The Respiratory Complex I (NDH I) from *Klebsiella pneumoniae*,
a Sodium Pump*

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The electrogenic NADH-Q oxidoreductase from the enterobacterium *Klebsiella pneumoniae* transports *Na*⁺ ions. The complex was purified with an increase of the specific *Na*⁺ transport activity from 0.2 μmol min⁻¹ mg⁻¹ in native membrane vesicles to 4.7 μmol min⁻¹ mg⁻¹ in reconstituted enzyme specimens. The subunit pattern resembled that of complex I from *Escherichia coli*, and two prominent polypeptides were identified as the NuoF and NuoG subunits of complex I. During purification the typical cofactors of complex I were enriched to yield −17 nmol mg⁻¹ iron, 24 nmol mg⁻¹ acid-labile sulfide, and 0.79 nmol mg⁻¹ FMN in the purified sample. The enzyme contained −1.2 nmol mg⁻¹ Q6 and 1.5 nmol mg⁻¹ Q8. The reduction of ubiquinone by NADH was *Na*⁺-dependent, which indicates coupling of the chemical and the vectorial reaction of the pump. The *Na*⁺ activation profile corresponded to the Hill equation with a Hill coefficient *K*₉(*Na*⁺) = 1.96 and with a half-maximal saturation at 0.33 mM *Na*⁺. The reconstituted complex I from *Klebsiella pneumoniae* catalyzed deamino-NADH oxidation, Q1 reduction, and *Na*⁺ translocation with specific activities of 2.6 units mg⁻¹, 2.4 units mg⁻¹, and 4.7 units mg⁻¹, respectively, which indicate a *Na*⁺/electron stoichiometry of one.

The electrogenic NADH-Q oxidoreductase (complex I or NDH I) catalyzes the electron transfer from NADH via FMN and multiple iron-sulfur clusters to Q (1–5). In mitochondria and various bacteria, electron transfer from NADH to Q by complex I is coupled to the translocation of protons across the membrane. It is generally assumed that the stoichiometry of this process is at least 2H⁺/e⁻. The mitochondrial NADH-Q oxidoreductase is a large and complex enzyme composed of more than 40 unlike polypeptides. Of these, seven are encoded by mitochondrial DNA (6) building the membrane part of the enzyme. The redox active centers that participate in the electron transfer reactions are one non-covalently bound FMN in the peripheral, NADH-oxidizing part and at least six iron-sulfur clusters (7, 8).

In bacteria like *Escherichia coli*, a simpler form of the enzyme exists composed of only 14 homologues of the mammalian subunits summing up to a molecular mass of ~530 kDa. It is regarded as the minimal catalytic structure of the proton-pumping NADH-Q oxidoreductase. *E. coli* complex I consists of two arms, which may be arranged perpendicular to each other or side by side (9). Upon purification the enzyme is prone to disruption into three fragments: a peripheral fragment, a connecting fragment, and a membrane fragment (10). It seems that the electrons originating from NADH are transferred from the iron-sulfur cluster N2 to the final electron acceptor Q located in the membrane (11–13). This exergonic reaction is coupled to the transport of H⁺ (or *Na*⁺) across the membrane (14). *Klebsiella pneumoniae*, a close relative of *E. coli*, possesses a respiratory NADH-Q oxidoreductase that was shown to be stimulated by *Na*⁺ ions (15). Inhibitor studies in native membranes and partial purification of the enzyme led to the proposal that this respiratory enzyme is of the complex I (NDH I) type (16). Sequencing studies revealed that 84% of the amino acids of complex I from *K. pneumoniae* and *E. coli* are identical (14, 17). These enterobacterial enzymes differ from the complex I of a-proteobacteria or eukaryotes with respect to subunits and Fe/S centers (10). It was not surprising therefore that our preliminary evidences with bacterial membrane vesicles and complex I-specific inhibitors indicated that the NADH-Q oxidoreductase from *K. pneumoniae* or *E. coli*, which we tentatively ascribed to complex I catalyzed *Na*⁺ transport (16, 18). Up to now, the purification and reconstitution of a eukaryotic or bacterial complex I in an active state, which allows the determination of H⁺ (or *Na*⁺) transport stoichiometries, have not been reported.

