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Full recovery of the NADH:ubiquinone activity of complex I (NADH:ubiquinone oxidoreductase) from *Yarrowia lipolytica* by the addition of phospholipids

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Dedicated to Karlheinz Altendorf (Osnabrück) on the occasion of his 60th birthday.

Abstract

NADH:ubiquinone oxidoreductase (complex I) is the largest multiprotein complex of the mitochondrial respiratory chain. His-tagged complex I purified from the strictly aerobic yeast *Yarrowia lipolytica* exhibited electron transfer rates from NADH to *n*-decylubiquinone of less than 2% when compared to turnover numbers calculated for native mitochondrial membranes from this organism. Reactivation was observed upon addition of asolectin, purified phospholipids and different phospholipid mixtures. Maximal activities of 6–7 μmol NADH min^{-1} mg^{-1} were observed following incubation with a mixture of 76% phosphatidylcholine, 19% phosphatidylethanolamine and 5% cardiolipin. For full reactivation, 400–500 phospholipid molecules per complex I were needed. This demonstrated that the inactivation of complex I from *Y. lipolytica* by general delipidation could be fully reversed simply by returning the phospholipids that had been removed during the purification procedure. Thus, our homogeneous and highly pure complex I preparation had retained its full catalytic potential and no specific, functionally essential component had been lost. As the purified enzyme was also found to contain only substoichiometric amounts of ubiquinone-9 (0.2–0.4 mol/mol), a functional requirement of this endogeneous ubiquinone could also be excluded.

Keywords: Mitochondria; Complex I; Yeast; Phospholipid; *Yarrowia lipolytica*

1. Introduction

As the first multiprotein complex involved in the build-up of the proton-motive force in mitochondria, NADH:ubiquinone oxidoreductase (EC 1.6.99.3, complex I) has a central position in oxidative phosphorylation. Mitochondrial complex I catalyses a two-electron transfer from NADH to ubiquinone linked to the translocation of four protons across the inner mitochondrial membrane [1]. In contrast to its central role in oxidative phosphorylation and the discovery of an increasing number of human diseases linked to this enzyme [2,3], neither the details of the molecular structure nor the molecular mechanism coupling electron transfer to proton transport across the inner mitochondrial membrane are known. As a new approach to solve some of these open questions, we have established the strictly aerobic yeast *Yarrowia lipolytica* as a model system that offers a set of molecular genetic tools similar to those known for the yeast *Saccharomyces cerevisiae*: we constructed *Y. lipolytica* strains, in which genes encoding essential nuclear encoded subunits of complex I were deleted, allowing us to investigate the effect of mutations in the respective subunit expressed on a single copy *Escherichia coli*/*Y. lipolytica* shuttle vector [4–8]. Furthermore, we attached a His_6-tag to the C-terminus of the 30 kDa subunit (encoded by the

Abbreviations: 6:0 PC, 1,2 dihexanoyl-*sn*-glycero-3-phosphocholine; 8:0 PC, 1,2 dioctanoyl-*sn*-glycero-3-phosphocholine; CL, cardiolipin; EY, egg yolk; DBQ, *n*-decylubiquinone; DDM, *n*-dodecyl-β-D-maltoside; HAR, hexaammineruthenium(III)-chloride; HPLC, high-performance liquid chromatography; OG, *n*-octyl-β-D-glucopyranoside; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, 'polar extract' of the total chloroform/methanol extract of soybean; PI, phosphatidylinositol; TLC, thin-layer chromatography

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NUGM gene) which allows for rapid isolation of highly pure and homogeneous complex I from *Y. lipolytica* [9,10]. As judged by the complex I specific electron transfer from NADH to the artificial electron-acceptor hexaammineruthenium(III)-chloride (HAR), the specific activity is increased by a factor of 70 during purification from mitochondrial membranes [9]. However, the specific electron transfer rate from NADH to the artificial acceptor *n*-decylubiquinone (DBQ) does not increase concomitantly, but is even reduced when compared to native mitochondrial membranes. Since the NADH:HAR activity of complex I was assigned to a three-subunit subcomplex (flavo protein 'FP') of the so-called peripheral arm [11], while the NADH:DBQ activity represents the activity of the whole complex, one might conclude that an important factor was lost upon purification. We already excluded the loss of non-covalently bound FMN or EPR-detectable iron sulfur clusters [4,10]. As there are also no indications for the loss of subunits, further candidates are bound phospholipids or a tightly bound 'functional' ubiquinone that had been postulated as an integral part in several hypothetical models for the catalytic mechanism [12–14]. Therefore, we have determined the phospholipid and ubiquinone content in highly pure preparations of His_6-tagged complex I of *Y. lipolytica* and have analyzed the effects of phospholipids on catalytic activity of isolated complex I.

