The Escherichia coli NADH:Ubiquinone Oxidoreductase (Complex I) Is a Primary Proton Pump but May Be Capable of Secondary Sodium Antiport*  

Stefan Stolpe and Thorsten Friedrich†  
From the Institut für Organische Chemie und Biochemie, Albert-Ludwigs-Universität Freiburg, Albertstrasse 21, D-79104 Freiburg, Germany

The NADH:ubiquinone oxidoreductase (complex I) couples the transfer of electrons from NADH to ubiquinone with the translocation of protons across the membrane. Recently, it was demonstrated that complex I from Klebsiella pneumoniae translocates sodium ions instead of protons. Experimental evidence suggested that complex I from the close relative Escherichia coli works as a primary sodium pump as well. However, data obtained with whole cells showed the presence of an NADH-induced electrochemical proton gradient. In addition, Fourier transform IR spectroscopy demonstrated that the redox reaction of the E. coli complex I is coupled to a protonation of amino acids. To resolve this contradiction we measured the properties of isolated E. coli complex I reconstituted in phospholipids. We found that the NADH:ubiquinone oxidoreductase activity did not depend on the sodium concentration. The redox reaction of the complex in proteoliposomes caused a membrane potential due to an electrochemical proton gradient as measured with fluorescent probes. The signals were sensitive to the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP), the inhibitors piericidin A, dicyclohexylcarbodi-imide (DCCD), and amiloride derivatives, but were insensitive to the sodium ionophore ETII-157. Furthermore, monensin acting as a Na+/H+ exchanger prevented the generation of a proton gradient. Thus, our data demonstrated that the E. coli complex I is a primary electrogenic proton pump. However, the magnitude of the pH gradient depended on the sodium concentration. The capability of complex I for secondary Na+/H+ antiport is discussed.

The NADH:ubiquinone oxidoreductase, also known as respiratory complex I, couples the transfer of electrons from NADH to ubiquinone with the translocation of protons across the membrane (1–3). The bacterial complex consists, in general, of 18 different subunits named NuoA to NuoN. Seven peripheral subunits bear all known redox groups of the complex, namely one FMN and up to nine iron-sulfur (Fe/S) clusters. The remaining seven subunits are mostly hydrophobic proteins predicted to fold into 54 α-helices across the membrane (4). These hydrophobic subunits do not contain a conserved sequence motif for the binding of a cofactor, nor has a cofactor been identified in these subunits as yet. However, because of their location within the membrane, they should be involved in proton translocation. The 14 subunits present in bacteria are called the minimal subunits because their homologues are found in the complex I of all species investigated as yet (2, 4, 5). They comprise the minimal structural and functional framework for redox-driven proton translocation in complex I. In addition to the homologues of the 14 minimal subunits the mitochondrial complex I of eukaryotes contains at least 32 extra proteins but no additional redox groups (6). It has been shown that both the bacterial and the mitochondrial complex I have a proton to electron stoichiometry of 4H+/2e− (7–11).

Until recently it was believed that complex I works exclusively as a proton pump. It has been elegantly demonstrated that the Klebsiella pneumoniae complex I acts as a Na+/H+ pump, with a stoichiometry of 2Na+/2e− (12–14). It was also shown that the K. pneumoniae complex I is not capable of proton translocation (14). Recent experimental evidence disclosed that complex I from Escherichia coli, a close relative of K. pneumoniae, acts as a sodium pump as well (15, 16). This proposal is based on experiments with the E. coli strain EP432 lacking the antiporter genes nhaA and nhaB, which leads to an inability to grow under elevated Na+ concentrations (17). However, growth on glycerol and fumarate enabled growth of strain EP432 at about 450 mM NaCl (15). Under these conditions, the expression of the nua genes coding for the subunits of complex I is 2-fold increased (18, 19). Inverted membrane vesicles catalyzed NADH-driven Na+ uptake, which was not cancelled by addition of the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP, Sigma) but by the addition of rotenone, a specific complex I inhibitor (15). It was also shown that the overproduced subunit NuoL of the E. coli complex I mediates Na+ uptake when reconstituted into proteoliposomes (20). This transport was inhibited by the addition of EIPA, an inhibitor of Na+/H+ antiporters (21).

However, data obtained with E. coli wild-type cells and membrane vesicles indicated the presence of an NADH-induced proton gradient (10, 22, 23). In addition, FT-IR spectroscopy has shown that the redox reaction of the E. coli complex I is associated with the protonation of tyrosines and acidic amino acids (24, 25). As activity measured in whole cells and membrane vesicles are sometimes hard to assign, we reconstituted the isolated E. coli complex I in proteoliposomes, measured the effect of Na+ ions on enzyme activity, and determined the type

* This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 49-(0)761-203-6096; Fax: 49-(0)761-203-6096; E-mail: tfriedri@uni-freiburg.de.

