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ORIGINAL PAPER

NADH oxidation drives respiratory Na^+ transport in mitochondria from *Yarrowia lipolytica*

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Abstract It is generally assumed that respiratory complexes exclusively use protons to energize the inner mitochondrial membrane. Here we show that oxidation of NADH by submitochondrial particles (SMPs) from the yeast *Yarrowia lipolytica* is coupled to protonophore-resistant Na^+ uptake, indicating that a redox-driven, primary Na^+ pump is operative in the inner mitochondrial membrane. By purification and reconstitution into proteoliposomes, a respiratory NADH dehydrogenase was identified which coupled NADH-dependent reduction of ubiquinone (1.4 μmol min^{-1} mg^{-1}) to Na^+ translocation (2.0 μmol min^{-1} mg^{-1}). NADH-driven Na^+ transport was sensitive towards rotenone, a specific inhibitor of complex I. We conclude that mitochondria from *Y. lipolytica* contain a NADH-driven Na^+ pump and propose that it represents the complex I of the respiratory chain. Our study indicates that energy conversion by mitochondria does not exclusively rely on the proton motive force but may benefit from the electrochemical Na^+ gradient established by complex I.

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Keywords Na^+ transport · NADH dehydrogenase · Respiration · Mitochondria

Abbreviations
Smp Submitochondrial particle
Q Ubiquinone

Introduction

Mitochondria are vital for the synthesis of ATP, the regulation of programmed cell death, and the modulation of intracellular Ca^{2+} concentration. These processes are governed by mitochondrial respiration which creates a proton motive force (Δp) consisting of ΔΨ, the transmembrane voltage, and ΔpH, the proton concentration gradient (Mitchell 1961). Respiring mitochondria maintain a Δp of 170 mV which essentially consists of ΔΨ as ΔpH contributes less than 10 mV (Murphy and Brand 1987). Three electron transfer reactions from NADH to quinone, quinole to ferricytochrome *c*, and ferrocytochrome *c* to O_2 provide the driving force for the generation of Δp by the respiratory complexes I, III and IV, respectively. The stoichiometries of translocated protons per transferred electrons in each of these respiratory segments were determined with intact mitochondria and inside-out vesicles of mitochondrial membranes, or submitochondrial particles (SMPs). H^+/e^- stoichiometries were also studied with the purified complexes reconstituted into artificial membrane systems. By this approach, consistent ratios for the mitochondrial complexes III (the bc_1 complex) and IV (the cytochrome c oxidase) were found in many different laboratories (for review, see Hunte et al. (2003), Ruitenberg et al. (2002) and references therein). Studies with eukaryotic (Walker 1992;

Brandt 2006) or bacterial model organisms (Sazanov and Hinchliffe 2006) revealed that complex I is L-shaped, with a NADH-oxidizing arm that protrudes in the mitochondrial matrix or the bacterial cytoplasm, and a cation-translocating arm embedded in the membrane. Compared to complexes III and IV, very little is known about the mechanism of redox-driven proton transport by complex I.

It is generally assumed that the transmembrane voltage established during mitochondrial respiration exclusively results from the electrogenic transport of protons. In contrast, bacterial respiratory chains may contain a Na^+-translocating NADH:quinone oxidoreductase (Na^+-NQR) not related to complex I (Türk et al. 2004) or terminal oxidases (Efiok and Webster 1990) which catalyze redox-driven Na^+ transport. The enterobacterium *Klebsiella pneumoniae* seemed to be an exception when we showed that its Na^+-translocating NADH dehydrogenase belongs to the complex I family of respiratory enzymes. We determined the cofactor composition and Na^+/electron transport stoichiometry of the *K. pneumoniae* complex I (Gemperli et al. 2002) and showed that NADH-driven Na^+ transport is electrogenic (Gemperli et al. 2003). Our views were challenged by Bertsova and Bogachev who claimed that Na^+ transport observed with our complex I preparation from *K. pneumoniae* was due to a contamination with the Na^+-NQR (Bertsova and Bogachev 2004). However, Bogachev and colleagues recently showed that the Na^+-NQR is not present in anaerobically grown *K. pneumoniae* cells (Fadeeva et al. 2007) which we used as source for the purification of complex I (Gemperli et al. 2002). We also corroborated our previous notion of Na^+ transport by complex I from *K. pneumoniae* and identified a functional Na^+ binding site in its membrane-embedded part (Vgenopoulou et al. 2006). The coupling ion specificity (Na^+ vs. H^+) of complex I has important implications for its transport mechanism. Our finding that the smaller complex I from some enterobacteria transports Na^+ rather than H^+ raised the question whether the larger mitochondrial complex I also has the capacity to translocate Na^+. Primary Na^+ pumps couple an exergonic reaction to the translocation of Na^+, whereas Na^+ translocation by secondary Na^+/H^+ antiporters depends on the proton motive force generated by respiratory chain complexes. Here we show that oxidation of NADH by SMPs from the yeast *Yarrowia lipolytica* is coupled to protonophore-resistant Na^+ uptake, indicating that a redox-driven, primary Na^+ pump is operative in the inner mitochondrial membrane. This is the first report on the use of Na^+ as respiratory coupling ion in mitochondria. NADH dehydrogenase enriched from solubilized mitochondrial membranes was reconstituted into liposomes to unequivocally assign the redox-driven Na^+ translocation activity to the NADH:quinone segment of the respiratory chain. It is proposed that Na^+ transport is an intrinsic catalytic property of complex I from *Y. lipolytica*.

Materials and methods

Growth of *Yarrowia lipolytica*

Yarrowia lipolytica strain po1t (Madzak et al. 2000) was grown at 30°C in 1 L YPD media (1% Bacto-yeast extract, 2% Bacto-peptone and 2% glucose) in a 5 L baffled Erlenmeyer flask for 18–22 h to the late exponential phase. The cells were harvested by centrifugation and washed with 100 mL deionised water. The cell suspension was frozen in liquid nitrogen and stored at −80°C.