2. Materials and methods

2.1. Materials

The following phospholipids were purchased from Sigma (all as solid powders): cardiolipin (CL, sodium salt, from bovine heart, purity >98%), phosphatidylethanolamine (PE, type III, from egg yolk, purity >98%) and phosphatidylcholine (PC, type XVI-E from fresh egg yolk, purity >99%). All other lipids were purchased from Avanti Polar Lipids (Alabaster, AL) as solid powder (asolectin=total soybean extract with 20% lecithin; referred to in general as 20%; two partially purified soybean extracts with 45% and 95% lecithin, usually referred to as 45% and 95%, respectively) or chloroform solutions ('polar extract' of the total chloroform/methanol extract of soybean; EYPC, purity >99%; EYPE, purity >99%; Plant PC from soybean, purity >99%; 6:0 PC, purity >99%; 8:0 PC, purity >99%). Dodecyl-β-D-maltoside (DDM) and octyl-β-D-glucopyranoside (OG) were obtained from BIOMOL and chelating Sepharose from Pharmacia. Silica 60 TLC plates were purchased from Merck, Darmstadt (Germany). All other chemicals were purchased from Sigma.

2.2. Analytical methods

Protein concentrations were determined according to a modified Lowry protocol [15]. The phospholipid content of purified complex I was determined as total organic phosphate content following the protocol of Ref. [16]. For the analysis of bound phospholipids, about 100–200 μg of purified complex I (pools after Ni-affinity chromatography and subsequent gel filtration, respectively; see below) were extracted with 20 volumes of chloroform/methanol (2:1). Thin-layer chromatography (TLC) of the chloroform/methanol extract and subsequent phospholipid staining by molybdenum spray was performed as detailed in Ref. [17]. For the determination of the ubiquinone content, ubiquinone was extracted from 7 to 8 mg of purified complex I according to a protocol in Ref. [18], modified by replacing light petroleum with *n*-hexane. In some cases, complex I was degraded by the addition of 1–2 units of proteinase K (Sigma P-8044, EC 3.4.21.64) prior to hexane extraction. Proteolysis took place for about 12 h at 56 °C. The residue of the hexane phase was dissolved in ethanol and analyzed by reversed phase HPLC (detection at 275 nm) according to Ref. [19], using a HiBar® prepacked Column RT 250-4, LiChropher® 100 RP-18 (5 nm) from Merck and the following solution as mobile phase: 7 g $NaClO_4$ H_2O in 1000 ml ethanol/methanol/70% $HClO_4$ (700:300:1). The column was calibrated by varying amounts of Q_9 from *Torulopsis utilis* (former *Candida utilis*) (90–95% purity) or synthetic Q_{10} (minimal purity 98%) from Sigma. The actual concentrations of the standards were determined spectrophotometrically at 275 nm, $\varepsilon_{red\text{-}ox}$=12,500 M^{-1} cm^{-1} [20]. The stoichiometry between bound ubiquinone and complex I was calculated assuming a molecular mass of 890 kDa for complex I [4].

2.3. Yeast growth and preparation of mitochondrial membranes

Y. lipolytica strain PIPO 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). The construction of this strain is detailed elsewhere [10]. Briefly, this strain contains a chromosomal copy—introduced by homologous recombination with plasmid pNK1.2 as described in Ref. [9]—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 Ref. [21] with the modification detailed in Ref. [9].

2.4. Purification of complex I

For the purification of complex I, mitochondrial membranes of *Y. lipolytica* strain PIPO were solubilized with dodecyl maltoside as described previously [9]. Purification was achieved by Ni-affinity chromatography, followed by gel filtration as detailed in Ref. [9] with a 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 were concentrated to 3–10 mg/ml by centrifugation through Vivaspin cartridges (MWCO 100,000; Vivascience, UK) and stored in liquid nitrogen.

