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Proton pumping by complex I (NADH:ubiquinone oxidoreductase) from *Yarrowia lipolytica* reconstituted into proteoliposomes

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Abstract

The mechanism of energy converting NADH:ubiquinone oxidoreductase (complex I) is still unknown. A current controversy centers around the question whether electron transport of complex I is always linked to vectorial proton translocation or whether in some organisms the enzyme pumps sodium ions instead. To develop better experimental tools to elucidate its mechanism, we have reconstituted the affinity purified enzyme into proteoliposomes and monitored the generation of ΔpH and $\Delta\psi$. We tested several detergents to solubilize the asolectin used for liposome formation. Tightly coupled proteoliposomes containing highly active complex I were obtained by detergent removal with BioBeads after total solubilization of the phospholipids with n-octyl-β-D-glucopyranoside. We have used dyes to monitor the formation of the two components of the proton motive force, ΔpH and $\Delta\psi$, across the liposomal membrane, and analyzed the effects of inhibitors, uncouplers and ionophores on this process. We show that electron transfer of complex I of the lower eukaryote *Y. lipolytica* is clearly linked to proton translocation. While this study was not specifically designed to demonstrate possible additional sodium translocating properties of complex I, we did not find indications for primary or secondary Na^+ translocation by *Y. lipolytica* complex I.

Keywords: Mitochondria; Complex I; Reconstitution; H^+-pumping; Yarrowia lipolytica

1. Introduction

NADH:ubiquinone oxidoreductase (complex I) is in most organisms the first multiprotein complex in oxidative phosphorylation of the bacterial cytoplasma membrane and the mitochondrial inner membrane. Despite its central role in the generation of the protonmotive force Δp and the discovery of an increasing number of human diseases linked to this enzyme, neither its molecular structure nor the mechanism by which electron transfer is coupled to proton translocation across the inner mitochondrial membrane are known (for a recent review, see [1]). This is in part due to the complexity of mitochondrial complex I comprising 46 subunits in bovine heart [2] and at least 37 subunits in fungi [3]. A number of hypothetical mechanisms have been proposed how complex I might pump protons (see [4] for a review). The idea that iron–sulfur cluster N2 may be a critical part of the proton pump [4–6] and the observation of several different semiquinone species [7] has stimulated proposals [4,8], that are based on the reversal of the protonmotive Q-cycle operating in the cytochrome bc_1 complex (see [9] for a review). However, recent findings [1,10–12] rather favour a conformational mechanism of proton pumping. A pumping mechanism that operates by redox linked conformational changes has been put forward already some years ago [13].

A major obstacle towards the elucidation of the proton pumping mechanism of complex I has been that experimental systems suitable to analyze this process are scarce. For mitochondrial complex I, proton-translocation has been measured only in isolated rat liver mitochondria [14] and

Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; DBQ, *n*-decylubiquinone; LM, *n*-lauryl-β-D-maltoside; ETH 157, *N*,*N*′-dibenzyl-*N*,*N*′-diphenyl-1,2-phenylenedioxydiacetamide; FCCP, carbonyl-cyanide-*p*-trifluoro-methoxy-phenylhydrazone; HAR, hexaammineruthenium(III)-chloride; OG, *n*-octyl-β-D-glucopyranoside; Q-1, 2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone; TX-100, Triton X-100

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submitochondrial particles derived from bovine heart [15]. In both studies, a $H^+/2e^-$ stoichiometry of 4 was determined. Translocation of protons by the prokaryotic enzyme has been reported for different species including *Paracoccus denitrificans* [16], *Escherichia coli* [17] and *Rhodobacter capsulatus* [18]. Only recently, it has been shown that purified and reconstituted complex from *E. coli* pumps protons [19]. However, in a similar system, it was reported that complex I of the closely related enterobacterium *Klebsiella pneumoniae* pumps sodium ions rather than protons [20,21], a claim that has now been challenged by other authors [22].

In recent years, we have developed the strictly aerobic yeast *Yarrowia lipolytica* as a powerful tool to analyze the structure and function of mitochondrial complex I. A large number of functional mutations have already been generated in this organism [23–26]. We have shown previously that purified *Y. lipolytica* complex I can be reactivated by the addition of phospholipids [27] and that after reconstitution into proteoliposomes, ubiquinone reductase activity can be stimulated by the addition of uncoupler [28]. In this study, we have analyzed how variation of the reconstitution protocol affects the functional properties of complex I proteoliposomes and demonstrate that electron transfer of complex I from a lower eukaryote is linked to the generation of a proton motive force.

2. Material and methods

2.1. Materials

Asolectin (=total soy bean extract with 20% lecithin) was purchased from Avanti Polar Lipids (Alabaster, AL). *n*-lauryl-β-D-maltoside was obtained from Glycon (Luckenwalde, Germany) and octyl-β-D-glucopyranoside from BIOMOL (Hamburg, Germany). 9-amino-6-chloro-2-methoxyacridine (ACMA) and Oxonol VI (bis-(3-propyl-5-oxoisoxazol-4-yl) pentamethine Oxonol) were purchased from Molecular Probes Europe (Leiden, The Netherlands). Chelating Sepharose was from Pharmacia. The ionophores valinomycin, nigericin (Antibiotic K 178), monensin, ETH 157 (sodium ionophore II) and FCCP and all other detergents and chemicals were from Sigma. ACMA and Oxonol VI were dissolved in dimethylsulfoxide; ubiquinone derivatives, inhibitors and ionophores in ethanol.

2.2. Analytical methods

Protein concentrations were determined according to a modified Lowry protocol [29]. Phospholipid content of purified complex I was determined as total organic phosphate following a procedure described previously [27].