In this study we report purification of NADH-Q oxidoreductase from *K. pneumoniae* with retention of the Q reduction and *Na*⁺ transport activities. Inhibition studies, cofactor analysis, and results from N-terminal sequencing are all consistent with the identity of the enriched enzyme with complex I. For the first time, transport by complex I has been studied in artificial membranes. It is shown here that the enzyme transports *Na*⁺ and that the transport stoichiometry is one *Na*⁺ translocated per electron transferred.

**EXPERIMENTAL PROCEDURES**

*Growth of K. pneumoniae—Klebsiella pneumoniae* from laboratory stock culture was grown anaerobically at 37 °C as described (19) with 46 mM glycerol as carbon source.

**Preparation and Solubilization of Membrane Vesicles under Exclusion of Air—** Cells of *K. pneumoniae* (50 g wet weight) were resuspended in 50 ml of air-saturated buffer (50 mM HEPES-KOH, pH 7.0, 50 mM KCl) containing 7% dimethylsulfoxide immediately after harvesting. The cell suspension was stored for less than 72 h at −20 °C. Using dimethylsulfoxide as antifreezing protectant, the NADH-Q oxidoreductase activities were as high as in fresh cells. After adding 0.1 mM diisopropylfluorophosphate and a trace of DNase, the suspension was passed once through a French pressure cell at 83 mPa, and the eluate was collected under a stream of N₂. Unbroken cells and large debris were removed by centrifugation at 35,000 × g for 20 min. If not indicated otherwise, all subsequent manipulations were performed in an anaerobic chamber with N₂/H₂ (95:5%) as gas phase and at 4 °C. The

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membranes were collected by ultracentrifugation (150,000 × g, 1 h) and were washed once with 10 mM Tris-HCl buffer, pH 7.5, containing 50 mM KCl and 10% glycerol. Membranes isolated by this procedure contained ~20-fold higher NADH-driven Na+ uptake activities than after preparation under air. Most probably, exposure of the NADH oxidoreductase to the atmosphere caused its inactivation. The purification and characterization of an iron-sulfur cluster 1 complex from *Thermus thermophilus* (20).

**Purification of NADH:Q Oxidoreductase**—The NADH:Q oxidoreductase was solubilized by stirring a suspension of membranes (2000 mg of protein) in 30 ml of 10 mM Tris-HCl buffer, pH 7.5, containing 50 mM KCl, 10% glycerol, and 15 mM DHPC1 or 2.2% (v/v) Triton X-100 for 30 min on ice in sealed vessels. Some loss of NADH:Q oxidoreductase activity (20%) was observed upon solubilization with Triton X-100, which is known to act as competitive inhibitor of eukaryotic complex I (21, 22). After centrifugation at 250,000 × g for 1 h the dark yellow supernatant was loaded onto a Fractogel TSK DEAE-650 column (Amersham Biosciences, 1.5 × 8.5 mm). Anionic buffers were connected to the FPLC system under an overpressure of N2, and fractions eluting from the column were collected under a stream of N2. After washing the column with 3 volumes of buffer A (50 mM HEPES-NaOH, 200 mM NaCl, pH 7.5, 10% glycerol, 0.1 mM EDTA) containing either 1 mM DHPC or 0.1% (v/v) Triton X-100, the enzyme was eluted with a linear gradient of 200–380 mM NaCl in buffer A with the detergent (total volume 380 ml) at a flow rate of 1.5 ml/min. When DHPC was used as detergent its concentration was increased to 1.5 mM (cmc = 1.5 mM) (23) immediately after elution of the enzyme. Subsequently, the enzyme (9 ml) was concentrated by ultracentrifugation (150,000 × g, 15 h). The dark yellow pellet was resuspended in 0.45 ml of 10 mM Tris-HCl buffer, pH 7.5, containing 50 mM KCl, and each 0.15-ml aliquot was applied to a gradient from 5 to 20% sucrose in 4.6 ml of 10 mM Tris-HCl buffer, pH 7.5, containing 50 mM KCl and 15 mM DHPC or 0.1% Triton X-100. After ultracentrifugation (150,000 × g, 8 h, Beckman SW55Ti), fractions (0.2 ml) were collected from the top. The Q reductase activity was enriched at 12–14% sucrose, which also has been reported for *E. coli* complex I (24). No disruption of the enzyme complex into a NADH dehydrogenase fragment and a NADH:Q oxidoreductase was observed. Activity was combined (22-fold concentration), concentrated (20–40 mg of protein ml−1). In some purifications all buffers contained KCl instead of NaCl, which reduced the endogenous Na+ concentration of the enzyme to 30–50 μM.