Isolation of mitochondria

All steps were carried out at 4°C. *Y. lipolytica* cells (0.5 g mL^{-1}) in 20 mM HEPES/KOH, pH 7.4, 0.6 M D-mannitol, 1 mM DL-dithiotreitol, 2 mM phenylmethanesulfonyl fluoride (PMSF), and 3 mM benzamidine were mixed with an equal volume of acid-washed glass beads (425–600 microns) and broken in a mix mill (TissueLyser, Qiagen) at 30 Hz for 15 min. The supernatant was combined with 15 ml buffer used to rinse the glass beads, and PMSF was added to a final concentration of 4 mM. Non-broken cells were removed by centrifugation (4,300×*g*, 20 min), and the crude extract was centrifuged at 36,000×*g* for 30 min. The supernatant was centrifuged at 150,000×*g* for 90 min to collect the mitochondria which were resuspended to a concentration of 30 mg protein mL^{-1} and stored in liquid N_2.

Preparation of submitochondrial particles and enrichment of complex I

All steps were carried out in the anaerobe chamber (COY Laboratory; 95% N_2, 5% H_2). Mitochondria were diluted fivefold with Tris buffer (10 mM Tris–HCl, pH 7.4, 50 mM KCl, 10% glycerol, 1 mM PMSF). The suspension was passed through the French Press Cell (AMICON) flushed with N_2. Ultracentrifugation (150,000×*g*, 80 min) yielded SMPs in the pellet and a soluble fraction containing cytochrome *c* released during the breakage of mitochondria. SMPs for Na^+ transport measurements were resuspended in Tris buffer. To enrich complex I, SMPs (20 mg protein mL^{-1}) in MOPS buffer (20 mM MOPS/KOH, pH 7.0, 50 mM KCl, 1 mM K_2EDTA, 1 mM PMSF) were solubilized with 0.5 g dodecylmaltoside (DDM) g^{-1} protein (Djafarzadeh et al. 2000) and centrifuged at 150,000×*g* for 60 min. The supernatant was loaded on a 15 mL anion exchange chromatography column (DEAE-Sepharose CL-

6B, Amersham Biosciences) equilibrated with buffer (25 mM MOPS/KOH, pH 7.0, 1 mM K_2EDTA, 0.05% DDM) containing 70 mM KCl. The column was washed at a flow rate of 1.5 mL min^{-1} with two bed volumes of buffer containing 70 and 100 mM KCl, respectively. A linear gradient of 30 mL from 100 to 500 mM KCl was applied to elute complex I around 300 mM KCl. Fractions which exhibited rotenone-sensitive quinol formation activity were combined and reconstituted into proteoliposomes.

Preparation of proteoliposomes

Enriched complex I from *Y. lipolytica* or the F1F0 ATPase from *Escherichia coli* were reconstituted by a dilution protocol under exclusion of O_2 (Gemperli et al. 2002). To a lipid film of 20 mg L-α-phosphatidylcholine (from soybean, Type II-S, 14–23% as choline, Sigma), the solubilized proteins were added drop-wise under stirring until the lipids were fully dispersed. The protein-lipid-detergent mixture was diluted at least tenfold by adding buffer (10 mM Tris–HCl, pH 7.4, 50 mM KCl, 10% glycerol) at 30 drops min^{-1}. Below the critical micelle concentration of DDM, proteoliposomes were formed which were collected by ultracentrifugation at 150,000×*g* over night and resuspended in 1–2 mL buffer. Enriched complex I was also reconstituted with buffer containing 5 mM NaCl. The protein to lipid ratio was 1:40 for enriched complex I, and 1:50 for the F1F0 ATPase purified according to Ishmukhametov et al. (2005). Prior to reconstitution, the ATPase was desalted using a NAP-10 column (GE Healthcare).

Transport measurements

If not indicated otherwise, Na^+ transport was followed in 0.3 mL 10 mM Tris–HCl, pH 7.4, 50 mM KCl, 10% glycerol within less than 3 h after the preparation of SMPs or proteoliposomes. To SMPs (1.7 mg protein mL^{-1}) or proteoliposomes (0.2 mg protein I mL^{-1}), 5 mM Na^+ or 5 mM $^{22}Na^+$ and 0.1 mM NADH were added to start the reaction. At indicated times, aliquots (70 µl) were passed through a Dowex cationic exchange column, and the internal Na^+ content of the vesicles was determined by atomic absorption spectroscopy (Gemperli et al. 2002) or γ-counting using 5 mM ^{22}NaCl (0.37 MBq) (Gemperli et al. 2002). Generation of a transmembrane potential (ΔΨ) by reconstituted complex I was followed using the voltage-sensitive dye oxonol VI. Proteoliposomes were prepared in buffer (100 mM potassium phosphate, pH 7.5, 0.05 mM EDTA, 10% glycerol, 0.07 mM Na^+) as described above. To 41 µg reconstituted complex I in 1 mL buffer containing 10 µM oxonol VI, 0.1 mM ubiquinone-1 (2,3-dimethoxy-5-methyl-6-isoprenyl-1,4-benzoquinone; Q1), 5 mM Na^+ or 10 µM monensin were added as indicated. The reaction mixtures were kept in the anaerobe chamber for 6 min prior to the measurements which were performed under air. To start the reaction, NADH (0.16 mM) was added to the stirred assay mixture, and the formation of ΔΨ was estimated from the difference in absorbance at 625–587 nm on a Shimadzu UV-3000 dual-wavelength spectrophotometer. If not indicated otherwise, proton transport was followed by fluorescence quenching of 1 µM 9-amino-6-chloro-2-methoxyacridin (ACMA) in 10 mM HEPES-KOH, pH 7.5, 0.1 M KCl, 5 mM $MgCl_2$ (Laubinger and Dimroth 1988). The proton-translocating F1F0 ATPase which was used in control reactions exhibited a specific ATP hydrolysis activity of 0.18 µmol min^{-1} mg^{-1}. Upon modification with 0.5 mM N,N′-di-cyclohexylcarbodiimide (DCCD) (Weber and Senior 2003), the ATPase activity decreased to 0.06 µmol min^{-1} mg^{-1}, indicating that 66% of the hydrolytic activity was coupled to proton transport. Assuming a ratio of 4 protons translocated per ATP hydrolyzed (Weber and Senior 2003), the calculated proton transport activity was $(0.12 \times 4 = 0.48)$ µmol min^{-1} mg^{-1}. During ATP hydrolysis, a transmembrane voltage of at least 30 mV was generated (Gemperli et al. 2003), demonstrating that a proton gradient was maintained across the liposomal membrane. Please note that transport of H^+ and Na^+ into proteoliposomes was followed in the presence of chloride which promotes cation accumulation by acting as an electrophoretic counter-ion, while measurements of ΔΨ were performed in the presence of phosphate which does not diffuse through the liposomal membrane (Gemperli et al. 2002).