Table 1
Effect of solubilization method on proteoliposome formation

	Complex I recovery [a] (%)	Inside-out [a] %	RCR [a, b]
Onset	46–57	70–100	1.7–2.2
Total	49–60	40–65	3.1–5.8 [c]

[a] The range reflects the values obtained with different detergents (octylglucoside, Triton X-100, $C_{12}E_8$, laurylmaltoside) that were used to solubilize asolectin. The protein to phospholipids ratio was 1:50 (w/w) throughout.

[b] No respiratory control ratios (RCR) were obtained for $C_{12}E_8$. See text for details.

[c] These values were determined with different batches of proteoliposomes made with one representative complex I preparation. In some measurements with another preparation, even higher RCRs were observed (see Fig. 2).

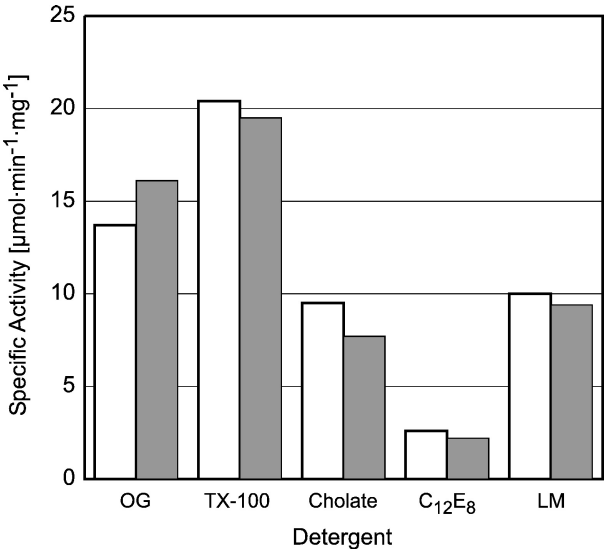

Fig. 1. Specific activities of complex I after reconstitution with different detergents. Proteoliposomes were prepared using 'onset' (open columns) or 'total' conditions (grey columns) and specific NADH:ubiquinone oxidoreductase activities of FCCP-uncoupled proteoliposomes were measured in 20 mM K^+-Mops, 50 mM KCl, pH 7.4 as detailed in Materials and methods. For the calculation of the specific activities, the complex I recovery and the percentage of inside-out oriented pumps were taken into account.

2.3. Yeast growth and preparation of mitochondrial membranes

Y. lipolytica strain PIPO [30] was grown overnight at 27 °C in a 10-l Biostat E fermenter (Braun, Melsungen) in modified YPD medium (2.5% glucose, 2% bactopeptone, 1% yeast extract). This strain contains a chromosomal copy (introduced by homologous recombination with plasmid pNK1.2 as described in [28]) of the modified *NUGM* gene, encoding a C-terminally his-tagged version of the 30-kDa subunit. Mitochondrial membranes from strain PIPO were prepared following the protocol of [31] with the modification detailed in [28].

2.4. Purification of complex I

Complex I was purified from *Y. lipolytica* strain PIPO as published previously [28] with one minor modification: the imidazole concentration of the buffer used for the equilibration and washing of the Ni^{2+}–NTA column was reduced to 55 mM. The combined complex I containing peak fractions of the gel filtration (TSKgel 4000SW) were concentrated to 7–10 mg/ml by centrifugation through Vivaspin cartridges (MWCO 100,000; Vivascience, UK) and stored in liquid nitrogen.

2.5. Complex I proteoliposomes

Optimal conditions for the reconstitution of complex I were evaluated following a guideline detailed by Rigaud and coworkers [32]. In general, variations included the detergents used for solubilization of phospholipids, protein-to-lipid-ratios, state of phospholipid solubilization and other factors affecting detergent-removal (temperature, addition of Bio-Beads see below). For an empirical approach, preformed liposomes were generated: asolectin obtained as a solution in chloroform was placed into glass vials and the solvent was evaporated under a constant stream of argon. Traces of chloroform were removed under vacuum. The phospholipids were finally dissolved to a concentration of 10 mg/ml in buffer containing 1.6% OG (w/v). This detergent concentration was usually sufficient to solubilize the phospholipids completely. If this was not achieved (as judged by turbidity), further OG was added until the solution became clear. The OG-solubilized phospholipids were placed into dialysis tubes and were dialyzed against 100 volumes of the respective buffer at 4 °C. After 4 h, the buffer was exchanged and dialysis was continued over night.

