for NADH- Q_1 reductase activity in bovine SMP. As shown in Fig. 2, MIA worked as a non-competitive inhibitor against quinone. These results may indicate that the amiloride inhibitors do not affect the apparent K_m values for quinone, suggesting that they do not bind to the quinone-binding site but act somewhere between quinone and center N2 (of the highest E_m value of electron carriers in complex I). Interestingly, Gong et al. [27] reported in a recent paper that binding of a photoaffinity analogue of quinone to the *E. coli* NDH-1 was not influenced by addition of complex I inhibitors.

Amiloride and its derivatives are known to inhibit a wide variety of Na^+/H^+ exchangers from different sources such as bacteria and yeast in addition to mammals. The inhibitory action and potencies differ depending on the species. For example, amiloride is a potent inhibitor for an Na^+/H^+ antiporter (NhaA) in the marine bacterium *Vibrio parahaemolyticus* but displays no significant inhibitory effect on the *E. coli*

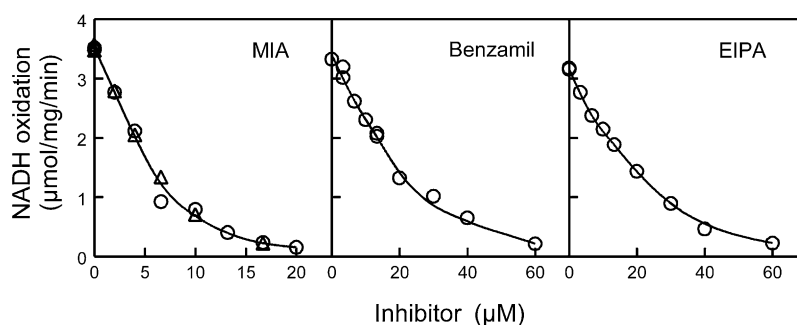


Fig. 1. Inhibition of NADH oxidase activity by amiloride derivatives in bovine SMP. SMP were incubated with the indicated concentrations of MIA (left), benzamil (center) or EIPA (right) for 5 min at 30°C and then assayed for NADH oxidase activity as detailed in Section 2. The SMP concentration was either 0.22 mg ml^{-1} (circles) or 0.11 mg ml^{-1} (triangles).

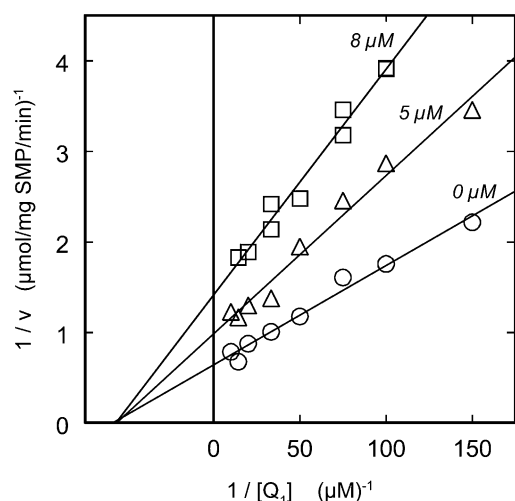


Fig. 2. Double reciprocal plots showing a non-competitive inhibition of NADH-quinone reductase activity by MIA against quinone in bovine SMP. SMP were incubated with 0 μM (circles), 5 μM (triangles), or 8 μM (squares) of MIA for 5 min at 30°C and then assayed for NADH-quinone reductase activity with 150 μM NADH and varied concentrations of Q_1 .