Analytical methods

NADH oxidation and reduction of Q1 were followed simultaneously on a diode array spectrophotometer (Agilent 8452A UV-visible system). The assay buffer (1 mL 50 mM Tris–HCl, pH 7.5, 10 mM KCl) containing 0.1 mM Q1, 0.1 mM NADH and 0.1 mM rotenone (if indicated) was repeatedly degassed under vacuum and flushed with N_2 using a quartz cuvette sealed with a rubber stopper. To study the influence of pH on activity, 50 mM potassium phosphate (pH 6.0, 6.5 or 7.0) or 50 mM Tris–HCl (pH 7.5 or 8.0) were used as buffers. The residual Na^+ concentration in assay buffers was 0.03 mM. The reaction was started by adding enriched complex I with a gas-tight syringe. Oxidation of NADH was followed at 340 nm ($\varepsilon_{340} = 6.22$ mM^{-1} cm^{-1}). Formation of ubiquinol-1 (QH_2) was determined from the difference in absorbance at the wavelength pair 248 and 268 nm ($\Delta\varepsilon_{248-268} = 7.8$ mM^{-1} cm^{-1}). Protein was determined by the bicinchoninic acid method (Smith et al. 1985) using reagent purchased from Pierce. Bovine serum albumin

was used as standard. Acid-labile sulfide was determined according to Beinert (1983). For UV-Vis spectroscopy, enriched complex I was concentrated with Ultrafree-MC filters (cutoff 30,000 Da, Millipore) in the anaerobe chamber. Heme concentrations were calculated from the difference in absorbance of the dithionite-reduced minus dehydroascorbate-oxidized forms using 10.4 mM^{-1} cm^{-1} at A_{605} (heme *a*) and 28.5 mM^{-1} cm^{-1} at A_{563} (heme *b*) as extinction coefficients (Rieske 1967). Tricine-buffered SDS-PAGE was performed with 10% polyacrylamide (Schägger and von Jagow 1987).

Mass spectroscopy

Gel pieces from SDS-PAGE containing proteins stained with Coomassie were washed twice with 100 µl 100 mM NH_4HCO_3 in H_2O:acetonitrile (1:1) and once with 50 µl acetonitrile. Trypsin (100 ng) in 10 µl buffer (10 mM Tris, pH 8.2, 2 mM $CaCl_2$) was added to the gel pieces. 10 µl buffer was added and proteins were digested over night at 37°C. The supernatant containing polypeptides was recovered and combined with extracts obtained by treatment of gel pieces twice with 100 µl 0.1% trifluoroacetic acid (TFA) in H_2O:acetonitrile (1:1). After evaporation of the solvent, the polypeptides were dissolved in 25 µl 0.1% formic acid. Matrix-assisted laser desorption/ionization tandem mass spectrometry (MALDI/MS/MS) was performed on a Ultraflex II mass spectrometer (Bruker). Aliquots of 10 µl were desalted using Ziptip C18 filters (Millipore) and mixed in a 1:1 ratio with matrix solution (5 mg ml^{-1} α-cyano − 4-hydroxycinamic acid in 0.1% TFA and 50% acetonitrile). Protein database searches were performed using the Mascot program, version 2.2.0 (Matrix Science).

Results

A redox-driven Na^+ pump in the respiratory chain from *Yarrowia lipolytica*

We first studied Na^+ transport by vesicles obtained from mitochondria (submitochondrial particles, or SMPs). In the presence of 50 µM rotenone, the Q reduction activity of SMPs was inhibited by 83 ± 17% ($n = 3$), indicating that the vesicles were predominantly oriented inside-out, with the NADH-oxidizing part of complex I exposed to the external buffer. In the absence of a respiratory substrate, there was some uptake of Na^+ by SMPs to a stable plateau of 11 nmol Na^+ mg^{-1} protein driven by the chemical Na^+ concentration gradient imposed at the start of the reaction. Addition of NADH significantly increased the amount of Na^+ entrapped in SMPs during 1 min to 100–120 nmol Na^+ mg^{-1} (Fig. 1), indicating that respiration stimulated Na^+ transport. Oxidation of succinate by SMPs did not result in an accumulation of Na^+ in the vesicles (electronic supplementary material, Fig. S1). The SMPs exhibited an NADH oxidation activity of 0.2 µmol min^{-1} mg^{-1}. In the presence of the Na^+-conducting ionophore gramicidin (Gemperli et al. 2002), the Na^+ content of the SMPs decreased to 6 nmol mg^{-1} protein, demonstrating that Na^+ did not bind unspecifically but was entrapped by the submitochondrial vesicles. Note that cytochrome *c* was not included in the assay, hence electron transfer from NADH to O_2 was catalyzed by complex I or the alternative NADH dehydrogenase (Kerscher et al. 2001) and the quinol oxidase (Akimenko et al. 2003) which is found in *Y. lipolytica* in addition to the cytochrome *c* oxidase. NADH-driven Na^+ accumulation was also observed with rat liver SMPs which exhibited a transport activity of 20–25 nmol Na^+ min^{-1} mg protein^{-1} (Douglas and Cockrell 1974). In this study, Na^+ uptake was completely abolished in the presence of the protonophore carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; 4 nmol/4.4 mg mitochondrial protein), and the authors concluded that the observed Na^+ transport was catalyzed by secondary Na^+/H^+ exchangers (Douglas and Cockrell 1974). With SMPs from *Y. lipolytica*, NADH-driven Na^+ transport was not inhibited by the protonophores carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Fig. 1) or FCCP (not shown) at concentrations of 30 nmol/0.5 mg protein. We searched for a homolog of the Na^+-translocating NADH:quinone reductase (Na^+-NQR) in the genome from *Y. lipolytica* (Dujon et al. 2004). With the exception of a putative NADH dehydrogenase