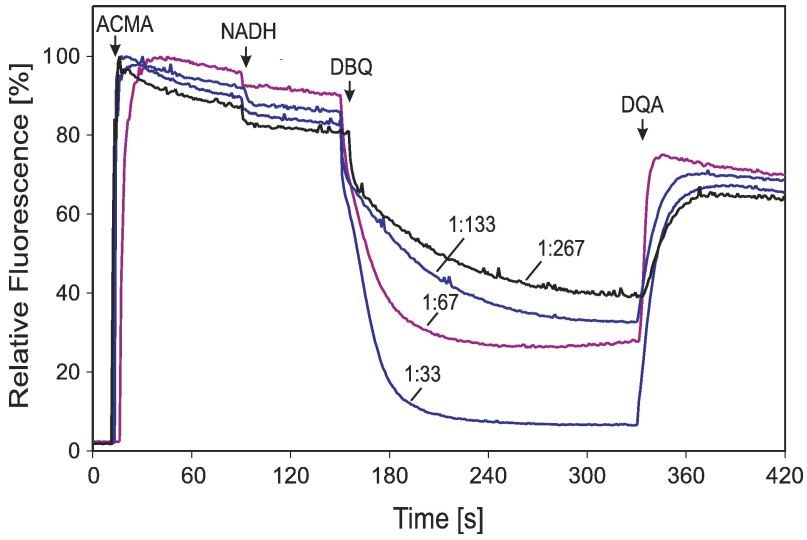

Fig. 2. Effect of lipid-to-protein ratio used for reconstitution on the magnitude of ACMA-quenching. Complex I was reconstituted using OG-total solubilized phospholipids at protein-to-lipid-ratios of 1:33, 1: 67, 1:133 and 1:267 (w/w). The specific activities were 2.9, 4.9, 3.1 and 2.6 μmol·min^{-1}·mg^{-1} and the coupling factors were 5.1, 9.2, 7.4 and 8.3 for these batches of proteoliposomes. Proteoliposomes were added at approximately the same amount of phospholipids in each measurement and the ACMA quenching was monitored as detailed under Materials and methods. 1 μM of DQA was added to inhibit complex I.

The resulting liposomes were frozen and stored in liquid nitrogen. Prior to further use (solubilization experiments or reconstitution), the preformed liposomes were pushed 19 times through polycarbonate membranes (pore size 200 nm) using a LiposoFast extruder (Avestin, Ottawa, Canada). Liposome solubilization using different detergents (OG, LM, Triton X-100, Na-cholate, $C_{12}E_8$) was monitored by measuring the optical density of the liposome-containing solution at 450 nm, thereby determining the 'onset' and 'total' solubilization conditions [32]. Detergent was added stepwise in small quantities to the liposome solution and the sample was stirred for 10 min before measuring the absorption.

For the reconstitution of complex I, preformed liposomes were solubilized by an amount of different detergents (OG, DDM, TX-100, Na-cholate, $C_{12}E_8$) that was just sufficient to result in an absorbance change at 450 nm ('onset'), or that resulted in a maximal absorbance change indicating complete solubilization ('total'). In some experiments at 'total solubilization', preparation of preformed liposomes was omitted and the phospholipid was directly dissolved in the respective detergent. Purified complex I (usually 100–200 μg) was added at a protein-to-lipid ratio of 1:50 (w/w) if not indicated otherwise. The detergents were removed by BioBeads SM2 (pretreated according to [33]), following the guidelines detailed by Rigaud and coworkers. As a standard procedure, detergents were removed at room temperature by the addition of a 20-fold excess of Bio Beads (by weight) that was applied stepwise as four equal aliquots in 1 h intervals. The Na^+ and K^+ concentration was checked in representative batches of proteoliposomes in a Roche Modular Analytics ISE900 analyzer. The readout was calibrated using standard solutions of NaCl and KCl in ultrapure water from Merck (Darmstadt, Germany).

2.6. Measurement of catalytic activity

NADH:HAR activity of purified and reconstituted complex I was measured at 30 °C in the same buffer that had been used for reconstitution (usually 20 mM K^+/Mops, 50 mM KCl, pH 7.4; in some experiments as indicated 20 mM Na^+/Mops, 50 mM NaCl, pH 7.4) using 2 mM HAR and 200 μM NADH as substrates. The fraction of inside-out oriented complexes (NADH binding site outside) and the total yield of reconstituted complex I was estimated based on the NADH:HAR activities before and after resolubilization of the proteoliposomes with 0.5 mM Na-cholate.

For the determination of NADH:ubiquinone oxidoreductase activity 100 μM DBQ (*n*-decylubiquinone) and 100 μM NADH were used as substrates, following the protocol described previously [34]. Measurements were done in the buffer that had been used during the reconstitution procedure. NADH-oxidation rates were recorded using a Shimadzu Multi Spec-1501 diode array spectrophotometer ($\varepsilon_{340-400\ nm}$=6.1 mM^{-1} cm^{-1}). The respiratory control ratio (RCR) of the proteoliposomes was determined as the ratio of NADH:DBQ activity of the reconstituted complex I in the presence and absence of 5 μM FCCP.

2.7. Measurement of proton gradient

Proteoliposomes containing 5–10 μg of complex I were added to 2 ml buffer (20 mM K^+/Mops pH 7.2, 50 mM KCl, 5 μM valinomycin if not stated otherwise) in a stirred cuvette. H^+-translocation was monitored as fluorescence change of ACMA that was added to a final concentration of 0.5 μM. Measurements were performed in a Shimadzu RF-5001 fluorimeter at an excitation wavelength of 430 nm and an emission wavelength of 475 nm (band pass 5 nm each, integration time 1 s). The reaction was started by the successive addition of DBQ (or Q-1) and NADH.

2.8. Measurement of the membrane potential

The generation of a membrane potential was recorded by measuring the absorption changes (623–604 nm) of the potential-sensitive dye Oxonol VI (2 μM) with a Shimadzu MultiSpec diode array spectrophotometer in a stirred 1 ml cuvette. Since a diode array spectrophotometer was used, NADH oxidation could be followed in parallel as absorbance change at 340 nm minus 400 nm. As in the case of the activity measurements, the experiments were performed in same buffer that had been used during reconstitution, i.e., K^+-containing proteoliposomes were added to 20 mM K^+/Mops pH 7.2, 50 mM KCl and Na^+-containing proteoliposomes to 20 mM Na^+/Mops pH 7.2, 50 mM NaCl.