The abbreviations used are: complex I, proton-pumping NADH:ubiquinone oxidoreductase; NQR, Na+-translocating NADH:quinone oxidoreductase; MES, 2-(N-morpholino)-ethanesulfonic acid; deetyl-ubiquinone, 2,3-dimethoxy-5-methyl-6-decylbenzoquinone; ACMA, 9-amino-6-chloro-2-methoxyacridin; ANS, 8-anilino-1-naphthalinesulfonic acid; CCCP, carbonylcyanide-m-chlorophenylhydrazone; DCCD, dicyclohexylcarbodi-imide; EIPA, 5-(N-ethyl-N-isopropyl)-amiloride; TMA, tetramethylammonium chloride.
of cation responsible for the electrochemical gradient generated by the redox reaction of complex I. Our data showed that the E. coli complex I is a primary proton pump but may be capable of secondary H+/Na+ antiport.

MATERIALS AND METHODS

Isolation of Complex I—E. coli complex I was isolated from the overproducing strain ANN003/pAR1219 similar to the procedure described (26) but treatment of the membranes with NaBr was omitted. All steps were carried out at 4°C. 50 g of cells were resuspended in the 5-fold volume 50 mM MES/NaOH, 0.1 mM phenylmethylsulfonyl fluoride, pH 6.0, with 10 μg/mL DNase I and 50 μg/mL lysozyme and disrupted by a single pass through a French Pressure cell (SLM Analytic). Complex I was purified by centrifugation at 360,000 × g, and cytoplasmic membranes were obtained by ultracentrifugation at 250,000 × g for 1 h. The membranes were resuspended in 50 mM MES/NaOH, 50 mM NaCl, 5 mM MgCl₂, pH 6.0 at a concentration of 80 mg/mL. Dodecyl maltoside (Glycon) was added to a final concentration of 3%, and the solution was gently homogenized and centrifuged for 20 min at 250,000 × g. The supernatant was applied to an HPQ-Sepharose 150 (Amersham Biosciences) column equilibrated in 50 mM MES/NaOH, 50 mM NaCl and 0.1% dodecyl maltoside, pH 6.0. The column was eluted with a 700-ml linear gradient of 50 mM MES/NaOH, 50 mM NaCl, 5 mM MgCl₂, and 0.1% dodecyl maltoside, pH 6.0 at a flow rate of 5 mL/min. Fractions containing NADH/ferricyanide reductase activity were combined, concentrated by precipitation with 9% (w/v) final concentration of 50–350 mM NaCl in 50 mM MES/NaOH, 50 mM NaCl and 0.1% dodecyl maltoside, pH 6.0 at a flow rate of 5 mL/min. Fractions containing NADH/ferricyanide reductase activity were pooled and stored at −80°C.

Reconstitution of Complex I—To determine the most suitable lipid for reconstitution experiments we examined the NADH:decyl-ubiquinone reductase activity of complex I in the presence of soybean L-α-phosphatidylcholine (Sigma), egg yolk L-α-phosphatidylcholine (Lipoid), E. coli polar lipid extract (Avanti), and total lipid extract (Avanti). Lipids (50 mg/ml) were dissolved in 50 mM MES/NaOH, 50 mM NaCl, 0.1% (w/v) dodecyl maltoside, pH 6.0 by sonication in five intervals of 20 s at 80% output level with a microtip (Branson sonifier). The suspensions were diluted to 3 mg/mL in the same buffer and either directly added to the assay or mixed in a 1:1 (w/v) ratio with complex I (1 mg/mL) and incubated for 20 min on ice before addition to the assay. Complex I was reconstituted in proteoliposomes by the BioBeads (Bio-Rad) method (27). 80 mg of E. coli polar lipid extract (Avanti) were suspended in 4 ml of 50 mM MES/NaOH, 50 mM NaCl, pH 6.0, and 800 μl of 20% (w/v) dodecyl maltoside were added. The lipids were dissolved by sonication of the suspension in five intervals of 20 s at 80% output level with a microtip (Branson sonifier). Complex I (2 mg/mL) and the lipid solution were mixed in a 1:4 (w/w) ratio. BioBeads with a capacity of absorbing 105 mg of dodecyl maltoside per g of beads (28) were added. The mixture was stirred for 3 h at 4°C and centrifuged for 30 min at 150,000 × g (Beckmann Airfuge) after separation of the beads. The sediment was resuspended in 50 mM MES/NaOH, 100 mM NaCl, pH 6.0. Various Na+ concentrations in the assay were obtained by dialyzing complex I overnight against the desired buffer at 4°C. In these cases the lipids and the proteoliposomes were resuspended in the corresponding buffer.