**Reconstitution of the NADH:Q Oxidoreductase**—*E. coli* lipids (30–150 mg) were dissolved in 1–4 ml of CHCl3/CH3OH (2:1) in a round-bottom flask, and the solvent was removed on a rotor evaporator. When electrophoretic analysis of membranes-bound NADH oxidoreductase was measured, the lipid solution was mixed with 600 nmol of Q2 (Sigma), 350 nmol of Q6 (Sigma), and 2 μmol of Q8 dissolved in 80 μl of diethylether. The NADH:Q oxidoreductase from different purification steps in DHPC was solubilized by stirring a lipid film (ratio of lipid/protein, 30; total volume, ~1 ml) in 10 mM Tris-HCl buffer, pH 7.5, containing 50 mM KCl and each 0.15-ml aliquot was applied to a gradient from 5 to 20% sucrose in 4.6 ml of 10 mM Tris-HCl buffer, pH 7.5, containing 50 mM KCl and 15 mM DHPC or 0.1% Triton X-100. After ultracentrifugation (150,000 × g, 8 h, Beckman SW55Ti), fractions of 0.2 ml were collected from the top. The Q reductase activity was enriched at 12–14% sucrose, which also has been reported for *E. coli* complex I (24). No disruption of the enzyme complex into a NADH dehydrogenase fragment and a NADH:Q oxidoreductase was observed. Activity was combined (22-fold concentration), concentrated (20–40 mg of protein ml−1). In some purifications all buffers contained KCl instead of NaCl, which reduced the endogenous Na+ concentration of the enzyme to 30–50 μM.

**Determination of Na+ Uptake**—Na+ uptake was followed by atomic absorption spectroscopy (16). Incubation mixtures contained in 0.3 ml of 0.1 M Tris-HCl, pH 7.5, containing 50 mM KCl, 10% glycerol, and 5 mM NaCl. If indicated CCCP (10 μM or 100 μM), antimycin V (50 μM), monensin (20 μM), valinomycin (50 μM), gramicidin (90 μg ml−1), mixture of gramicidin A, B, and C; Fluca) was added to the assay. The reaction was started by the addition of 0.1 ml of NADH (final concentra-

1 The abbreviations used are: DHPC, 1,2-dihexanoylphosphatidylcholine; CCCP, carbonyl cyanide m-chlorophenylhydrazone; ccm, critical micellar concentration; Na+-NQR, Na+-translocating NADH:quinone oxidoreductase; S2−, acid-labile sulfur; HPLC, high performance liquid chromatography; MALDI, matrix-assisted laser desorption/ionization. At different times, samples of 70 μl were applied to a 1-ml plastic syringe containing 0.6 ml of Dowex 50 (K+)-, equilibrated with assay buffer without NaCl. The outlet of the syringe was sealed with a HDPE membrane (high-pressure polyethylene, 0.35-μm pore diameter, Reichelt Chemie technik, Heidelberg, FRG). The proteopisomes were immediately passed through the Dowex material using the piston of the syringe (the total handling time, <1 s). The eluate was collected in plastic tubes, and the Na+ entrapped in the vesicles was determined by atomic absorption spectroscopy. In controls performed with substrate and 5 mM NaCl in buffer, the absence of membrane vesicles, Na+ was completely ab-
sent. Black dots obtained with vesicles in buffer without Na+ added corresponded to the internal Na+ content of the liposomes (3–8 nmol of Na+ per sample after passage through the Dowex column). Parallel experiments performed with 5 mM NaCl and analysis by atomic absorption spectroscopy or 5 mM 22NaCl (1000 cpm/ nmol) and analysis by γ-counting (19) yielded identical rates of NADH-driven Na+ uptake. The rates of Na+ transport varied by less than 10% with different preparations of complex I.