3. Results

3.1. Properties of complex I proteoliposomes

As a general approach, purified complex I from *Y. lipolytica* was added to detergent solubilized lipids or preformed liposomes. Proteoliposomes were formed by incubation with a large excess of BioBeads for several hours that removed the detergent molecules from the phospholipid detergent mixtures

[28]. Finally, the Bio Beads were removed and the proteoliposomes were collected by ultracentrifugation. To study the influence of the detergent to lipid ratio, lipid–detergents mixtures were prepared either with an amount of detergent just sufficient to destabilize the preformed liposomes ('onset' condition) or to completely solubilize the lipid ('total' condition, [32]). The appropriate amounts of the different detergents used were determined for every batch of preformed liposomes to account for variations and heterogeneity of the asolectin used. As judged by the NADH:HAR oxidoreductase activity following resolubilization of the liposomes with Na-cholate, the overall recovery of complex I was in the range of 45–60% and did not depend markedly on the detergent or solubilization method used (Table 1). Losses of complex I varied considerably from batch to batch and were likely due to binding of the protein to the Bio Beads.

The fraction of incorporated complex I that was accessible in intact proteoliposomes for its hydrophilic substrate NADH reflected the portion of enzyme that had been incorporated in 'inside-out' orientation with respect to intact mitochondria. Although this fraction varied considerably, the 'inside-out' orientation clearly was predominant if 'onset' conditions were used (Table 1). In contrast, complex I was found to be randomly oriented in the proteoliposomes, if 'total' conditions were applied. Although the type of detergent used to solubilize the lipids seemed to have no major influence on the orientation of complex I in the membrane, the fraction of 'inside-out' oriented complexes tended to be somewhat higher if $C_{12}E_8$ or LM rather than OG or TX-100 were used (data not shown). While 'onset' conditions seemed to result in more favourably oriented proteoliposomes, 'total' conditions seemed to result in tighter sealed membranes: this was evident from the significantly higher respiratory control ratios that were observed when the lipids were completely solubilized prior to the addition of complex I. Typically, addition of the uncoupler FCCP to proteoliposomes prepared using 'total' conditions resulted in a 3-6 fold increase in NADH:ubiquinone oxidoreductase activity. In contrast, this rate was only roughly doubled when the proteoliposomes had been prepared by the 'onset' protocol (Table 1). Also respiratory control ratios were hardly affected by the detergent used to solubilize the lipids. When lower protein to lipid ratios were used, even higher respiratory control ratios were obtained (see below). Only when $C_{12}E_8$ was used, rather low electron transfer activities (Fig. 1) that exhibited no respiratory control were observed. This indicated that significant amounts of this polyoxyethylene detergent that is also known to act as a rather efficient inhibitor of bovine complex I [35] were still bound to complex I. In fact, we determined an I_{50} value for $C_{12}E_8$ of about 10 μM for purified and relipidated complex I from *Y. lipolytica* (data not shown). Remarkably, residual inhibition was not observed with TX-100, the other polyoxyethylene detergent used in this study that had been shown to inhibit bovine complex I [36]. At around 400 μM, the I_{50} value for TX-100 indicated a rather low affinity of this detergent for *Y. lipolytica* complex I (data not shown). In fact, the highest specific activities were observed when TX-100 was used to solubilize the lipids (Fig. 1).

In contrast to other properties of complex I proteoliposomes, specific activities were essentially the same, no matter whether