Determination of Complex I Activity—The NADH:decyl-ubiquinone oxidoreductase activity of complex I was measured with a Perkin Elmer 156 dual-wavelength spectrophotometer at 340 and 400 nm using an ε of 0.3 mM⁻¹ cm⁻¹ in a final volume of 1 ml (29). To obtain various Na+ concentrations in the assay, the E. coli polar lipid extract (30 mg/mL) was suspended in the desired buffer containing 0.1% (w/v) dodecyl maltoside. The enzyme (20 mg/mL) was dialyzed for 16 h against 50 mM MES/LiOH, 100 mM LiCl, pH 6.0 at 4°C, mixed in 1:1 (v/v) ratio with the lipid and incubated for 20 min on ice. 3 μl of this mixture were incubated for 5 min at room temperature in the assay buffer (50 mM MES/LiOH, 100 mM LiCl, pH 6.0). The Na⁺ concentration of the buffer was varied from 25 μM to 100 mM by adding the corresponding amount of 50 mM MES/LiOH, 100 mM NaCl, pH 6.0. 50 μM decyl-ubiquinone was added to the assay, and the reaction was started by addition of 50 μM NADH (potassium salt, Sigma). The sodium content of the assay buffer was determined by Christine Höwer, Institut für Mineralogie, Petrologie und Geochemie, Albert-Ludwigs-Universität Freiburg by atomic absorption spectroscopy.

For inhibitor titrations of the isolated and reconstituted E. coli complex I an ethanol solution of 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) and an aqueous solution of benzamid (both from Sigma) was added to the assay. The samples were preincubated for 5 min at room temperature with inhibitors in the enzyme assay. Measurements were performed either in 50 mM MES/NaOH, 100 mM NaCl, pH 6.0 or 50 mM MES/KOH, 100 mM KCl, pH 6.0. The final ethanol concentration was kept constant in each enzyme assay and did not exceed 2%.

Determination of a Membrane Potential (Δψ)—The generation of Δψ by complex I was monitored by 8-anilino-1-naphthalene-sulfonic acid (ANS, Sigma; Ref. 30) at various NaCl concentrations. The ′transient′ nature of the assay buffer was kept constant by adding the corresponding amount of tetramethylammonium chloride (TMA). The respective ion concentrations are given in the figure legends. 3 μl of proteoliposomes, 1 μM ANS, and 50 μM decyl-ubiquinone were added to the assay buffer (5 mM MES/KOH, 80 mM NaCl, pH 6.0) and preincubated for 5 min at 25°C. The reaction was started by addition of 50 μM NADH (Gerbu) in a final volume of 1 ml. The increase of ANS fluorescence caused by the generation of Δψ was followed with an SFM 25 spectrofluorometer (Kontron), using an excitation wavelength of 390 nm and an emission wavelength of 480 nm. When desired, the proton ionophore CCCP, the Na⁺–specific ionophore N,N,N-dibenzyl-N,N,N-triethyl-2-phenylenediacetate (ETH-157, Fluka; Ref. 31), and the specific complex I inhibitor piericidin A were added to a final concentration of 10 μM, respectively.

Effect of Dicyclohexylcarbodi-imide (DCCD)—The effect of DCCD on the NADH:decyl-ubiquinone oxidoreductase activity of complex I and its ability to generate a membrane potential and a proton gradient was measured as described above. Complex I reconstituted in phospholipids was incubated for 5 min at 4°C with various amounts from a 0.1 mM DCCD (Sigma) stock solution in ethanol. The final ethanol concentration was kept constant and did not exceed 1% (v/v). After incubation the enzyme was immediately used for NADH:decyl-ubiquinone oxidoreductase activity measurement in 50 mM MES/NaOH, 100 mM NaCl, pH 6.0. Addition of 10 μM CCCP had no effect on the measurement. Complex I reconstituted into proteoliposomes was incubated for 10 min with 0.3 mM DCCD final concentration at room temperature and immediately used for measurements in 5 mM MES/KOH, 80 mM NaCl, pH 6.0. Prolongation of the incubation time had no effect.