**Analytical Methods and Enzyme Activities**—Protein was determined by the bicinchoninic acid method using the reagent obtained from Pierce, or by the microbiuret method (25) after trichloroacetic acid/ deoxycholate precipitation (26). In the presence of detergents, this method gives more reliable values than the bicinchoninic acid method. Buffers were pH 7.5 ± 0.1.

**SDS-PAGE** was performed with 10% polyacrylamide according to Ref. 27. Enzyme assays were performed in 50 mM Tris-HCl, pH 8.0 with 50 μM to 50 mM NaCl or deamin-NADH, 0.1 mM NaCl and 0.1 mM Q1 (Sigma) at 25 °C. The NADH or deamin-NADH oxidation was followed at 340 nm (εmax = 6.22 mM−1 cm−1). The Q reductase activity correspond- ing to the formation of ubiquinol-1 from Q1 was determined from the difference in absorbance at the wavelength pair 248 and 268 nm (Δεmax = 7.8 mM−1 cm−1) (28). The reduction of ferricyanide with NADH or deamin-NADH as electron donor was followed at 420 nm (εmax = 1.0 mM−1 cm−1). 1 unit is defined as the amount of enzyme consuming 1 μmol of substrate or forming 1 μmol of product per minute. In order to study the inhibition of the NADH:Q oxidoreductase by Ag+, buffers containing sulfate instead of chloride were used for the isolation of complex I and the subsequent incubations with AgNO3 (29). Iron was determined colorimetrically using 3-(2-pyridyl)-5,6-bis-(5-sulfo-2-furyl)-1,2,4-triazine, disodium salt as iron chelator (30). Acid-labile sulfur was determined according to (31). Phospholipids were determined as phosphate concentrations according to (32).

Flavins from protein samples were extracted under light protection by 80% methanol in water (3:1). The precipitate was re-

**Determination of the Na+ Transport and Q Reductase Activities** by proteoliposomes containing NADH:Q oxidoreductase, both types of experiments

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Purification of the Na⁺:Q oxidoreductase from Klebsiella pneumoniae

Starting material was 50 g (wet weight) cells, DHPC was used as detergent. Q₁ reduction was followed with dNADH as electron donor. The values represent at least four measurements

| Protein                  | dNADH oxidation (mg units mg⁻¹) | NADH oxidation (units mg⁻¹) | Q₁ reduction after reconstitution (units mg⁻¹) | Yield Q₁ reduction (%) | Na⁺ translocation (units mg⁻¹) | Yield Na⁺ translocation (%) |
|--------------------------|---------------------------------|-----------------------------|---------------------------------------------|------------------------|-------------------------------|----------------------------|
| Membrane fraction        | 0.4 ± 0.1                       | 0.8 ± 0.3                   | 0.1 ± 0.03                                  | 100                    | 0.2 ± 0.03                    | 100                        |
| Solubilized membranes    | 3.4 ± 0.4                       | 8.0 ± 0.3                   | 0.2 ± 0.1                                  | 53                     | 0.3 ± 0.1                     | 40                         |
| Fractogel TSK            | 0.1 ± 0.4                       | 1.0 ± 0.4                   | 0.2 ± 0.2                                  | 48                     | 2.4 ± 1.3                     | 47                         |
| Sucrose density-gradient | 10.0 ± 1.1                      | 2.0 ± 0.5                   | 1.2 ± 0.7                                  | 45                     | 4.7 ± 1.6                     | 47                         |

