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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).

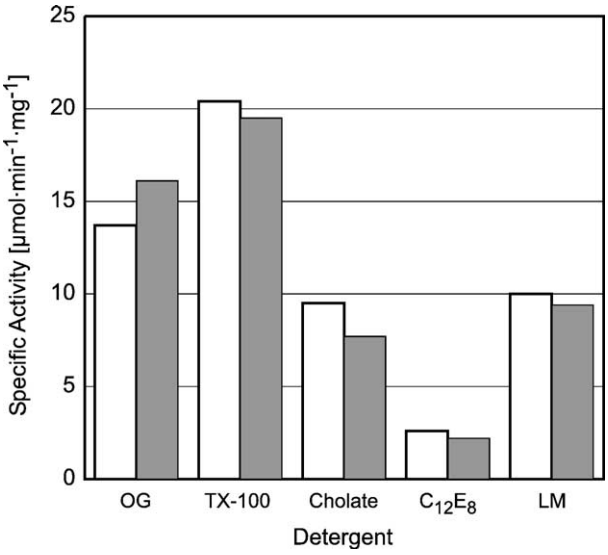

Fig. 1. Specific activities of complex I after reconstitution with different detergents. Proteoliposomes were prepared using 'onset' (open columns) or 'total' conditions (grey columns) and specific NADH:ubiquinone oxidoreductase activities of FCCP-uncoupled proteoliposomes were measured in 20 mM K^+-Mops, 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.