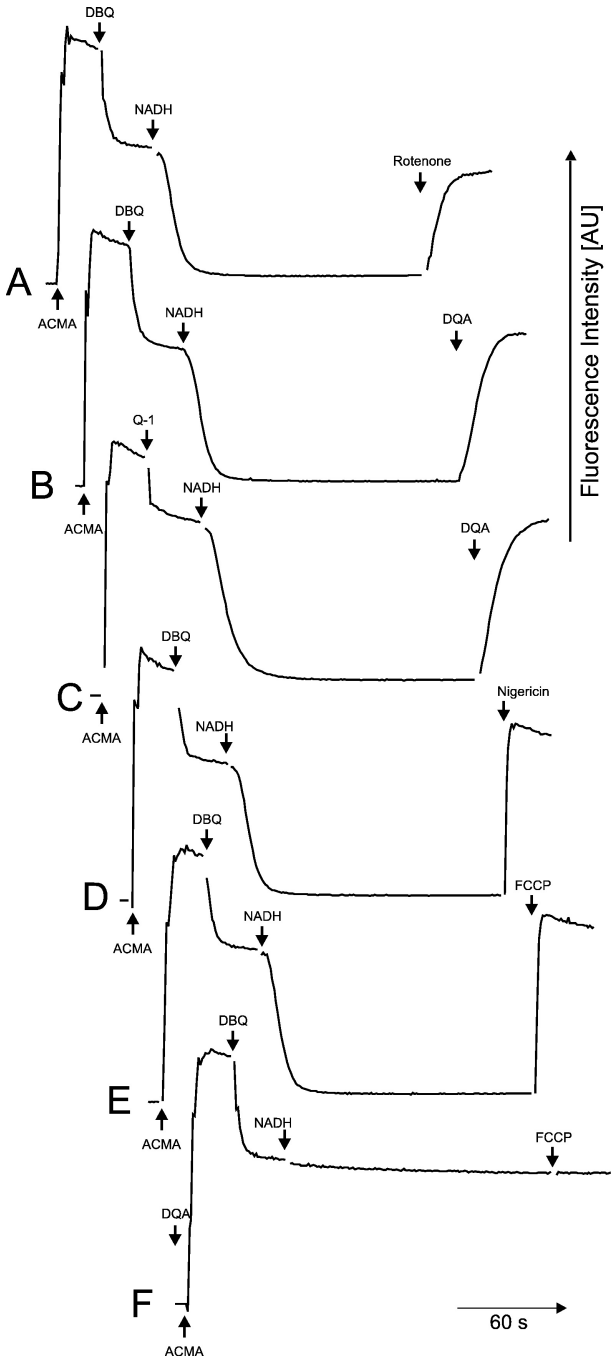

Fig. 3. Proton pumping of reconstituted complex I. Purified complex I (NADH:HAR activity 39.1 μmol·min^{-1}·mg^{-1}) was reconstituted as detailed in Materials and Methods using OG-total solubilized asolectin at protein-to-lipid-ratios of 1:50. Uncoupled NADH:DBQ activity was 4.1 μmol·min^{-1}·mg^{-1} and uncoupled NADH:Q_1 activity was 4.8 μmol·min^{-1}·mg^{-1}. Respiratory control ratios were 3.0 and 3.2, respectively. All measurements were done in presence of 50 mM KCl and 5 μM valinomycin with 9.8 μg of reconstituted complex I. 0.5 μM ACMA, 60 μM DBQ or Q_1 and 100 μM NADH, 1 μM DQA, 12.5 μM nigericin, 1 μM rotenone, 1 μM FCCP were added as indicated. In panel F, 1 μM DQA was added before starting the measurement.

'onset' or 'total' conditions were used (Fig. 1). Using cholate and LM resulted in specific activities that were only about half of those obtained with TX-100. With a specific NADH: ubiqunone oxidoreductase activity of around 15 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, proteoliposomes that had been prepared using OG as a detergent exhibited about 75% of the rates observed with TX-100. Despite of the higher activities obtained with TX-100, we decided to use octylglucoside as a detergent to completely avoid any specific interference of residual detergent with the ubiquinone binding pocket of complex I. As only the 'inside-out' oriented enzymes could react with NADH and tightness of the liposomal membranes is the more important parameter when measuring proton translocation, proteoliposomes were routinely prepared using octylglucoside and 'total' conditions for all subsequent experiments. No specific efforts were made to remove sodium-ions from the proteoliposomes, but Na^+ determinations in representative batches revealed that the Na^+ concentration was well below 0.3 mM when the proteoliposomes were prepared in 50 mM KCl.

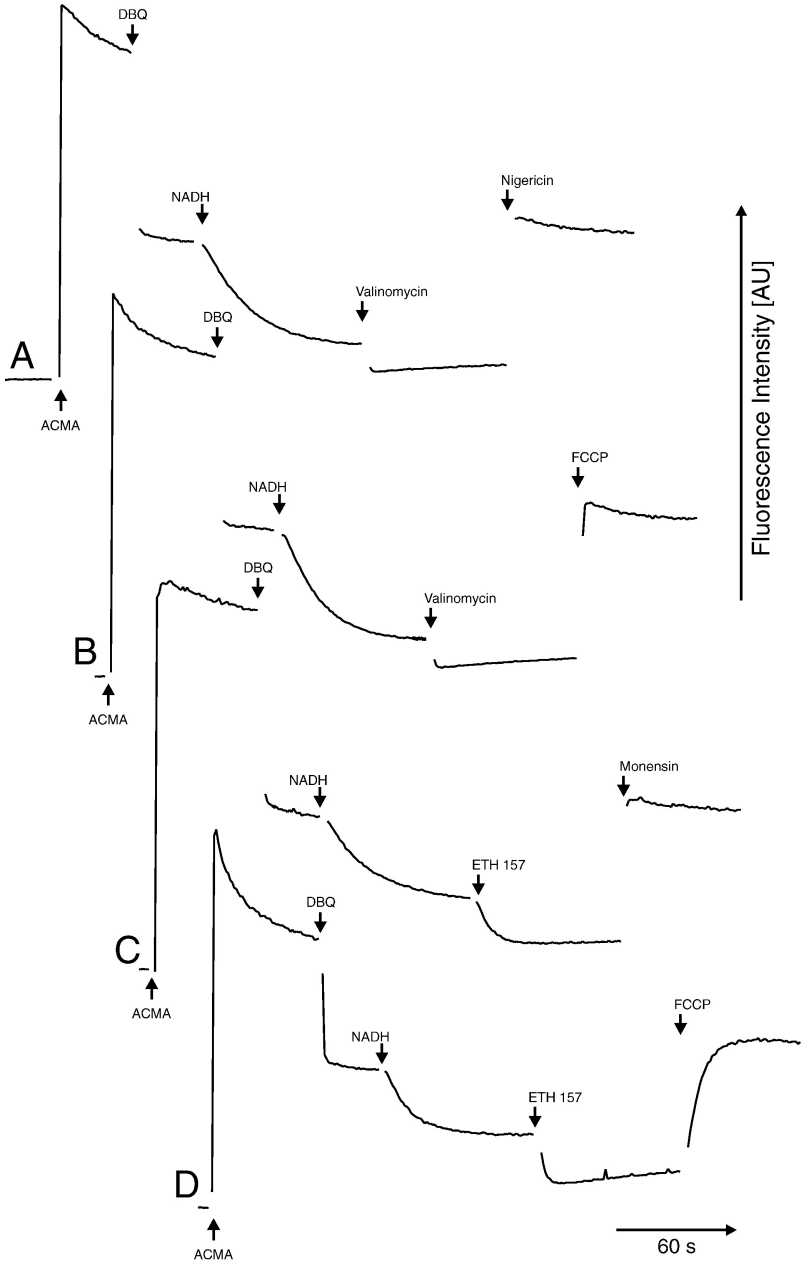

Fig. 4. Effect of K^+/valinomycin and Na^+/ETH157 on proton pumping of reconstituted complex I. Purified complex I (NADH:HAR activity 65,5 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) was reconstituted either in 1 mM Na^+/Mops, 50 mM NaCl, pH 7.4 or 1 mM K^+/Mops, 50 mM KCl, pH 7.4 using OG-total solubilized asolectin at protein-to-lipid-ratios of 1:50. Uncoupled NADH:DBQ activity was 17.0 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ in the K^+-buffer and 12.5 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ in Na^+-buffer. Respiratory control ratios were 4.7 and 3.9, respectively. Measurements were done in the respective buffers (traces A and B: K^+-buffer; traces C and D: Na^+-buffer). Additions: 0.5 μM ACMA, 60 μM DBQ, 100 μM NADH, 0,5 μM valinomycin, 1 μM nigericin, 1 μM monensin, 1 μM FCCP, 1 μM (trace C) or 5 μM (trace D, to ensure a fast collapse of $\Delta\Psi$, comparable to valinomycin) ETH 157.