The presence of complex I in our enzyme preparation was further confirmed by N-terminal sequencing of major polypeptides present in the Na⁺:Q oxidoreductase from the final sucrose centrifugation step. Upon separation by SDS-PAGE, two polypeptides with apparent molecular masses of 100 and 50 kDa were identified as the NuoG subunit (MATHYDGEKEYEV) and the FMN-carrying NuoF subunit (MKNVIRTAETHPLT) of complex I (Fig. 1). The N-terminal sequences of the NuoF and the NuoG subunits are identical to the sequences derived from the nuoF and nuoG genes of complex I from K. pneumoniae (14, 16). Note that there is only one nuo operon on the genome of K. pneumoniae (17), which excludes the possibility that K. pneumoniae possesses a Na⁺-dependent complex I in addition to a H⁺-dependent one. The SDS-PAGE of complex I from K. pneumoniae reveals a polypeptide pattern that is very similar to the one observed in the related complex I from E. coli (3) and allows the putative assignment of the fused NuoCD subunit (68 kDa), and the NuoM and NuoN subunits (42–45 kDa) in complex I from K. pneumoniae (Fig. 1).

Kinetic Properties—A comparison of the initial rates of Q reduction and Na⁺ transport allows an estimation of the Na⁺/electron stoichiometry of redox-driven Na⁺ transport by complex I, if the redox pump works in a fully coupled manner. Uncoupled quinol formation, e.g. reduction of Q not linked to Na⁺ transport, would result in an underestimation of the actual Na⁺/electron ratio. We determined the stimulation of Q reductase activity of complex I by Na⁺ ions using an enzyme preparation that contained little endogenous Na⁺. Essentially no Q reduction occurred in the absence of Na⁺ ions (Fig. 2), which is reminiscent of other primary sodium pumps (35). The addition of Na⁺ resulted in a drastic increase of the Q reductase activity of complex I by Na⁺ ions using an enzyme preparation that contained little endogenous Na⁺. Essentially no Q reduction occurred in the absence of Na⁺ ions (Fig. 2), which is reminiscent of other primary sodium pumps (35). 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type from *Vibrio*. The NADH:Q oxidoreductase activity of native membranes from *K. pneumoniae* was completely inhibited with the complex I-specific inhibitors annonin VI (12 μM) and piericidin A (16 μM). In the presence of rotenone (50 μM), Q reduction activity of membranes dropped by 90% (Fig. 3). Rotenone binds close to a Q binding site of complex I (5, 36–38). A residual activity in the presence of rotenone is also observed for bovine complex I (39, 40). The residual Q reduction activity of membrane-bound complex I from *K. pneumoniae* in the presence of rotenone with 50 mM NaCl or with 0.5 mM NaCl did not differ significantly over a concentration range from 0.02 to 0.15 mM Q1. Note that half-maximal activation of reconstituted complex I is already observed at ~0.3 mM NaCl.

Ag⁺ acts as specific inhibitor of the Na⁺-translocating NQR from *Vibrio alginolyticus* (29, 41). Q reduction by our purified complex I preparation was not inhibited in the presence of 600 nM Ag⁺ in chloride-free buffer. These results further support the notion that the Na⁺-translocating NADH:Q oxidoreductase from *K. pneumoniae* is identical to complex I.