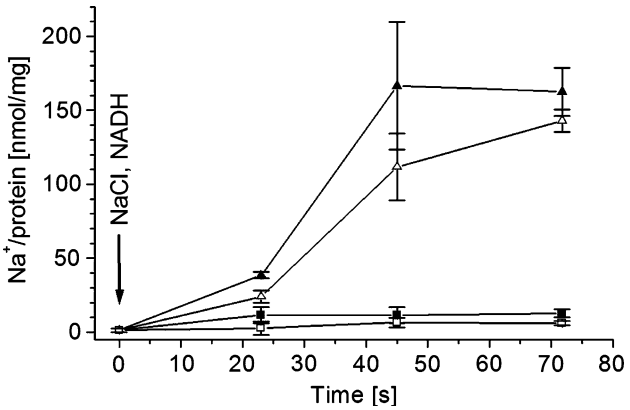

Fig. 1 Respiratory NADH oxidation drives Na^+ translocation. Na^+ uptake by submitochondrial particles in the presence of 5 mM NaCl was followed without added NADH (*closed square*), with 0.1 mM NADH (*open triangle*), with 0.1 mM NADH and 0.1 mM CCCP (*closed triangle*), or with 0.1 mM NADH and gramicidin (5 µg mg^{-1} protein; *open square*). The Na^+ content of the particles prior to addition of NaCl was 2 nmol mg^{-1} protein. Mean values from three experiments are presented

Table 1 Search for homologs of subunits of the Na^+-translocating NADH:quinone oxidoreductase (Na^+-NQR) in *Yarrowia lipolytica*

Query	Subject Accession number (function)	Partial amino acid sequence of the *Y. lipolytica* protein (total number of amino acids)
NqrA	YALI0C21131g(putative nuclear matrix protein)	559 **KKPDLPSIGTPQVYIDKLSELDKV** 582 (724)
NqrB	YALI0F16940g (unknown)	1381 **N**TQV**V**GII**TGSP** 13921425 **A**FEVKG**NV**VDKNG**NP**QWTVGG**HWH** 1448 (1582)
NqrC	YALI0E15554g (putative histidine kinase)	353 **TPHAI**PQQPHLQ**TPG**QDAFPGP**PS**AT**D** 379
		384 **YDEDTKPARK**RRDI**G**SAGS**SQHSV**A**G** 409 (913)
NqrD	YALI0B18502g (unknown)	110 **WYNPSSSTLLAYSAASLVAIVPYTLIVMKP** 139 (188)
NqrE	YALI0E33583g(putative urea transport protein)	51 **MV**AN**R**AVGVGLT**A**SAV**F**SSWMWA**N**ETLY**G**A**V** 81
		84 **YNF**GM**S**GPFW**F**AA**G**LSFHI**A**LMTVV**GI**QV**KLK** 115 (761)
NqrF	YALI0D11330g (putative NADH dehydrogenase)	81 **L**TK**Y**VTPKGSNVV**RPY**TPVSD**P**DSK**G**EFE**L**VV 113
		118 **GKMS**KHI**H**E**LKEGD**TLSFK**GP**IIKYQWQPN 147
		148 LHK**E**ITLI**G**A**G**T**G**I**TP** 163
		168 **I**SAIN**K**NPED**KTK**VNLF**YG** 186
		200 **D**AIAKA**K**PQQ**F**NV**H**YF**L** 216
		219 **PSDNWKG**EN**GFI** 230
		232 **E**EFIKGNSPAA**D**SDNVKVFV**CGPP**PFYKA**ISG** 263 (291)

The six subunits (NqrA to NqrF) of the Na^+-NQR from *Vibrio alginolyticus* (accession number BAA22910) were compared with annotated and hypothetical proteins encoded by the genome from *Yarrowia lipolytica* strain CLIB122 (Dujon et al. 2004) using the BLASTP program version 2.2.6 (Altschul et al. 1997) at http://cbi.labri.fr/Genolevures/. Only *Y. lipolytica* proteins exhibiting the highest similarities to Nqr subunits are shown. Bold letters indicate amino acids in the *Y. lipolytica* protein which are also present in the corresponding Nqr subunit used as query sequence in the BLASTP search (*Y. lipolytica* numbering). The total lengths of the *Y. lipolytica* proteins are given in brackets

(YALI0D11330g) which is related to the flavin-binding domain of NqrF, no homologs of Nqr subunits were found in *Y. lipolytica* (Table 1). Our conclusion that *Y. lipolytica* does not contain a respiratory complex of the Na^+-NQR-type is further supported by the observation that NADH oxidation by mitochondrial membranes was not inhibited by 100 μM Ag^+, a specific inhibitor of the Na^+-NQR (not shown). These findings prompted us to speculate that NADH-driven Na^+ transport in SMPs was catalyzed by complex I. To test this hypothesis, we obtained a complex I preparation from *Y. lipolytica* which coupled the oxidation of NADH to the two-electron reduction of Q, reconstituted the complex into proteoliposomes, and analyzed its coupling cation specificity.