2.5. Preparation of lipid–detergent mixtures

Two different protocols were used for the preparation of solubilized phospholipids. For larger quantities, 70 mg of solid phospholipids where sonicated in 7 ml buffer (20 mM K^+/Hepes pH 7.2 or 20 mM K^+/Mops pH 7.2, respectively, containing 50 mM KCl and 1.6% OG; no buffer-specific differences were observed) until the solution was clear. Dilutions of this stock solution were prepared with the same buffer. For smaller quantities (and when the lipids were supplied as solutions in chloroform), an aliquot of the phospholipids in chloroform was added to a glass vial and the solvent was evaporated under a stream of argon. After another hour under vacuum the phospholipids were then dissolved to a concentration of 10 mg/ml in the above-mentioned buffer. Dilutions were prepared in the same buffer.

2.6. Determination of catalytic activity

NADH:HAR activity of purified complex I was measured at 30 °C in a buffer containing 20 mM Na^+/Hepes pH 8.0, 250 mM sucrose, 2 mM EDTA and 2 mM NaN_3 using 2 mM HAR and 200 μM NADH as substrates. For the determination of NADH:ubiquinone oxidoreductase activity, 60 μM DBQ and 100 μM NADH were used as substrates, following the protocol described previously [4]. NADH oxidation rates were recorded with a Shimadzu Multi Spec-1501 diode array spectrophotometer ($\varepsilon_{340-400\ nm}$=6.1 mM^{-1} cm^{-1}[22]). To analyze the effect of the different phospholipids and lipid mixtures, aliquots of different stock solutions were added to the buffer after starting the reaction with DBQ (controls) or to the complex I preparation prior to the addition of the enzyme to the assay buffer. In the latter case, the concentrated enzyme–lipid–detergent mixture (usually 5 μl) was preincubated for 20 min on ice, if not indicated otherwise.

3. Results

3.1. Phospholipid and ubiquinone content of purified complex I from Y. lipolytica

Complex I purified from the *Y. lipolytica* strain PIPO carrying a chromosomal copy of the gene coding for the His-tagged 30 kDa subunit exhibited very low NADH:ubiquinone oxidoreductase activities (0.1–0.2 μmol min^{-1} mg^{-1}). Similar values were found previously for a strain carrying a plasmid-borne copy of the His-tagged 30 kDa subunit [9] and for the wild-type complex purified by conventional chromatographic techniques [4]. Since activity could be largely restored by reconstitution of the complex into asolectin proteoliposomes [9], delipidation seemed to be the most likely explanation for inactivation. However, asolectin is a crude lipid mixture derived from soybean by total chloroform/methanol extraction and partitioning against deionized water. In addition to various phospholipids, it contains a number of poorly characterized lipophilic components. Therefore, it still seemed possible that rather than the phospholipids of asolectin, a quinoid component was responsible for the observed reactivation.

We analyzed the phospholipid and ubiquinone content of our complex I preparation. The phospholipid content determined as total organic phosphate varied between 26 and 66 mol phosphate per mol complex I in different batches (n=4). The analysis of a chloroform/methanol extract of our preparation by TLC revealed that the phospholipids bound to complex I after detergent solubilization and purification represented the three major classes of phospholipids of the inner mitochondrial membrane/CL, PC and PE (results not shown).

For the determination of ubiquinone content, about 9 nmol of purified complex I were extracted with *n*-hexane/methanol/70% $HClO_4$ (69%/29.5%/1.5%). The residue of the hexane phase was analyzed by reversed phase HPLC (Fig. 1). Since *Y. lipolytica* contains ubiquinone-9 (Q_9) [23], commercially available Q_9 from *T. utilis* was used as a standard. When the Q_9 standard from *T. utilis* was applied to