RESULTS

Purification of Complex I—Our standard purification method was modified to circumvent the washing of the membranes with NaBr, which led to an incubation of complex I with 1.4 M Na⁺ (26). Complex I was extracted from the membranes with 3% dodecyl maltoside and eluted by anion exchange chromatography on Source 15Q at 280 mM NaCl. The final size-exclusion chromatography on Sepharyl S-300 showed a homogenous peak coeluting with the complex I activity (Fig. 1). Residual traces of ATP synthase were present in the late fractions of the peak. These fractions were discarded. The molecu-
lar mass of the preparation was estimated by comparison of the elution volume with standards of known molecular mass to 600,000 Da. Upon SDS/PAGE the preparation was resolved into 12 subunits corresponding to the proteins encoded by the \( \text{nuo} \) operon (Fig. 1; Refs. 26, 35, and 36). The subunits NuoE and D were not separated by SDS-PAGE as reported previously (26, 35). Approximately 25 mg of complex I were obtained from 55 g of cells (Table I).

**Effect of Phospholipids on Enzymatic Activity**—To choose a convenient lipid for the reconstitution of the \( \text{E. coli} \) complex I, the effect of soybean \( \text{l}-\alpha\)-phosphatidylylcholine, egg yolk \( \text{l}-\alpha\)-phosphatidylcholine, and \( \text{E. coli} \) polar and total lipid extracts on the NADH:decyl-ubiquinone reductase activity was examined (Table II). Addition of soybean \( \text{l}-\alpha\)-phosphatidylcholine and \( \text{E. coli} \) polar lipid extract to the enzyme assay doubled the enzymatic activity, whereas addition of the other lipids had only a minor effect. However, the NADH:decyl-ubiquinone oxidoreductase activity, whereas addition of the other lipids had no contribution to the membrane potential from a primary reaction of complex I as the signal was completely cancelled by addition of piericidin A, a specific complex I inhibitor (Fig. 3). Addition of an equal volume of ethanol had no effect on the ANS fluorescence signal. The ANS fluorescence signal was completely sensitive to the membrane potential arising from the \( \text{NADH}:\text{decyl-ubiquinone oxidoreductase activity} \) of complex I reconstituted in proteoliposomes was monitored with ANS in 50 mM MES/NaOH, pH 6.0, and 80 mM NaCl. Addition of NADH to the proteoliposomes incubated with decyl-ubiquinone and ANS showed a 90% enhanced fluorescence signal (Fig. 3). Thus, complex I reconstituted in proteoliposomes generated a membrane potential. This membrane potential was due to the redox reaction of complex I as the signal was completely cancelled by addition of piericidin A, a specific complex I inhibitor (Fig. 3).

**Detection of \( \Delta \Psi \)—**The membrane potential arising from the NADH:decyl-ubiquinone oxidoreductase activity of complex I reconstituted in proteoliposomes was monitored with ANS in 50 mM MES/NaOH, pH 6.0, and 80 mM NaCl. Addition of NADH to the proteoliposomes incubated with decyl-ubiquinone and ANS showed a 90% enhanced fluorescence signal (Fig. 3). Thus, complex I reconstituted in proteoliposomes generated a membrane potential. This membrane potential was due to the redox reaction of complex I as the signal was completely cancelled by addition of piericidin A, a specific complex I inhibitor (Fig. 3). Addition of an equal volume of ethanol had no effect on the signal. The ANS fluorescence signal was completely sensitive to the membrane potential generated by the \( \text{E. coli} \) complex I to the protonophore CCCP indicating that the membrane potential is established as an electrochemical proton gradient (Fig. 3). 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 (Fig. 3).

**Determination of \( \Delta \rho H \)—**The sensitivity of the membrane potential generated by the \( \text{E. coli} \) complex I to the protonophore CCCP indicated the presence of a proton gradient. We determined the presence of a pH gradient directly by using the fluorophore ACMA. The specificity of ACMA to protons was determined by addition of either HCl or NaCl to liposomes without enzyme. While HCl caused a 70% rise in fluorescence, NaCl had no effect on the fluorescent signal (data not shown). Addition of NADH to the proteoliposomes incubated with decyl-ubiquinone and ACMA showed a 40% quenched fluorescence signal (Fig. 4). The ACMA fluorescence signal was completely sensitive both to the preincubation and the addition of the protonophore CCCP demonstrating that it was generated by a proton gradient (Fig. 4). The ACMA signal was also sensitive to the Na\(^+\)/H\(^+\) exchanger monensin again showing the presence of a proton gradient. The ACMA signal was completely cancelled by adding piericidin A. Addition of an equal volume

![Fig. 1. Isolation of \( \text{E. coli} \) complex I from strain ANN003/pAR1219. A, chromatography on Source 15Q; B, chromatography on Sephacyr S-300; absorbance at 280 nm (—); NADH/ferricyanide reductase activity (●); NaCl gradient (—). C, SDS/PAGE of the indicated fractions from size exclusion chromatography.](http://www.jbc.org/content/1839/25/18379)
ethanol had no effect on the signal. This demonstrated that the E. coli complex I is a primary proton pump.