Properties of enriched complex I

As observed previously with a bacterial complex I (Gemperli et al. 2002), highest quinol formation activities of complex I from *Y. lipolytica* were achieved by excluding O_2 during the purification. Anoxically prepared SMPs exhibited a quinol formation activity of 0.20 μmol min^{-1} mg^{-1} (electronic supplementary material, Table S1), compared to 0.06 μmol min^{-1} mg^{-1} observed with SMPs prepared under air. We used dodecylmaltoside to solubilize the mitochondrial membrane proteins which were separated on a weak anionic exchange column in the anaerobe chamber. NADH oxidation activity was detected in several fractions, but only proteins eluting around 0.3 M KCl exhibited NADH dehydrogenase activity coupled to the two-electron reduction of Q1 (Fig. 2). In these fractions, formation of quinol was completely inhibited by rotenone (50 μM), indicating that quinone reduction was catalyzed by complex I. This was further confirmed by SDS-PAGE and mass spectroscopic analysis of the fractions showing rotenone-sensitive NADH:Q1 oxidoreductase activity. A prominent polypeptide with a molecular weight of 75 kDa represented the NUAM subunit of the *Y. lipolytica* complex I (Djafarzadeh et al. 2000), the homolog of the 75 kDa subunit from bovine complex I (electronic supplementary material, Fig. S2). The NUAM subunit was also identified in the NADH dehydrogenase fraction which did not bind to the anionic exchange column, indicating partial dissociation of the peripheral fragment of complex I. The fraction showing rotenone-sensitive NADH:Q1 reductase activity obtained by anionic exchange chromatography contained 1.2 nmol heme *a* mg^{-1} protein and 0.1 nmol heme *b* mg^{-1} protein, indicating the presence of the heme *a*-containing complex IV and the heme *b*-containing complex III. Approximately 50% of the heme iron was in the Fe^{2+} state (Fig. 3), suggesting that complex I enriched in the anaerobe chamber also was (partially) reduced.

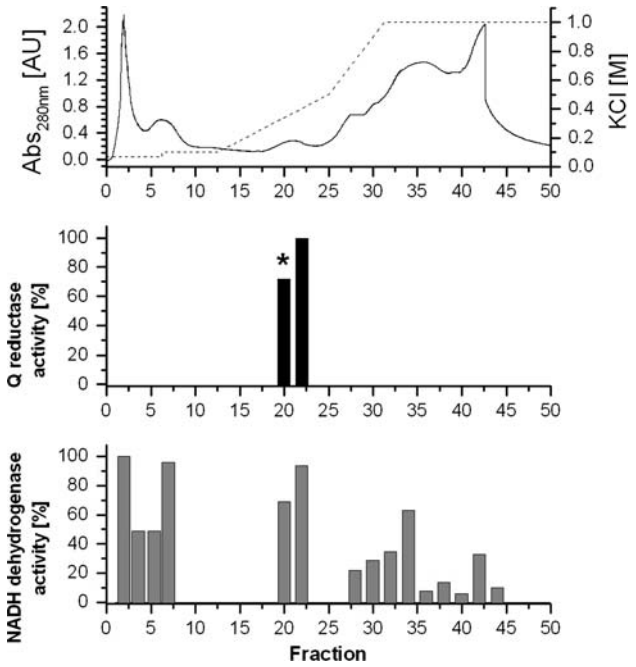

Fig. 2 Enrichment of complex I by anionic exchange chromatography. *Upper panel*, solubilized mitochondrial proteins (*black trace*) were eluted from the DEAE column with a gradient from 0.07–1.0 M KCl (*dashed trace*) under exclusion of O_2. The absorbance at 280 nm was monitored. *Middle panel*, Q reductase activity of fractions (monitored as ubiquinol formation activity). Hundred percent activity corresponds to 2 μmol min^{-1} mg^{-1}. *Lower panel*, NADH dehydrogenase activity of fractions. Hundred percent activity corresponds to 3 μmol min^{-1} mg^{-1}. Only fractions with Q reductase activity which could be inhibited by rotenone (*) were used for transport studies

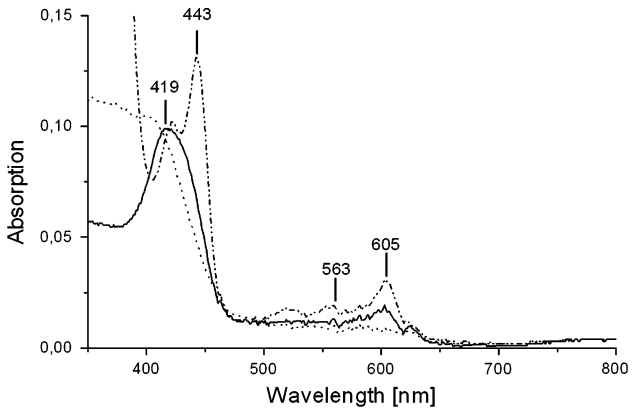

Fig. 3 Visible absorption spectra of enriched complex I from DEAE anionic exchange chromatography. *Solid line*, enriched complex I (3.2 mg ml^{-1}) as isolated; *dotted line*, with excess dehydroascorbate; *dashed line*, with excess dithionite

Na^+ stimulates NADH oxidation by enriched complex I

In Na^+-translocating NADH dehydrogenases, electron transfer from NADH to quinone is strongly stimulated by Na^+ (Gemperli et al. 2002; Hayashi and Unemoto 2004) which suggests binding of Na^+ to a specific site relevant for

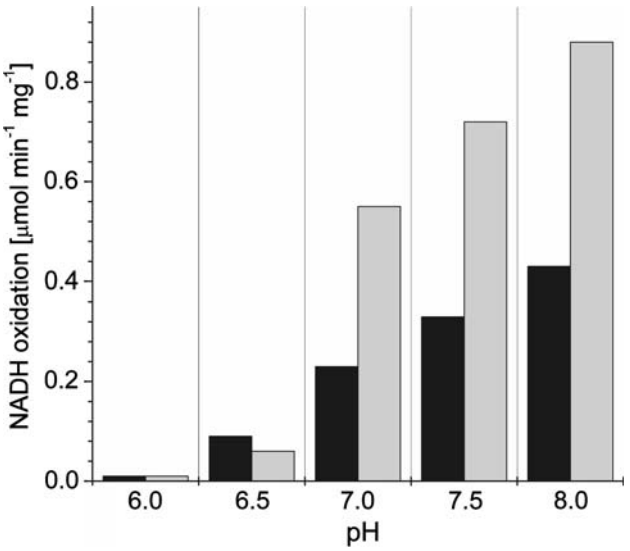

Fig. 4 Na^+ stimulation of NADH dehydrogenase activity of enriched complex I NADH dehydrogenase activities were determined in the presence of 0.03 mM Na^+ (*black bars*) or 10 mM Na^+ (*gray bars*). Ubiquinone-1 served as electron acceptor