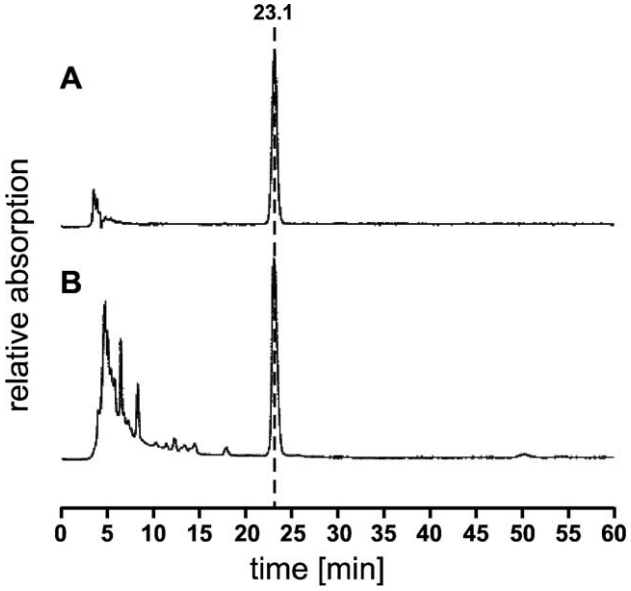

Fig. 1. Ubiquinone-9 extracted from purified complex I from *Y. lipolytica*. A ubiquinone-9 standard from *T. utilis* and the residue of the *n*-hexane extract of a complex I preparation from *Y. lipolytica* were applied to reversed phase HPLC as detailed under Materials and methods. The retention profiles were monitored at 275 nm with identical sensitivity. Two representative chromatograms are presented in scale: (A) 0.155 nmol ubiquinone-9 standard; (B) 1/10 of the total *n*-hexane extract of 8.7 nmol complex I.

reversed phase HPLC, one prominent peak detected at 275 nm with a retention time of 23.1 min was observed (Fig. 1A). Minor fluctuations of the automatically determined peak values occurred in different runs of the same standard. A peak with essentially the same retention time was detected in the *n*-hexane extract of purified complex I (Fig. 1B). Other peaks were observed with retention times between 3.5 and 18 min. None of these peaks could be related to ubiquinone-10 (Q_{10}), which was found to elute with a retention time of around 31.2 min (not shown). Unfortunately, the Q_9 content in our preparation was too low for spectroscopic characterization. Based on a calibration of the peak areas with different Q_9 standard runs, we initially calculated a Q_9 content of 0.07 mol per mol complex I for our preparation. However, we observed a precipitation of protein in the hexane phase that interfered with complete separation of the hexane from the methanol phase. Probably, the majority of the hydrophobic subunits of complex I stayed in the hexane phase. This could be largely overcome by total proteolysis of complex I. Following 12 h degradation of complex I with proteinase K prior to hexane–methanol-extraction, much less precipitated protein was found in the hexane phase. All major subunits of complex I were degraded as judged by polyacrylamide electrophoresis (not shown). After proteolysis, the Q_9 content determined in the same preparation increased to 0.2 mol per mol complex I. In a different batch of protein 0.4 mol per mol complex I were found. In summary, we conclude that complex I purified from *Y. lipolytica* contained amounts of Q_9 that were clearly substoichiometric.

3.2. Activation of purified complex I by detergent solubilized asolectin

In a first experiment, we investigated the effect of asolectin on the NADH:DBQ activity of complex I purified from *Y. lipolytica* mitochondrial membranes (Fig. 2). The basal NADH:DBQ activity in this representative experiment was about 0.2 μmol min^{-1} mg^{-1} and did not increase markedly upon addition of 2.5 μl of 1.6% OG (final concentration as in all other measurements 0.004%; trace A, 0.25 μmol min^{-1} mg^{-1}), the detergent used for solubilization of the phospholipids. If solubilized asolectin was added at a lipid to protein ratio of about 3.5:1(w/w) during the assay, only a minor activation to 0.5 μmol min^{-1} mg^{-1} could be observed (trace B). However, a pronounced stimulating effect was observed, if the purified complex I was preincubated at a concentration of 1.4 mg ml^{-1} with 3.5:1 g/g solubilized asolectin (trace C) prior to addition to the cuvette. Activation was also observed when complex I purified in the presence of 0.1% dodecylmaltoside was preincubated with preformed liposomes (not shown).