**Effect of DCCD on E. coli Complex I**—It is well known that DCCD simultaneously inhibits electron transfer and proton translocation of the mitochondrial complex I (38, 39). To examine whether the same holds true for the bacterial complex I, we titrated the NADH:decyl-ubiquinone reductase activity of the isolated complex I with DCCD (Fig. 5). DCCD inhibited 90% of the enzyme activity with an apparent IC₅₀ of 0.25 mM, which was comparable to the value reported for the mitochondrial enzyme (38, 39). Incubation of complex I proteoliposomes with DCCD completely prohibited the generation of a membrane potential (Fig. 5).

**Effect of Sodium Concentration on the ∆pH**—It has been reported that the overproduced and isolated subunit NuoL facilitates Na⁺ transport across the membrane (20, 40). Devoid of the residual complex I subunits, NuoL is capable of mediating a passive Na⁺ uptake along a concentration gradient, which coincides with an increased proton permeability of the vesicles. This is interpreted in terms of an H⁺/Na⁺ antiport (20). We therefore addressed the question, whether this is an intrinsic property of NuoL assembled in the complex I as well.

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**Proton Pumping of E. coli Complex I**

**TABLE I**

<table>
<thead>
<tr>
<th>Volume</th>
<th>Protein</th>
<th>NADH/ferricyanide reductase activity</th>
<th>Yield</th>
</tr>
</thead>
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<tr>
<td></td>
<td>ml</td>
<td>µmol min⁻¹</td>
<td>µmol min⁻¹ mg⁻¹</td>
</tr>
<tr>
<td>Membranes</td>
<td>64</td>
<td>2600</td>
<td>8200</td>
</tr>
<tr>
<td>Extract</td>
<td>57</td>
<td>1500</td>
<td>6500</td>
</tr>
<tr>
<td>Source 15Q</td>
<td>26</td>
<td>78</td>
<td>2300</td>
</tr>
<tr>
<td>Sephacryl S-300</td>
<td>18</td>
<td>25</td>
<td>1200</td>
</tr>
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</table>

**TABLE II**

<table>
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<th>Incubation with phospholipids</th>
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</thead>
<tbody>
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<td></td>
<td>Specific activity</td>
<td>Activation</td>
</tr>
<tr>
<td></td>
<td>µmol/min/mg</td>
<td>%</td>
</tr>
<tr>
<td>Without</td>
<td>0.30</td>
<td>–</td>
</tr>
<tr>
<td>Phosphatidylcholine (soybean)</td>
<td>0.66</td>
<td>119</td>
</tr>
<tr>
<td>Polar lipid extract (E. coli)</td>
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<td>103</td>
</tr>
<tr>
<td>Total lipid extract (E. coli)</td>
<td>0.45</td>
<td>56</td>
</tr>
<tr>
<td>Phosphatidylcholine (egg yolk)</td>
<td>0.38</td>
<td>27</td>
</tr>
</tbody>
</table>

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a C. Hägerhall, personal communication.
Proton Pumping of E. coli Complex I

Fig. 4. Generation of a pH gradient by the redox reaction of E. coli complex I reconstituted in proteoliposomes monitored by ACMA fluorescence. The assay contained 20 μg of purified complex I in 5 mM MES/KOH, 80 mM NaCl, 50 μM decyl-ubiquinone, pH 6.0. The reaction was started by addition of 50 μM NADH indicated by an arrow. The ACMA fluorescence was detected at an excitation wavelength of 410 nm and an emission wavelength of 480 nm. A, no further additions. Signal after additions of 10 μM piericidin A (B), 20 μM CCCP (C), 10 μM monensin (D), and 20 μM CCCP (E) added during enzyme turnover are shown.

were added to an ACMA assay in 5 mM MES/KOH, 1 mM NaCl, 79 mM TMA, and in 5 mM MES/KOH, 80 mM NaCl, respectively. The reaction was started by addition of NADH (Fig. 6). The amplitude of the ACMA signal was ~20% larger in the buffer containing 1 mM Na+ than in the buffer containing 80 mM Na+ (Fig. 6). The amplitude of the ACMA signal was independent of the applied Na+ gradient when the proteoliposomes were preincubated with 10 μM ETH-157 prior to the addition of NADH (Fig. 6). Thus, our data are in line with the idea that complex I is capable of secondary Na+/H+ antiport coupled to its redox reaction.