cation pumping. The activation by Na^+ is expected to be more prominent under slightly alkaline conditions, since protons may compete with Na^+ for the binding to Na^+ pumps (Dimroth 1997). At pH 6.0 and 6.5, the NADH dehydrogenase activity of enriched complex I from *Y. lipolytica* was very low and not influenced by the Na^+ concentration. In the pH range from 7.0–8.0, Na^+ stimulated the NADH dehydrogenase activity approximately twofold, with 0.23 μmol min^{-1} mg^{-1} (0.03 mM Na^+) versus 0.55 μmol min^{-1} mg^{-1} (10 mM Na^+) at pH 7.0, and 0.43 μmol min^{-1} mg^{-1} (0.03 mM Na^+) versus 0.88 μmol min^{-1} mg^{-1} (10 mM Na^+) at pH 8.0 (Fig. 4). It is noteworthy that the matrix pH of mitochondria is around 8 (Nicholls and Ferguson 2002). We concluded that binding of Na^+ to enriched *Y. lipolytica* complex I promoted electron transfer from NADH to Q1, as reported earlier for complex I from the enterobacterium *Klebsiella pneumoniae* (Gemperli et al. 2002).

Na^+ transport

We now asked whether our complex I preparation which was stimulated by Na^+ in the electron transfer reaction could also use Na^+ as a coupling ion in the transport reaction performed at pH 7.5. The complex was reconstituted into proteoliposomes to follow NADH-driven Na^+ uptake. As observed previously with a bacterial complex I (Gemperli et al. 2002), endogenous quinones from the soy bean lipids used for reconstitution served as electron acceptors. Addition of NADH led to a rapid uptake of 0.5 μmol Na^+ mg^{-1} which was inhibited by rotenone (Fig. 5a). Note that uptake was

followed in the presence of a Na^+ concentration gradient ($Na^+_{out} \gg Na^+_{in}$), since the analysis by atomic absorption spectroscopy required low internal Na^+ concentration of the proteoliposomes at the start of the reaction. We also followed Na^+ transport at $Na^+_{in} = Na^+_{out} = 5$ mM and added $^{22}Na^+$ as radioactive tracer to the external lumen. Again, the oxidation of NADH by reconstituted complex I was coupled to the translocation of $^{22}Na^+$ (Fig. 5b). Uptake of $^{22}Na^+$ did not collapse in the presence of the protonophore CCCP, demonstrating that the observed transport did not result from the combined action of a proton-translocating complex I and a Na^+/H^+ antiporter (Fig. 5b).

No uptake of Na^+ was observed at pH 6.5 (data not shown). Next we investigated whether the transport of Na^+ during NADH:quinone oxidoreduction by the reconstituted NADH dehydrogenase from *Y. lipolytica* resulted in the formation of a transmembrane potential ($\Delta\Psi$) using the voltage-sensitive dye oxonol VI. Generation of $\Delta\Psi$ was dependent on Na^+ (5 mM) present in the external lumen of the proteoliposomes, and was stimulated by adding the electron acceptor Q1. No $\Delta\Psi$ was established in the presence of 0.07 mM Na^+, or 5 mM Na^+ and 10 μM monensin (Fig. 5c). Monensin is a Na^+-specific ionophore that normally catalyzes Na^+/H^+ exchange across a membrane. It has been shown that monensin which does not collapse a Na^+-coupled $\Delta\Psi$ generated by a Na^+/H^+ antiporter dissipates a Na^+-coupled $\Delta\Psi$ generated by a primary Na^+ pump (Efiok and Webster 1990). We conclude that *Y. lipolytica* mitochondria contain an electrogenic NADH dehydrogenase which specifically requires sodium ions for the build-up of a transmembrane potential. The NADH-dependent Na^+ transport activity increased significantly upon purification from 0.12 μmol min^{-1} mg^{-1} in SMPs (Fig. 1) to 2.0 μmol min^{-1} mg^{-1} with the reconstituted NADH dehydrogenase (Fig. 5b), concurrent with an increase in Q reduction activity (electronic supplementary material, Table S1). A comparison of the specific quinol formation activity of reconstituted complex I (1.4 μmol min^{-1} mg^{-1}) with the rate of Na^+ translocation (2.0 μmol min^{-1} mg^{-1}) gave a transport ratio of 0.8 Na^+/electron transferred. The alternative NADH dehydrogenase (NDH II) of *Y. lipolytica* which is attached to the inner mitochondrial membrane does not act as a cation pump (Kerscher et al. 2001). Kerscher and coworkers also demonstrated that complex I and NDH II are the only respiratory NADH dehydrogenases found in mitochondria from *Y. lipolytica* (Kerscher et al. 2001). We