To explain the large difference between addition of and preincubation with asolectin, one has to consider that upon addition of the detergent–phospholipid mixture to the large

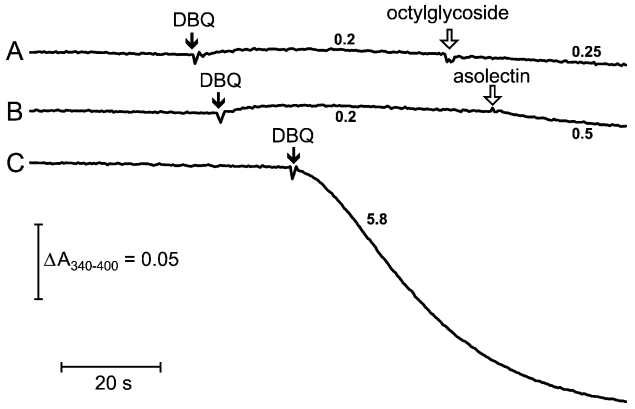

Fig. 2. Stimulating effect of asolectin on the NADH:DBQ-activity of complex I. For the representative measurements shown, the activity of purified complex I was monitored at 30 °C. Total spectra in the range from 340 to 400 nm were recorded (1 nm intervals). Time courses of the difference A_{340} minus A_{400} are shown. Enzyme (6.7 μg) was added to 1 ml of assay buffer containing 100 μM NADH before the measurement. The reaction was started by the addition of 60 μM DBQ (black solid arrows). OG or asolectin in OG were added as indicated. (A) Control with addition of 0.004% OG. (B) Addition of 25 μg solubilized asolectin and 0.004% OG. (C) Complex I preincubated with 25 μg asolectin for 20 min on ice. The numbers indicate activities in μmol min^{-1} mg^{-1}.

volume of the assay buffer, the OG- and DDM-concentrations (0.14 mM and <25 μM, respectively) drop far below their critical micellar concentration (20–25 mM, equivalent to 0.6–0.7% for OG and 0.1–0.6 mM, equivalent to 0.03–0.005% for DDM) and liposomes or mixed micelles are instantly formed. Therefore, the interaction between complex I present in the assay buffer and subsequently added phospholipids is limited. A different situation occurs when the lipids are preincubated with the enzyme. Upon dilution into the assay buffer, detergent-containing proteoliposomes or at least mixed protein–detergent–phospholipid micelles are formed in which a thorough interaction between complex I and phospholipids is possible. While we can't distinguish between these two possibilities, we can at least exclude that tightly coupled proteoliposomes were formed since the addition of uncouplers (FCCP) had no effect on the NADH:DBQ activity (results not shown). The highest NADH:DBQ activity observed in the presence of asolectin (about 7 μmol min^{-1} mg^{-1}) was comparable to the highest value observed previously following reconstitution of *Y. lipolytica* complex I into asolectin proteoliposomes by the detergent removal technique (6.7 μmol min^{-1} mg^{-1}, [9]). Therefore, the procedure described here can be considered a fast and material-saving method to investigate the effect of different phospholipids on complex I activity. Activation of purified complex I by preincubation with solubilized asolectin was essentially complete after a few seconds. However, analysis of the time dependence of activation revealed an additional slight increase of activity by extending the preincubation time up to 20 min (not shown). Therefore, a 20 min preincubation time was used routinely in further experiments.

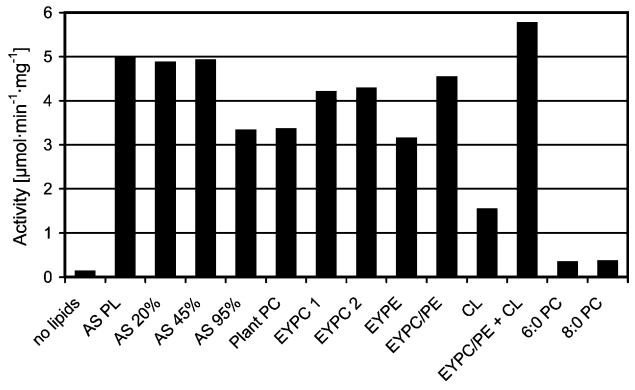

Fig. 3. Stimulating effects of different asolectins and purified phospholipids on the NADH:DBQ activity of complex I. Lipids (5 μg) were preincubated with 6.8 μg of purified complex I. For experimental conditions see legend of Fig. 2. AS PL, 'polar lipid extract' of asolectin ('total' chloroform/methanol extract of soybean containing 20% lecithin, precipitated with acetone and then extracted with diethyl ether); AS 20%, AS 45% AS 95%, different asolectin fractions containing 20%, 45%, 95% of lecithin (PC), respectively; EYPC 1, egg yolk PC from Avanti; EYPC 2, egg yolk PC from Sigma; EYPE, egg yolk PE; EYPC/PE, 80% EYPC/20% EYPE; CL, cardiolipin; EYPC/PE+CL, 76% EYPC/ 19% EYPE/5% CL; 6:0 PC, 1,2 dihexanoyl-*sn*-glycero-3-phosphocholine; 8:0 PC, 1,2 dioctanoyl-*sn*-glycero-3-phosphocholine. For details see Results.