Effect of Amiloride Inhibitors on E. coli Complex I—It has been shown that the electron transfer of mitochondrial and bacterial complex I is inhibited by amiloride derivatives, inhibitors of Na+/H+ antiporters (41, 42). In addition, these substances prevent labeling of the homologue of NuoL in bovine heart complex I with a photofluor analogue of fenpyroximate, an inhibitor binding to the ubiquinone site of complex I (41). The Na+ transport mediated by the overproduced and isolated subunit NuoL was shown to be sensitive to EIPA, an amiloride inhibitor (20). To test the effect of these inhibitors on the isolated E. coli complex I we monitored the NADH:decyl-ubiquinone reductase activity at various EIPA and benzamil concentrations (Fig. 7). The inhibitor concentrations needed for half-maximal inhibition were 100 and 70 μM for EIPA and benzamil, respectively. The residual activity was less than 5% at 500 μM EIPA and 300 μM benzamil. These values are comparable to those obtained for the enzyme in the bacterial membrane of >100 μM and 45 μM, respectively (42). The IC50 did not depend on the Na+ concentration (Fig. 7). Comparable values were obtained in the presence of 25 μM and 100 mM NaCl, respectively. Thus, these inhibitors did not act at a hypothetical Na+ binding site in complex I. Beside their effect on electron transfer we also measured the influence of EIPA and benzamil on proton translocation by complex I. At concentrations needed for 95% inhibition of the electron transfer activity, the NADH-induced ACMA signal of complex I proteoliposomes was completely gone (Fig. 7). This showed that amiloride inhibitors affect both electron transfer and proton translocation of complex I.

DISCUSSION

Because of its enormous complexity in combination with the lack of structural data, knowledge about the mechanism of complex I is rather limited. The mitochondrial complex I is made up of at least 46 different subunits (6) seven of which are encoded on the mitochondrial genome (1, 2). Although the bacterial complex shows a simpler composition of 14 different subunits, this number is still quite large. In addition, its exact cofactor composition is still under debate (43–46). While it is generally accepted that the mitochondrial complex I translocates four H+ across the membrane for every NADH oxidized (7–11), it has recently been shown that complex I from K. pneumoniae pumps two Na+ per NADH oxidized but is not capable of translocating protons (14). The different type of ions translocated and the different stoichiometries have general consequences for models of the enzyme mechanism (47). It needs to be established whether Na+ translocation by the K. pneumoniae complex I is a unique exception to the rule or whether it represents a general, so far overlooked feature of complex I.

As a substantiation of the latter, it was reported that the E. coli complex I is capable of pumping Na+ as well (15). This proposal was derived from data obtained with inverted membrane vesicles. Because measurements with membrane vesicles may contain enzyme activity obscuring the activity in question we set out to advance measurements with the isolated complex I reconstituted in artificial phospholipid vesicles. Therefore, a suitable mixture of phospholipids and a suitable method for reconstitution had to be established. The best results in terms of enzyme activity and amount of enzymes reconstituted into liposomes were obtained with E. coli polar lipid extract reconstituted by the BioBeads method (Ref. 27 and Table 2). It is a well known property of E. coli membrane proteins that they show the highest activity when reconstituted into E. coli phospholipids (48).

As isolated in detergent, the E. coli complex I has a very low activity of 0.3 μmol of NADH/(min·mg). This low activity can be stimulated by addition of endogenous phospholipids up to 8-fold (Table 2) as described in the literature (34, 36, 49, 50). As pointed out for the Yarrowia lipolytica enzyme, complex I has to be preincubated with phospholipids to achieve sufficient interaction, which is limited in the diluted assay buffer (50). Reconstitution in proteoliposomes led to an enzymatic activity of about 5.5 μmol of NADH/(min·mg) in the presence of CCCP, which is close to the estimated turnover of the enzyme in the bacterial membrane of 6 μmol of NADH/(min·mg) (29). Titration of the NADH:decyl-ubiquinone oxidoreductase activity with E. coli polar lipid extract revealed that the full activity of the E. coli complex I is recovered when incubated with the 4-fold amount (w/w) of phospholipids (data not shown). This was in accordance with previously reported values (36).