### Na⁺-Translocating NADH:Q Oxidoreductase (Complex I)

| Cofactors and flavins present in the NADH:Q oxidoreductase from Klebsiella pneumoniae at different purification steps | FMN | FAD | Riboflavin | Q6 | Q8 | Fe | FMN:Fe | S⁻² | S⁻²:Fe |
|---|---|---|---|---|---|---|---|---|---|
| Solubilized membranes | 0.036 | 0.008 | 0.088 | 0.2 | 0.4 | 8 | 1:220 | 11 | 1:2.1 |
| Fractogel TSK | 0.09 | 0.007 | 0.025 | 0.6 | 0.9 | 16 | 1:500 | 21 | 3:1.1 |
| Sucrose density-gradient | 0.79 ± 0.15 | 0.046 ± 0.011 | 0.016 ± 0.007 | 1.20 ± 0.15 | 1.5 ± 0.2 | 17 ± 7 | 1:32 | 24 ± 7 | 1:3.1 |

**TABLE II**

**Fig. 1.** SDS-PAGE of the NADH:Q oxidoreductase from *K. pneumoniae*. Major polypeptides present in the NADH:Q oxidoreductase after sucrose density gradient centrifugation (80 g) were stained with Coomassie Blue and N-terminally sequenced. The polypeptides around 100 and 50 kDa were unambiguously identified as NuoG and NuoF subunits of complex I, respectively. The putative assignment of the fused NuoCD, the NuoM and NuoN subunits (in brackets) is based on a comparison with the SDS-PAGE of complex I from *E. coli* (3).

**Fig. 2.** Activation profile of NADH:Q oxidoreductase by Na⁺ ions. The reduction of Q1 was followed in the presence of 50 μM to 5 mM Na⁺. Experimental data were fitted using the Hill equation (upper panel). A Hill coefficient of 1.96 was calculated as a slope of the corresponding plot in linear coordinates (lower panel).
of NADH (Fig. 4, upper panel), or in the presence of the Na⁺-conducting ionophores monensin or gramicidin, demonstrating that this activity is not caused by passive diffusion through the liposome membrane (Fig. 4, lower panel). NADH-driven Na⁺ transport was not inhibited by the uncoupler CCCP, but continued for about 30 s reaching internal concentrations of 1800 nmol/mg of protein (Fig. 4, upper panel). This approximately double increase of Na⁺ uptake in the presence of the protonophore clearly indicates that the transport was accomplished by a primary Na⁺ pump and not by the combined action of a proton pump and a Na⁺/H⁺ antiporter. The internal volume of the reconstituted *E. coli* lipid vesicles is ~7 μl/mg of lipid (42), and hence internal Na⁺ concentrations of 40 mM are established with NADH and CCCP. This is eight times higher than the external Na⁺ concentration and thus in accord with an active transport system. The higher levels of Na⁺ accumulation in the presence of CCCP are consistent with an electrogenic Na⁺ pump where the generated membrane potential is inhibitory for further Na⁺ pumping. In the presence of the uncoupler, the membrane potential is dissipated by compensating H⁺ movements and Na⁺ transport proceeds without interference with the membrane potential.

The observed transport of Na⁺ into proteoliposomes without added protonophore is due to compensating fluxes of chloride through the phospholipid bilayer (43). Using proteoliposomes prepared in potassium phosphate buffer, no NADH-driven Na⁺ transport by complex I occurred unless the membrane potential was dissipated by the addition of K⁺/valinomycin (Fig. 4, lower panel). We conclude that the membrane potential established during the initial phase of Na⁺ translocation inhibited further transport and accumulation of Na⁺. Most importantly, no Q reduction by reconstituted complex I was observed under these conditions unless valinomycin was added (not shown). These results demonstrate full coupling of the chemical (NADH:Q oxidoreduction) and the vectorial (Na⁺ transport) reaction of the reconstituted complex I.

The complex I-specific inhibitor annonin VI (50 μM) led to a decrease of Na⁺ transport activity by 30–50% (Fig. 4, upper panel). The sensitivity toward annonin VI is less in reconstituted complex I compared with the membrane-associated complex (36). As annonin VI acts partially competitive with respect to Q (36), endogenous quinones present in the *E. coli* lipids might have prevented the complete inhibition of Na⁺ uptake by our enzyme.