3.3. Phospholipid specificity of reactivation

Using the protocol established for asolectin, the effect of different partly purified asolectins and purified phospholipids on the NADH:DBQ activity of complex I was investigated. Depending on the batch of purified complex I, maximal activities of 3–7 μmol min^{-1} mg^{-1} could be reached. Therefore, the effects of different phospholipids were compared within the same batch of enzyme. Fig. 3 summarizes the results for a typical preparation exhibiting a maximal activity of 5.8 μmol min^{-1} mg^{-1}. Comparable results were obtained with other complex I preparations. At a protein-to-lipid-ratio (w/w) of about 1:1, activation by preincubation with the 20%, 45% and 'polar extract' asolectin was comparable, almost reaching 5 μmol min^{-1} mg^{-1}. According to the manufacturer, these 'soybean lipid extracts' all contain variable amounts of PC, PE, phosphatidylinositol (PI), phosphatidic acid (PA) and lyso PC as well as up to 37% (in the case of the 'total extract') components which are listed as 'other'. In addition, 'asolectins' contain between 10% and 25% CL [24,25], which could be easily detected by TLC and molybdenum staining (results not shown). When asolectin with a higher PC content (95%) was used, an activity of only 3.2 μmol min^{-1} mg^{-1} was observed, which compared well with activities of 3.1–4.2 μmol min^{-1} mg^{-1} that were obtained using HPLC-purified PC from different sources or PE from egg yolk. Slightly higher activities around 4.5 μmol min^{-1} mg^{-1} were observed if an 80:20 mixture of PE and PC was used. If CL alone was used for activation, a maximal activity of only 1.5 μmol min^{-1} mg^{-1} was obtained. However, preincubation with a mixture of 76% PC, 19% PE and 5% CL, the three main phospholipids of the inner mitochondrial membrane [26], resulted in maximal activation to 5.8 μmol min^{-1} mg^{-1} for this batch of purified complex I. In contrast, almost no stimulation was observed after addition of the synthetic short-chain phospholipids 6:0 PC and 8:0 PC.

From these data we conclude that complex I from *Y. lipolytica* purified by affinity chromatography can be fully reactivated simply by returning PC, PE and CL, the phospholipids removed by the purification procedure. Thus, no specific factor required for NADH:ubiquinone oxidoreductase activity was lost during purification and had caused the observed inactivation.

3.4. Amount of phospholipid required for activity

In our initial experiments with different asolectin fractions, we observed that the stimulation of NADH:DBQ activity depends on the amount (ratio) of added lipid. For a detailed analysis, we measured the dependence of activity on the lipid-to-protein-ratio for several purified lipids (Fig. 4). With different phospholipids, we found that the maximal activity was reached at a ratio between 450 and 900 mol of phospholipids added per mol complex I. The value for different PCs (purified from egg yolk (two different sources) and from soybean) was around 450:1, while with egg yolk PE the maximal activity was reached at a ratio of around 870:1. At higher ratios (>1000:1), some reduction of the NADH:DBQ activity was observed in most cases. A somewhat different picture emerged for purified cardiolipin (Fig. 5). In this case maximal activity was reached when about 100 mol of cardiolipin were added per mole of purified complex. At higher concentrations, a sharp drop in activity