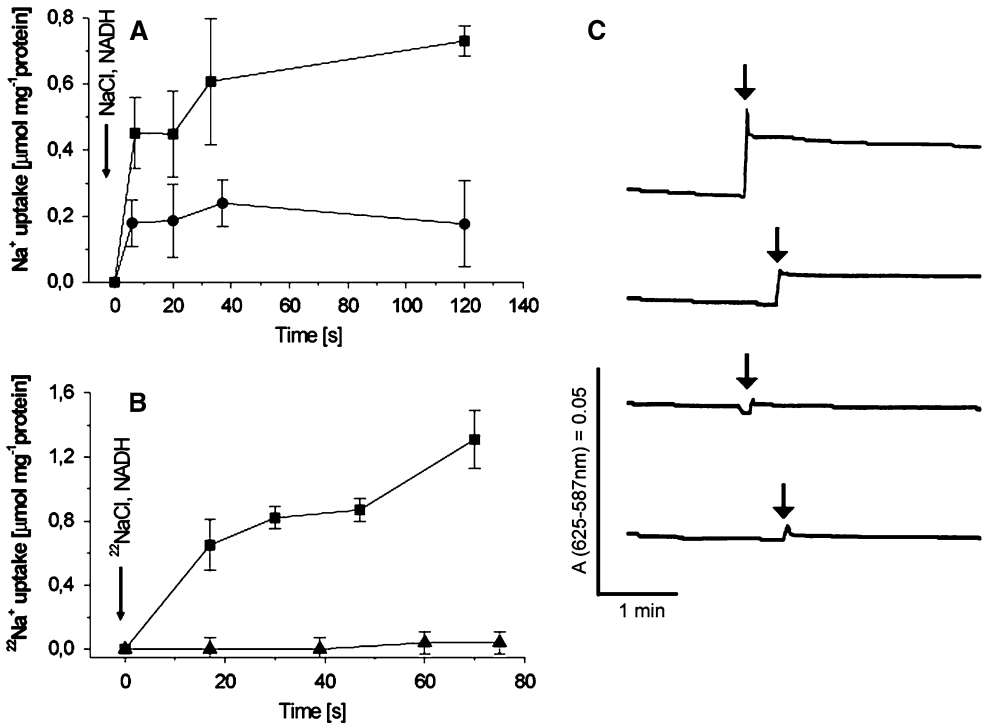

Fig. 5 Electrogenic Na^+ transport by reconstituted complex I. Na^+ transport was followed by atomic absorption spectroscopy (**a**) or γ-counting of $^{22}Na^+$ (**b**) in the presence of 5 mM NaCl. Mean values from three experiments are presented. **a**, with 0.1 mM NADH in the absence (*square*) or presence of 0.1 mM rotenone (*circle*). In **a**, the data were corrected for the endogenous Na^+ content of the proteoliposomes in the absence of substrates (0.42 μmol mg^{-1} protein). **b**, without (*triangle*) or with 0.1 mM NADH and 0.1 mM CCCP (*square*). In B, the internal Na^+ concentration at the start of the reaction was 5 mM. In **c**, the absorption difference of 0.01 mM oxonol VI (625–587 nm) indicated the formation of a transmembrane potential during oxidation of 0.16 mM NADH (*arrow*) by reconstituted complex I. The assay contained (traces from *top* to *bottom*): 5 mM Na^+ and 0.1 mM ubiquinone-1 (Q1); 5 mM Na^+; 0.07 mM Na^+ and 0.1 mM Q1; 5 mM Na^+, 0.1 mM Q1 and 10 μM monensin

therefore propose that the rotenone-sensitive, Na^+-translocating, electrogenic NADH dehydrogenase identified in mitochondria from *Y. lipolytica* represents the complex I of the respiratory chain.

Proton transport

To investigate whether complex I translocates H^+, it was crucial to show that the proteoliposomes used for Na^+ transport experiments were also suited for the analysis of transmembrane proton movements. Proton transport was followed by ACMA quenching using the H^+-translocating F1F0 ATPase as control. The pH of the internal and external lumen was 7.5. Addition of ATP elicited quenching of the fluorescence signal, indicating that a pH gradient (acidic inside) was established by the ATPase. CCCP restored the signal, demonstrating that the pH gradient across the liposomal membrane resulted from the vectorial transport of protons from the external to the internal lumen of the proteoliposomes (Fig. 6, upper trace). In contrast, hardly any quenching was observed upon addition of NADH to proteoliposomes containing complex I. Assuming a coupling ratio of 3.6 H^+ translocated/2 electron transferred by the *Y. lipolytica* complex I (Galkin et al. 2006), we would expect a proton transport activity of (3.6 × 0.33 =) 1.19 μmol min^{-1} mg^{-1} based on the quinol formation activity of our complex I preparation. Considering that the ATPase had an activity of 0.48 μmol H^+ translocated min^{-1} mg^{-1}, proton transport by complex I should have quenched the fluorescence of ACMA to an extent observed with the ATPase in the control reaction. We also tested whether reductive activation of complex I would be required to observe proton transport (Kotlyar and Vinogradov 1990) and added a second aliquot of NADH, but no significant quenching of ACMA fluorescence was observed unless Q1 was also added. However, this Q1-dependent quenching reaction was not accompanied by transmembrane proton movements since it could not be reversed by CCCP (Fig. 6, middle trace). In fact, Q1 strongly decreased the fluorescence signal even in the absence of NADH, and again, addition of NADH did not significantly quench the remaining fluorescence (Fig. 6, lower trace). Proton transport studies with reconstituted complex I performed at pH 6.5 gave similar results (data not shown).It is concluded that there is no evidence for proton transport by reconstituted complex I in this artificial membrane system which allows following NADH-driven Na^+ transport (Fig. 5).

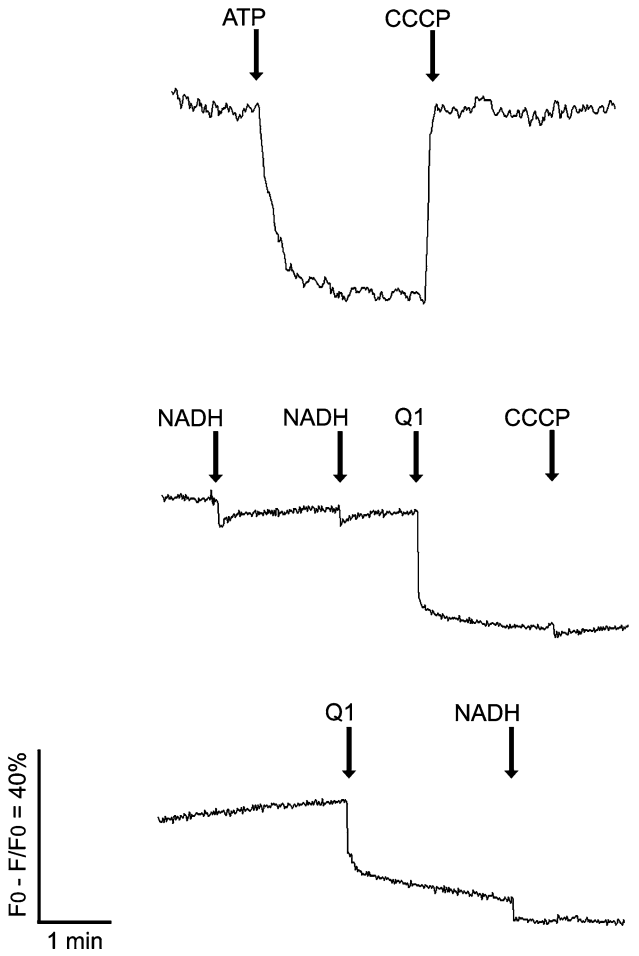

Fig. 6 Proton transport followed by quenching of ACMA fluorescence. To 48 μg reconstituted ATPase, 2 mM ATP and 10 μM CCCP were added as indicated (*upper trace*). To 42 μg reconstituted complex I, 0.6 mM NADH (two subsequent additions), 0.1 mM Q1 and 1 μM CCCP (*middle trace*), or 0.1 mM Q1 and 0.6 mM NADH (*lower trace*) were added as indicated