The reduction of ubiquinone by NADH was Na⁺-dependent which further indicates coupling of the chemical and the vectorial reaction of complex I. Moreover NADH oxidation and Q reduction were catalyzed by reconstituted complex I with almost equal rates (Fig. 5, A and B). The Q reductase and Na⁺ transport activities of reconstituted complex I from *K. pneumoniae* were followed at saturating Na⁺ concentrations and under exactly the same conditions using proteoliposomes prepared in the presence of chloride (Fig. 5B). The initial rates for Na⁺ uptake and Q reduction were 4.7 ± 1.6 units mg⁻¹ and 2.4 ± 1.3 units mg⁻¹, respectively, corresponding to a 2:1 ratio. This ratio did not change during the entire observation period of 30 s (Fig. 5C) and was consistently found with different preparations of the enzyme, hence indicating a Na⁺ to electron stoichiometry of 1. The observed transport of two Na⁺ per two electrons transferred by complex I is in accordance with kinetic data, demonstrating cooperative binding of at least two Na⁺ ions during quinone reduction (Fig. 2).
Na\textsuperscript{+}-translocating NADH-Q Oxidoreductase (Complex I)

**FIG. 5.** Comparison of NADH oxidation, Q reduction, and Na\textsuperscript{+} transport catalyzed by reconstituted NADH-Q oxidoreductase. NADH oxidation (panel A), Na\textsuperscript{+} uptake, and Q reduction (panel B) were measured under exactly the same conditions. Note that the ratio of Na\textsuperscript{+} transport to Q reduction did not change during the entire observation period (panel C). For details see “Experimental Procedures.”

**DISCUSSION**

One of the most important aspects of this work is the isolation of a new respiratory Na\textsuperscript{+} pump from the enterobacterium *K. pneumoniae* in a fully functional state. The Na\textsuperscript{+} pump operates in the respiratory chain segment from NADH to ubiquinone and according to previous biochemical data with bacterial membrane vesicles, it was concluded that it is identical with complex I (16). Another respiratory Na\textsuperscript{+} pump within the NADH-Q segment has been identified in several marine *Vibrio* species, which was termed NQR and has clearly distinct properties with respect to subunit composition, cofactor content, and inhibition studies (44, 45). Incidentally, a similarity search using the NQR sequences (accession number AB008030) from *Vibrio alginolyticus* (44, 45) led to the identification of NQR genes (*nqra–F*) on the *K. pneumoniae* genome (17). Hence, there was the theoretical option that the Na\textsuperscript{+}-pumping activity we had observed in the *K. pneumoniae* membranes was due to this enzyme. However, based on inhibitor studies and N-terminal sequences of the partially purified enzyme (16), this does not seem to be the case. Inhibitor studies indicate that complex I possesses distinct Q binding sites (37, 53). A quinone binding site is located in the vicinity of the NuoD (49 kDa; bovine heart mitochondria) subunit of complex I (5, 16). The presence of the Na\textsuperscript{+} pump we isolated is identical with complex I.

Here we have improved the purification of the respiratory Na\textsuperscript{+} pump with retention of coupling between electron and Na\textsuperscript{+} transport activities. All properties of the purified enzyme confirm our previous conclusion that the Na\textsuperscript{+} pump is of the complex I type and not of the NQR type (16). These include: (i) migration of the Na\textsuperscript{+} pump on sucrose density gradient centrifugation, which was the same as that of complex I from *E. coli* (24) under otherwise identical conditions; (ii) a very similar subunit pattern as complex I purified from *E. coli* (3); (iii) identification of two prominent polypeptides as NuoF and NuoG by N-terminal sequencing; (iv) the typical cofactors of complex I in the expected molar ratios, (v) sensitivity toward the complex I specific inhibitors annomyn VI and rotenone. In contrast, the Na\textsuperscript{+}-NQR (*M*, 210) is much smaller than complex I (*M*, 530) and would therefore migrate more slowly in sucrose density centrifugation. The NQR consists of only 6 polypeptides with clearly distinct molecular masses and N-terminal sequences than the 13 polypeptides of complex I. The NQR has FAD as cofactor, which was not detectable in our purified enzyme. It is specifically inhibited by Ag\textsuperscript{+}, in contrast to our enzyme. Hence, all these data provide compelling evidence that the Na\textsuperscript{+} pump we isolated is identical with complex I.