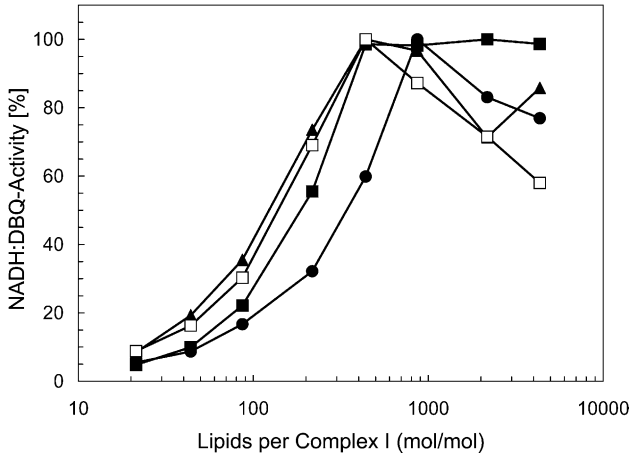

Fig. 4. Dependence of the NADH:DBQ activity on the amount of added phospholipids. Increasing amounts of purified phospholipids were preincubated with 6.8 μg of purified complex I. Experimental conditions as indicated in Fig. 2. ■ EYPC from Avanti (100%=3.52 μmol min^{-1} mg^{-1}) □EYPC from Sigma (100%=3.85 μmol min^{-1} mg^{-1}) ▲ soybean PC (100%=3.53 μmol min^{-1} mg^{-1}) ● EYPE (100% = 4.19 μmol min^{-1} mg^{-1}).

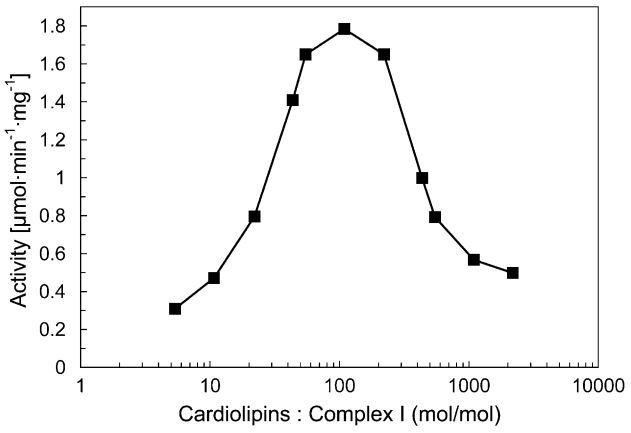

Fig. 5. Dependence of the NADH:DBQ activity on the amount of added cardiolipin. Increasing amounts of cardiolipin from bovine heart were preincubated with 6.8 μg of purified complex I. Experimental conditions as indicated in Fig. 2.

was observed, resulting in an almost bell-shaped curve in the half-logarithmic diagram (Fig. 5). This inhibitory effect of higher concentrations might be responsible for the relatively low maximal activity obtained with cardiolipin that is specifically enriched in the inner mitochondrial membrane of eukaryotic cells [27].

4. Discussion

There are several recent reports that describe the purification of largely delipidated complex I from mitochondrial membranes of different sources [4,9,28–30]. Preparations with low lipid contents are a prerequisite for crystallisation of complex I. Thus, their functional integrity is critical for the interpretation of any X-ray structures that will be based on such preparations. In all cases however, the catalytic activity—as judged by electron transfer from NADH to artificial ubiquinone derivatives (usually Q_1 or DBQ)—was reduced dramatically upon solubilization by various non-ionic detergents (e.g. Triton X-100 or dodecylmaltoside) and subsequent purification of the complex. In some reports, partial reactivation to varying extents by the addition of phospholipids or by reconstitution into proteoliposomes was mentioned as a side remark [4,9,29,30]. More detailed studies on the phospholipid requirements of bovine complex I date back more than 20 years [31–33] and were performed with enzyme preparations using ionic detergents like cholate which always leave a rather large number of phospholipid molecules and other hydrophobic components like ubiquinone bound to the complex. Still, these preparation protocols also result in marked activity loss and full recovery by the addition of phospholipids was never observed [32,33]. It remained unclear at the time whether this was due to some irreversible damage to the complex or partial removal of subunits or an unknown factor like a special phospholipid or endogeneous ubiquinone that was not replenished with the phospholipid mixtures used for reactivation.

The proposal that complex I might contain a still unidentified redox cofactor ‘X’ in its hydrophobic part [34] and the even greater activity loss observed with monodisperse preparations needed for crystallisation prompted us to carefully analyze the correlation between lipidation state, ubiquinone content and catalytic activity for complex I purified from *Y. lipolytica* mitochondria.