In the absence of Na+ ions, primary sodium pumps show apparently no turnover (51). The activity of the Na+-pumping complex I from K. pneumoniae drops to 50% of the Vmax at 332 μM Na+ (13). The enzymatic activity of the Na+-translocating NQR from V. cholerae is lowered by 50% at ~10 mM Na+ (37). However, the NADH:decyl-ubiquinone reductase activity of the isolated E. coli complex I did not depend on the Na+ concentration (Fig. 2), indicating that this enzyme is not involved in primary Na+ translocation.

This was confirmed by measurements of the membrane potential using ANS as a fluorescent probe. An NADH-induced membrane potential was detected using complex I reconstituted into liposomes (Fig. 3). No signal was detected when either NADH or decyl-ubiquinone was omitted from the assay or when complex I was preincubated with the specific inhibitor...
primary sodium pump (15, 16). It is more than unlikely that a discrepancy exists between data derived from different materials used for different studies. Proton pumping has been reported for the complex in whole cells as mentioned above, and the intactness of our preparation was demonstrated by the complete sensitivity of the NADH-decyl-ubiquinone reductase activity with EIPA (A) and benamil (B) monitored as the decrease of the NADH concentration at 340–400 nm. The assay contained 15 μg of reconstituted complex I in either 50 mM MES/NaOH, 100 mM NaCl, pH 6.0 (closed squares) or 50 mM MES/KOH, 100 mM KCl, pH 6.0 (closed circles), and 50 μM decyl-ubiquinone. C, effect on the pH gradient generated by complex I monitored as ACMA fluorescence at 410 nm (excitation) and 480 nm (emission). The assay contained 20 μg of purified complex I in 5 mM MES/KOH, 80 mM NaCl, 50 μM decyl-ubiquinone, pH 6.0. The reaction was started by addition of 50 μM NADH indicated by an arrow. The upper curves in B and C were control experiments without DCCD, and the lower curves were obtained in the presence of 100 μM DCCD.

Fig. 5. Effect of DCCD on the isolated E. coli complex I reconstituted in E. coli polar lipids. A, inhibition of the NADH:decyl-ubiquinone reductase activity monitored as the decrease of the NADH concentration at 340–400 nm. The assay contained 30 μg of purified complex I in 50 mM MES/NaOH, 100 mM NaCl, pH 6.0, 50 μM decyl-ubiquinone, and 50 μM NADH. B, effect on the membrane potential generated by complex I monitored as ANS fluorescence at 380 nm (excitation) and 480 nm (emission). The assay contained 20 μg of purified complex I in 5 mM MES/KOH, 80 mM NaCl, 50 μM decyl-ubiquinone, pH 6.0. C, effect on the pH gradient generated by complex I monitored as ACMA fluorescence at 410 nm (excitation) and 480 nm (emission). The assay contained 20 μg of purified complex I in 5 mM MES/KOH, 80 mM NaCl, 50 μM decyl-ubiquinone, pH 6.0. The reaction in B and C was started by addition of 50 μM NADH indicated by an arrow. The upper curves in B and C were control experiments without DCCD, and the lower curves were obtained in the presence of 100 μM DCCD.

Fig. 6. Influence of the Na+ concentration on the proton gradient. Complex I in proteoliposomes containing 5 mM MES/KOH and 80 mM NaCl was added to an assay buffer of 5 mM MES/KOH, 80 mM NaCl, pH 6.0, and 1 μM ACMA. A, proteoliposomes containing 80 mM NaCl were added to an assay buffer of 1 mM NaCl. B, proteoliposomes containing 1 mM NaCl were added to an assay buffer of 80 mM NaCl. At the time indicated by arrows 3 μl of proteoliposomes (PL), 50 μM decyl-ubiquinone (DQ), and 50 μM ACMA were added, respectively. C, proteoliposomes containing 80 mM NaCl were added to an assay containing 80 mM NaCl, D, proteoliposomes containing 80 mM NaCl were added to an assay containing 1 mM NaCl. Reactions in C and D were started by addition of 50 μM NADH. Addition of 10 μM ETH-157 to C prior to NADH led to a signal comparable with D.