The preparation described in this report is the first resulting in a fully energy-coupled complex I specimen suited for transport studies in artificial liposomes. The reduction of Q by NADH was strictly Na\textsuperscript{+}-dependent, which is reminiscent of other primary Na\textsuperscript{+} pumps, e.g. the Na\textsuperscript{+}-translocating ATP synthase of *Propionigenium modestum* (46) or the oxaloacetate decarboxylase of *K. pneumoniae* (47). This property by itself strongly indicates functional coupling of the Na\textsuperscript{+} pump, which was firmly assisted by Na\textsuperscript{+} transport measurements into reconstituted proteoliposomes. Here, we determined electron transfer from NADH to Q at a 1:1 ratio and with one Na\textsuperscript{+} translocated per electron transferred. A Hill coefficient of 1.96 indicates that at least two Na\textsuperscript{+} ions bind to complex I during quinol formation in a positively cooperative manner. Therefore, the transport of 2 Na\textsuperscript{+} per quinol formed by complex I is in excellent accordance with the kinetic data.

A transport ratio of 2Na\textsuperscript{+}/2e\textsuperscript{−} observed with reconstituted complex I may at first glance appear surprising, given the generally assumed stoichiometry of 4H\textsuperscript{+}/2e\textsuperscript{−} for complex I. This value is based on measurements with submitochondrial particles from bovine heart or rat liver (48, 49), i.e. enzymes with a significant phylogenetic distinction to complex I from enterobacteria. In another report, however, the solubilized and reconstituted complex I from bovine heart mitochondria was found to operate at a 2H\textsuperscript{+}/2e\textsuperscript{−} stoichiometry (50). For the *E. coli* complex, a transport stoichiometry of 1.5 H\textsuperscript{+}/e\textsuperscript{−} has been determined in whole cells (51), whereas experiments with native membrane vesicles indicate that complex I from *E. coli* also operates as a Na\textsuperscript{+} pump (18). An interesting question to be considered in this context is whether complex I from *K. pneumoniae* may transport protons instead of or besides Na\textsuperscript{+} ions under certain conditions. A switch from Na\textsuperscript{+} to H\textsuperscript{+} pumping has been observed for the ATP synthase from *P. modestum* at low environmental Na\textsuperscript{+} concentrations (52). These investigations are currently underway in our laboratory and will be reported elsewhere.

Another important finding is the presence of tightly bound quinones Q6 and Q8 in complex I from *K. pneumoniae*, which were copurified with the complex, not with the adhering phospholipids, in nearly 1:1 stoichiometry. These quinones may be involved in the electron transfer within the membrane-bound part of the complex. Inhibitor studies indicate that complex I possesses distinct Q binding sites (37, 53). A quinone binding site is located in the vicinity of the NuoD (49 kDa; bovine nomenclature), NuoB (PSST), and NuoH (ND1) subunit of complex I (5, 54). Quinones may also bind to the peripheral fragment of complex I from *E. coli* consisting of the subunits NuoE, F, and G that catalyze the reduction of quinones with NADH as electron donor (24). We assume that the low residual Q reductase activity of complex I from *K. pneumoniae* observed in the presence of rotenone is catalyzed by the peripheral fragment that does not participate in redox-driven Na\textsuperscript{+} transport. In contrast, Q reduction in the absence of rotenone is stimulated by Na\textsuperscript{+} ions. It will be important to test whether the Na\textsuperscript{+}
binding site of complex I is located in the vicinity of enzyme-bound quinones.

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The Respiratory Complex I (NDH I) from *Klebsiella pneumoniae*, a Sodium Pump

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