3.2. Proton pumping by reconstituted complex I

To monitor proton pumping of reconstituted *Y. lipolytica* complex I, we followed the fluorescence change of ACMA. Its fluorescence is quenched upon formation of a pH-gradient across the liposomal membrane due to redistribution of the dye following acidification of the vesicle lumen [37]. Valinomycin was added to complex I proteoliposomes prior to the start of the measurements to prevent formation of a membrane potential.

When NADH and the ubiquinone-derivative DBQ were added to liposomes previously loaded with ACMA, some of the fluorescence was quenched due to absorption by spectral overlap (Fig. 2). This immediate fluorescence change was followed by a further decrease in fluorescence reflecting pumping of H^+ by complex I into the lumen of the proteoliposomes. The signal reached a steady state plateau where the rate of proton pumping, which is controlled by the $\Delta\mu H^+$ across the membrane, was compensated by the proton leak rate of the liposomal membrane. The ACMA quenching was clearly due to the build-up of a $\Delta\mu H^+$ and not due to the consumption of 'scalar' protons during the oxidation of NADH ($NADH + H^+ \rightarrow NAD^+$). Even when 100 μM NaOH were added, no change in ACMA fluorescence was observed because of the high outside buffer capacity used in the experiments (not shown). Both, the rate at which the ACMA fluorescence was quenched and the level of the steady state plateau was strictly dependent on protein load of the liposomes (Fig. 2): At a protein to lipid ratio of 1:33 more than 90% of the total fluorescence was quenched within about 30 s, while at a ratio of 1:267 the maximal quench was only about 60% and was reached only after about 2 min. This suggested that despite the higher respiratory control ratio the smaller number of complex I molecules per vesicle was not sufficient to maintain a maximal steady-state proton gradient. The ACMA quench was rapidly abolished by the addition of the complex I inhibitors DQA or rotenone (Figs. 2, 3, traces A and B). Virtually identical results were obtained when Q-1, a ubiquinone derivative carrying a single isoprenoid group as a sidechain, was used as a substrate (Fig. 3, trace C). The proton gradient could also be collapsed by the addition of the H^+/K^+ antiporter nigericin or the uncoupler FCCP (Fig. 3, traces D and E). If the inhibitor DQA was added before the substrate, electron transfer did not result in a fluorescence quench and subsequent addition of uncoupler had no effect (Fig. 3, trace F). If valinomycin was omitted from the experiment, the magnitude of the ACMA quenching was lower and increased if the potassium ionophore was added subsequently (Fig. 4, traces A and B). These results clearly demonstrated that the substrate induced ACMA fluorescence quench indicated the formation of a proton gradient due to proton pumping by complex I. A very similar set of data was