Fig. 7. Effect of amiloride derivates on the isolated E. coli complex I reconstituted in E. coli polar lipids. Inhibition of the NADH-decyl-ubiquinone reductase activity with EIPA (A) and benamil (B) monitored as the decrease of the NADH concentration at 340–400 nm. The assay contained 15 μg of reconstituted complex I in either 50 mM MES/NaOH, 100 mM NaCl, pH 6.0 (closed squares) or 50 mM MES/KOH, 100 mM KCl, pH 6.0 (closed circles), and 50 μM decyl-ubiquinone. C, effect on the pH gradient generated by complex I monitored as ACMA fluorescence at 410 nm (excitation) and 480 nm (emission). The assay contained 20 μg of purified complex I in 5 mM MES/KOH, 80 mM NaCl, 50 μM decyl-ubiquinone, pH 6.0. The reaction was started by addition of 50 μM NADH indicated by an arrow. The upper curves in B and C were control experiments without EIPA, and the lower curves were obtained in the presence of 100 μM EIPA or 300 μM benzamil.
activity to picricidin A and by its capability to generate membrane potential. However, by using inverted membrane vesicles, it is possible that electrons from NADH entering the respiratory chain via complex I are distributed to other energy-converting enzymes, with one of these probably acting as primary sodium pump. This would also explain the rotenone sensitivity of the sodium gradient because rotenone prevents electrons from entering the respiratory chain. The same would hold true for the measurements performed with the E. coli strain deficient in complex I. The electron entry to the respiratory chain would be missing, and rotenone does not have any effect on this mutant.

Sequence comparisons have shown that the complex I subunits NuoL, -M, and -N are related to cation/proton antiporters (5, 53–57). Experiments with individually overproduced NuoL from E. coli and Rhodobacter capsulatus have shown that these subunits are capable of passive transport of sodium ions along a concentration gradient (20, 40). The sodium transport of the overproduced E. coli NuoL reconstituted into proteoliposomes is accompanied by an increase in the proton permeability of the membrane. This was interpreted as a Na⁺/H⁺ antiport (20). Overproduced NuoL and -M from R. capsulatus rescued Bacillus subtilis mutants lacking the Na⁺/H⁺ antiporter subunits MrpA and -D at elevated Na⁺ concentrations (40). Our data on complex I reconstituted into proteoliposomes showed that these subunits assembled into the entire complex do not function as a passive Na⁺/H⁺ antiporter (Fig. 6). A Na⁺ gradient across the membrane did not induce a proton translocation (Fig. 6).

However, the magnitude of the pH gradient generated by the redox activity of complex I varied in dependence on Na⁺ concentration (Fig. 6). If the Na⁺ concentration in the proteoliposomes was higher than in the assay buffer, the amplitude of the ACMA signal was larger than that obtained with equal Na⁺ in the assay buffer (20, 40). This was interpreted as a Na⁺ sensitivity of the sodium gradient because rotenone prevents the redox reaction of the enzyme. The efflux of Na⁺ coupled to a further proton uptake enlarging the pH gradient.

Overproduced NuoL and -M from E. coli (57). Experiments with individually overproduced NuoL units NuoL, -M, and -N are related to cation/proton antiporter subunits NuoM and -N, which are involved in the assembly of complex I (20, 40). NuoN, -M, and -N are related to cation/proton antiporter subunits NuoM and -N, which are involved in the assembly of complex I (20, 40).

Anchor peptides, which are present in the subunits of Na⁺/H⁺ antiporters, inhibit complex I from different sources although with different affinities (39, 40). The IC₅₀ value we measured for the isolated and reconstituted E. coli complex I is comparable to the values reported for the complex in the membrane (42). The inhibition was independent from the Na⁺ concentration in the measured range, which is further evidence for the finding that the E. coli complex I is not involved in primary Na⁺ translocation and that the amiloride type inhibitors do not act at a Na⁺ binding site. Here, we showed that in addition to their effect on electron transfer, EIPA and benzamil also prevent proton translocation (Fig. 7). Thus, their inhibitory action is comparable to that of DCCD. As inhibition of complex I by amiloride derivatives is not restricted to the bacterial complex (20, 40) but was also detected for the mitochondrial complex (41–42), it is possible that complex I in general may be capable of redox-driven secondary H⁺/cation antiport.

Acknowledgments—We thank Heinrich Jung, Osnabrück, and Peter Gruber, Freiburg, for advice in the preparation of liposomes and to Uli Brandt, Frankfurt, for his advice in measuring pH gradients. We thank Dr. Peter Heiss and Joachim Grams (Roche Applied Science, Penzberg) for the donation of the Kontron spectrophotometer.

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The *Escherichia coli* NADH:Ubiquinone Oxidoreductase (Complex I) Is a Primary Proton Pump but May Be Capable of Secondary Sodium Antiport

Stefan Stolpe and Thorsten Friedrich


doi: 10.1074/jbc.M311242200 originally published online February 17, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M311242200

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