The delipidating conditions during the purification procedure were found to leave only around 50 phospholipids bound to the essentially inactive complex (NADH:ubiquinone oxidoreductase activity <2%). Among these were all three major lipids of the inner mitochondrial membrane, namely PC, PE and CL. Complete reactivation was achieved by returning phospholipids as detergent–phospholipid mixed micelles. Although about 80% and 50–70% of the maximal activity were observed with different crude preparations of soybean phospholipid (asolectins) and highly pure PC or PE, respectively, optimal reactivation was observed with a mixture of 76% PC, 19% PE and 5% CL, roughly reflecting the relative abundance of these lipids in the inner mitochondrial membrane [26]. The specific activity reached by adding a saturating amount of this lipid mixture varied somewhat between different batches of protein, but often reached 6–7 μmol min^{-1} mg^{-1}, a value matching the estimated turnover number of *Y. lipolytica* complex I in mitochondrial membranes of 110–130 s^{-1} NADH oxidised per molecule of complex I.

When we titrated the number of phospholipid molecules needed for maximal reactivation, we found that it also depended on the composition of the lipid micelles added to the complex. With neutral PC about 450 lipid molecules per complex I were sufficient, while almost 900 were needed if acidic PE was used, which may be explained by electrostatic repulsion. Four hundred and fifty lipid molecules are sufficient to form a hydrophobic annulus around the hydrophobic domain of the complex (dimensions for *Y. lipolytica* complex I estimated from the preliminary low-resolution structure derived from single particle analysis, Zickermann et al., unpublished results), suggesting that such a layer of phospholipids shields the hydrophobic regions of the membrane arm from the aqueous phase and is needed for full activity with hydrophobic quinones like DBQ. PC and PE were also proposed to serve as a ‘solvent’ for ubiquinones in earlier studies with bovine complex I isolated in cholate [31,33,35]. A very similar phospholipid dependence was reported for bovine heart cytochrome bc_1 complex purified in Triton X-100 [36].

A specific cardiolipin requirement was reported for several complexes of the respiratory chain and the carriers of the inner mitochondrial membrane (for reviews see Refs. [26,27,37]). Recently, bound cardiolipin (in addition to one

PC, one PI and two PEs) was detected in the structure of the yeast cytochrome bc_1 complex [38], where it might be involved directly in proton uptake. The precise function of cardiolipin in complex I from *Y. lipolytica* is difficult to assess from our data. Since the highest activities were only obtained in the presence of cardiolipin (added as purified phospholipids or as component of asolectin), an essential role of cardiolipin, be it structural or catalytic, seems possible. As in earlier studies with the bovine enzyme [32,33], cardiolipin alone failed to regain full activity, probably because this phospholipid is not able to build a regular lipid bilayer, but rather forms precipitates in the reversed hexagonal state [37].

In summary, we found that the inactivation of complex I from *Y. lipolytica* by general delipidation could be fully reversed simply by returning the phospholipids that had been removed during the purification procedure. This demonstrates that our homogeneous and highly pure enzyme retained its full catalytic potential and that no specific, functionally essential component had been lost. Endogenous ubiquinone-9 also turned out to be non-essential, as the amount still bound was found to be clearly substoichiometric (0.2–0.4 mol/mol). The latter finding has some important implications for the catalytic mechanism of complex I (reviewed in Ref. [12]): as no known prosthetic group of complex I could be assigned to the membrane domain of complex I, the nature of the proton pump is still obscure. Therefore, several authors have proposed that prosthetic ubiquinone(s) [13,39–41] or a quinoid prosthetic group 'X' [34] are involved in electron transport and the proton translocation mechanism. Taking its substoichiometric content in the purified enzyme, endogenous ubiquinone-9 could only serve as a functional prosthetic group of complex I, if one assumes that it could be replaced instantly by added decylubiquinone (the conditions of our activity measurements). A similar result was obtained by Fato et al. [42] in investigations with bovine heart submitochondrial particles: after pentane extraction of most of the endogenous ubiquinone-10, the complex I-specific reduction of different quinone substrates (DBQ, CoQ_1) still reached its maximal rate. Based on these and our own results, it also seems unlikely that any non-covalently bound endogenous quinone is required for complex I activity, as one would have expected it to be partially extracted by the detergent, thereby preventing full reactivation merely by purified phospholipids. For the postulated but yet unidentified additional prosthetic group, this leaves a covalently bound quinone or quinoid group as the most likely alternative.

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