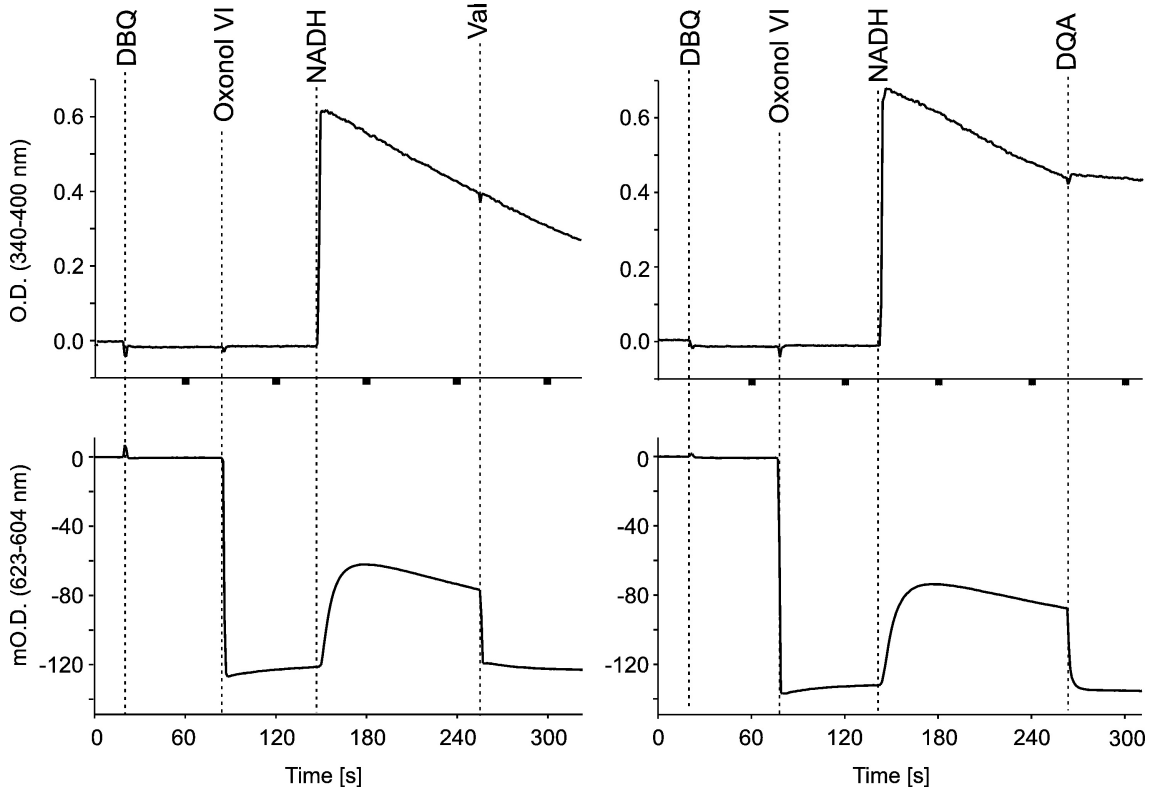

Fig. 5. Generation of $\Delta\psi$ by reconstituted complex I in the presence of potassium ions. Absorbance changes (623–604 nm) of the potential-sensitive dye Oxonol VI and the NADH oxidation (340–400 nm) were recorded simultaneously with a Shimadzu MultiSpec diode array spectrophotometer as detailed under Materials and methods. Proteoliposomes were reconstituted in 20 mM K^+/Mops, 50 mM KCl with OG-total solubilized asolectin. For each measurement, proteoliposomes (approx. 5 μg protein) were diluted in 1 ml of the same buffer. Substrates/effectors were added as indicated at the following concentrations: 2 μM Oxonol VI, 100 μM NADH, 100 μM DBQ, 5 μM valinomycin (Val), 1 μM DQA. Note, that the respiratory control ratios that can be deduced from the traces are somewhat lower than the values given in Table 1. This can be explained by the partial uncoupling effect of the dye oxonol VI.

obtained using sodium rather than potassium ions during reconstitution and proton translocation measurements except that the potassium-specific ionophore valinomycin was replaced by the sodium ionophore ETH 157 (Fig. 4). A somewhat lower NADH:ubiquinone oxidoreductase activity was the only significant difference observed if potassium was replaced by sodium and ACMA quenching in the absence of ionophores was less pronounced. Importantly, the sodium ionophore ETH 157 had the same effect on Na^+-loaded proteoliposomes as valinomycin on K^+-loaded proteoliposomes (Fig. 4 traces C and D): ΔpH increased due to the collapse of $\Delta\Psi$. If Na^+ would be transported by complex I, and the observed ΔpH would be only secondary, one would have expected a decrease following addition of ETH 157.

3.3. Generation of $\Delta\psi$ by reconstituted complex I

The generation of a membrane potential (inside positive) was monitored using the potential-sensitive dye Oxonol VI. In tightly coupled proteoliposomes or submitochondrial particles, a red shift of the absorption spectrum of this dye occurs in response to a positive-inside membrane potential [38]. When complex I turnover was started by the addition of DBQ and NADH to the proteoliposomes (Fig. 5 top), an increase of Oxonol VI absorption at 623 minus 604 nm was detected (Fig. 5 bottom) indicating the build-up of a membrane potential. The increase in Oxonol VI absorption leveled off after about 20 s, suggesting that – as in the ACMA fluorescence quench experiments – formation of the gradient was limited by the proton leak. Consistently, FCCP induced a rapid collapse of the membrane potential by uncoupling (not shown). Addition of the ionophore valinomycin (in presence of K^+) resulted in a rapid collapse of the membrane potential, but as $\Delta\psi$ but not ΔpH was dissipated, the rate of electron transfer monitored by the absorption change at 340–400 nm was hardly affected (Fig. 5 left panel). Addition of the complex I inhibitor DQA blocked electron transfer and, due to the proton leak of the proteoliposomes, resulted in the dissipation of the membrane potential (Fig. 5 right panel).

Again, rather similar results were obtained if potassium ions were exchanged by sodium ions during reconstitution and Oxonol VI measurements. The membrane potential generated by complex I turnover was sensitive to uncoupling by the protonophore FCCP. Abolishing the proton motive force completely resulted in an increase in the electron transfer rate. On the other hand, no effect on the electron transfer rate was observed following addition of the H^+/Na^+ antiporter monensin and even a transient increase of the positive inside membrane potential was observed (Fig. 6). This demonstrated that the turnover of reconstituted complex I was linked to the formation of primary proton gradient.

4. Discussion

Functional reconstitution of purified complex I in well coupled proteoliposomes is prerequisite to study the still elusive