Discussion

With the discovery of Na^+/H^+ antiporters in mitochondria (Mitchell and Moyle 1967), it became obvious that a Na^+ gradient is maintained across the inner mitochondrial membrane at the expense of the proton motive force. Respiring mitochondria from vertebrates established a Na^+ gradient of ≈ 8 ($Na^+_{out} \gg Na^+_{in}$) which collapsed when the proton motive force was dissipated by protonophores (Douglas and Cockrell 1974; Jung et al. 1992), suggesting that mitochondrial Na^+ transport is exclusively catalyzed by secondary Na^+ transporters. In the yeast *Yarrowia lipolytica*, protonophores did not inhibit respiration-driven Na^+ transport in mitochondrial membranes, indicating that a redox-driven Na^+ pump is operative in this lower eukaryote. Using solubilized mitochondrial proteins reconstituted into proteoliposomes, we confined the redox-driven Na^+ transport activity to the NADH:quinone segment of the respiratory chain from *Y. lipolytica*, and propose that it is an intrinsic catalytic property of complex I. As observed previously with complex I from *Klebsiella*

pneumoniae (Gemperli et al. 2003), Na^+ transport by complex I from *Y. lipolytica* was coupled to the generation of a transmembrane potential. We did not find evidence for proton transport by complex I under our experimental conditions. How can we reconcile our observations with previous investigations?

We are aware of three reports on proton transport using solubilized or purified complex I from mitochondria reconstituted into proteoliposomes (Ragan and Hinkle 1975; Dröse et al. 2005; Galkin et al. 2006). In these studies, the possibility that other cations are transported by complex I instead of (or in addition to) protons was not considered (Ragan and Hinkle 1975), or could not be excluded unequivocally (Dröse et al. 2005). The electron transfer reaction catalyzed by complex I results in the net consumption of one H^+ per NADH oxidized for the formation of ubiquinol (QH_2) from ubiquinone (Q). If Q like NADH binds at the matrix side of complex I, electron transfer by reconstituted complex I will be accompanied by an alkalization of the external lumen of the proteoliposomes. Indeed, the reconstituted complex I consumed 1 H^+ per NADH oxidized in the presence of a protonophore, demonstrating that proton uptake by complex I is not necessarily coupled to proton transport (Ragan and Hinkle 1975; Galkin et al. 2006). Brandt and coworkers followed proton uptake by the purified and reconstituted complex I from *Y. lipolytica* using the pH-sensitive fluorescence dye, ACMA. Notably, NADH-dependent quenching of ACMA by reconstituted complex I required short-chain quinones like Q1 (Dröse et al. 2005) which very efficiently quenched the fluorescence of ACMA even without added NADH (see Fig. 6 in this study, and Fig. 3 in Dröse et al. (2005)). Hence, it is questionable whether the quenching of ACMA fluorescence during electron transfer from NADH to short-chain quinones described in Dröse et al. (2005) exclusively resulted from vectorial proton translocation by complex I, as stated by the authors. Neither of the three studies on proton transport by reconstituted complex I (Ragan and Hinkle 1975; Dröse et al. 2005; Galkin et al. 2006) was based on a method to determine the proton concentration in the internal lumen of the proteoliposomes during NADH oxidation by complex I. In contrast, the results described here are based on the analysis of Na^+ entrapped in SMPs or proteoliposomes. NADH oxidation by proteoliposomes containing complex I from *Y. lipolytica* was clearly linked to Na^+ transport but did not result in a significant quenching of ACMA fluorescence. Under our experimental conditions, Na^+ rather than H^+ was the preferred coupling cation of complex I, but proton translocation should be considered nevertheless. Protons may be transported in addition to Na^+, or the cation selectivity may switch from Na^+ to H^+ under conditions of acidic pH and low [Na^+], as observed with Na^+-translocating F1F0 ATPases (Dimroth 1997). Neither proton nor Na^+ translocation was observed with reconstituted *Y. lipolytica* complex I at pH 6.5, but rates of NADH oxidation were very low under these conditions, and cation transport activities might have escaped detection. It is noteworthy that the bacterial complex I from *Klebsiella pneumoniae* exhibited NADH oxidation and Na^+ transport activities at pH 6.5 (Vgenopoulou et al. 2006).

The use of Na^+ as respiratory coupling cation in mitochondria seems to contradict the central tenet of bioenergetics which states that mitochondrial respiration is coupled to ATP synthesis via the proton motive force. On the other hand, bacterial respiratory chains provide many examples where H^+-dependent ATP synthases and respiratory proton and Na^+ pumps operate in the same membrane (Dimroth 1997). The proton and sodium ion cycles are linked by Na^+/H^+ antiporters which exchange H^+ and Na^+ across the membrane (Padan et al. 2004). They are involved in the homeostasis of H^+ and Na^+ and are found in membranes and organelles from cells of many different organisms. Based on studies with vertebrates, it has been assumed that the Na^+ gradient across the inner mitochondrial membrane solely results from the activity of Na^+/H^+ antiporters. Our study performed with the yeast *Y. lipolytica* indicates that the Na^+ cycle in mitochondria includes the NADH-driven extrusion of Na^+ which we propose to be catalyzed by complex I. It will be important to investigate whether the use of Na^+ as a coupling cation is restricted to complex I from lower eukaryotes, or whether the vertebrate complex I also shows the capacity to transport Na^+. The cation specificity (Na^+ vs. H^+) of complex I has fundamental implications for our understanding of its mechanism and its role in energy transduction and metabolism.The use of Na^+ as respiratory coupling cation might enable *Y. lipolytica* to efficiently adjust the mitochondrial proton and Na^+ concentrations in response to cellular demands.

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