NhaA [28,29]. Steuber's group demonstrated that *E. coli* and *K. pneumoniae* NDH-1 can pump Na^+ [11,30]. In addition, it has been reported that the expressed *E. coli* NuoL (ND5 homologue) subunit can transport Na^+ and this Na^+ transport is suppressed by EIPA [31]. Therefore, it is of interest to see if the amiloride derivatives inhibit energy-transducing activity of bacterial NDH-1. We have examined their effect on the NADH oxidase activity of several bacterial membranes (Table 1). All compounds inhibited the bacterial membranes but with different potencies. In the case of *P. denitrificans* membranes, MIA inhibited the NADH oxidase activity with an IC_{50} of 20 μM and displayed >90% inhibition at 50 μM . Succinate oxidation was not affected by MIA at least up to 40 μM (data not shown). The IC_{50} values of benzamil and EIPA for *P. denitrificans* membranes were also three to four times higher than those for bovine SMP. In contrast to *P. denitrificans* which contains the NDH-1 as the sole NADH dehydrogenase [32], *E. coli* has rotenone-insensitive NADH dehydrogenase (NDH-2) in addition to NDH-1. To measure the activity of NDH-1 in *E. coli*, we constructed a mutant lacking NDH-2 and used its membrane preparations. When compared to *P. denitrificans*, the IC_{50} value for benzamil in the *E. coli* NDH-1 was about the same but those for MIA and EIPA were twice as high. Also shown in Table 1 is the effect of amiloride derivatives on the NDH-2 enzyme. The inhibition of NDH-2 was assessed in two ways. One is to use the membrane from wild-type *E. coli* which has both NDH-1 and NDH-2. The activity of NDH-2 can be measured by addition of piericidin A which selectively inhibits NDH-1. Another source of NDH-2 is *B. subtilis* because it does not harbor NDH-1. The NDH-2 enzyme from either bacterium was much less sensitive to all compounds tested. This was most notable with EIPA which, at 100 μM , did not inhibit the *E. coli* NDH-2 and exhibited only 30% inhibition with *B. subtilis*. The difference in the inhibitory potencies between NDH-1 and NDH-2 and the species specificity observed for amiloride analogues are similar to those reported for common complex I inhibitors. *P. denitrificans* NDH-1 tends to show inhib-

itor sensitivities that are close to those of mitochondrial complex I whereas *E. coli* NDH-1 is in general significantly less sensitive to complex I inhibitors [33–35]. It should also be noted that complex I inhibitors such as rotenone and piericidin A partially inhibit NDH-2 but at much higher concentrations [33].

4. Discussion

The mechanism of energy coupling of complex I/NDH-1 is still speculative. Two types of coupling mechanism have been proposed [3]. One is direct coupling in which electron carriers such as quinones are involved in H^+ translocation and the other is indirect coupling which assumes energy transduction driven through dynamic conformation changes of the enzyme. Most recent reports on this subject seem to be in favor of the indirect coupling mechanism. First, complex I/NDH-1 consists of two distinct domains and all prosthetic groups are present in the peripheral domain and none is found in the membrane domain where the H^+ translocation takes place. Second, it was recently demonstrated that complex I/NDH-1 from *E. coli* and *K. pneumoniae* are capable of pumping Na^+ [11,12]; the observation that does not reconcile with the direct coupling mechanism. In fact, high sequence similarity has been reported between the ND5 subunit of complex I and the MnhA subunit of Na^+/H^+ antiporters from various bacterial sources [13,14]. If H^+ pumping and Na^+ pumping share a common mechanism, it is highly likely that the ND5 subunit constitutes a key member in the Na^+/H^+ translocation machinery. In agreement with this notion, our earlier results of the TDF labeling of the ND5 subunit indicated that this subunit is part of the proposed inhibitor-binding pocket of complex I that encompasses several subunits [15,36]. Furthermore, we showed that TDF binding to the ND5 subunit was suppressed by amiloride analogues and that the amiloride derivatives indeed inhibit energy-coupled activities of complex I and effective concentrations are similar to those for the Na^+/H^+ exchangers. The precise location of the amiloride binding site is still not clear. Kinetic data suggest that it is not the same as the quinone binding site [27]. Recently, Steuber reported that the truncated NuoL (ND5) subunit of *E. coli* NDH-1 reconstituted in proteoliposomes can transport Na^+ [31]. Interestingly, this Na^+ uptake was diminished by EIPA. This observation is in perfect agreement with our results that amiloride analogues, including EIPA, inhibit NADH oxidase activity of the *E. coli* NDH-1. Taken all available data together, we can envision that complex I/NDH-1 may pump H^+ (Na^+) by conformational change which is inhibited by amiloride derivatives and that the ND5 subunit plays an important role in H^+ (Na^+) translocation function of complex I/NDH-1.

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