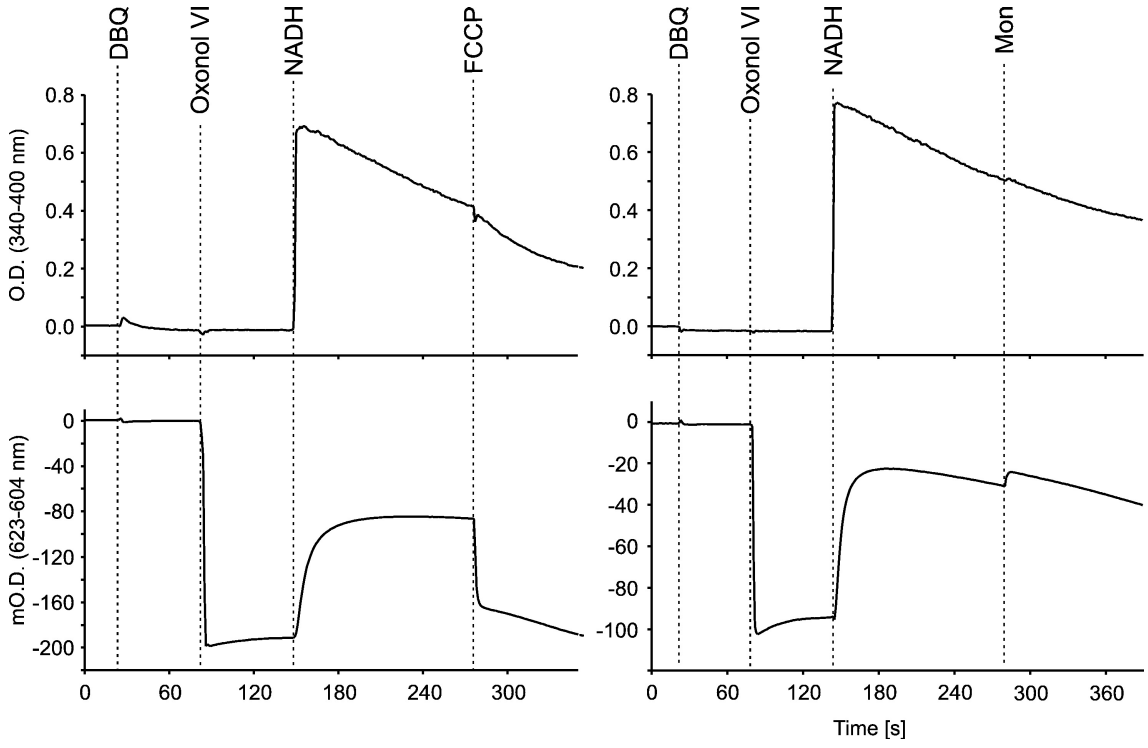

Fig. 6. Generation of $\Delta\psi$ by reconstituted complex I in the presence of sodium ions. The general conditions were as indicated in the legend of Fig. 5. For these experiments, complex I was reconstituted in 20 mM Na^+/Mops, 50 mM NaCl with OG-total solubilized asolectin. Measurements were performed in the identical Na^+/Mops buffer. Substrates/effectors were added as indicated at the following concentrations: 2 μM Oxonol VI, 100 μM NADH, 100 μM DBQ, 1 μM FCCP, 1 μM monensin (Mon).

mechanism of this major proton pump of the respiratory chain. We found that both the nature and amount of the detergent used to solubilize the phospholipids had marked effect on the properties of complex I proteoliposomes that were formed upon removal of the detergent with BioBeads. Proteoliposomes best suited for functional studies were obtained when the phospholipids were solubilized completely with octylglucoside resulting in highly active and well coupled complex I liposomes. Specific ubiquinone reductase activity was found to be highest when TX-100 was used for solubilization, but this detergent, as well as $C_{12}E_8$, the other polyoxyethylene detergent used, were found to act as inhibitors of *Yarrowia* complex I. As inhibition was observed with $C_{12}E_8$, the presence of residual inhibitory detergent in the proteoliposomes could also not be completely excluded for the weaker binding TX-100. Clearly, the well-coupled proteoliposomes that contain highly active complex I will be suited to measure proton to electron stoichiometries of wild-type and mutant enzyme in future studies.

So far, proton-translocation by mitochondrial complex I was only demonstrated directly in isolated rat liver mitochondria [14], submitochondrial particles derived from bovine heart [15] and bovine heart complex I reconstituted into proteoliposomes [39]. Here, we show that reconstitution of highly pure mitochondrial complex I from the yeast *Y. lipolytica* into proteoliposomes resulted in fully functional and inhibitor sensitive electron transfer activity that was linked to vectorial H^+-translocation. In agreement with the findings of Ragan and Hinkle [39], we observed that also reconstituted complex I from *Y. lipolytica* is only completely 'uncoupled' (as judged by the collapse of the ΔpH or by the stimulation of the NADH:DBQ activity) if ionophores that dissipate both components of the proton-motive-force (either FCCP or the combination of valinomycin and nigericin in K^+-containing buffer or ETH 157 and monensin in Na^+-containing buffer) are present. The $\Delta\Psi$-dissipating ionophores valinomycin or ETH 157 alone had no effect.

It has been reported that reconstituted complex I (NDH I) from the enterobacteria *Klebsiella pneumonia* [20,21] and *Escherichia coli* [40,41] exclusively pumps Na^+-ions rather than protons. This claim has been challenged by other authors for both organisms [19,22]. Thus, it remains unclear which ions are pumped by prokaryotic complex I. Our results analyzing the effects of different uncouplers and ionophores on ΔpH and $\Delta\psi$ generated by mitochondrial complex I unequivocally demonstrate that the enzyme from *Y. lipolytica* is a primary proton pump. With respect to the possibility that complex I from *Y. lipolytica* may in addition act as a Na^+ pump, it should be noted that rather than being stimulated NADH:DBQ oxidoreductase activity was found to be consistently lower in the presence of 50 mM Na^+ than in the presence of 50 mM K^+. Although we have made no efforts to remove sodium ions from the proteoliposomes, the Na^+ concentration in the proteoliposomes prepared with 50 mM KCl was found to be well in the submillimolar range. As the apparent K_m for the stimulatory effect on the activity of complex I from *K. pneumonia* was reported to be around 4 mM [42] we would have rather expected higher activities in the presence of 50 mM NaCl. Moreover, the effects of valinomycin in the presence of potassium ions and ETH157 in the presence of sodiums ions on the proton pumping activity were essentially the same (Fig. 4). Finally, adding the Na^+/H^+ antiporter monensin in presence of sodium ions caused a small increase (Fig. 5) which seems to rule out that complex I itself acts as a potent H^+/Na^+ antiporter. Overall, there were no indications suggesting that complex I functioned as a primary sodium pump or exhibited H^+/Na^+ antiporter activity.

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