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The proton pumping stoichiometry of purified mitochondrial complex I reconstituted into proteoliposomes

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Abstract

NADH:ubiquinone oxidoreductase (complex I) is the largest and most complicated enzyme of aerobic electron transfer. The mechanism how it uses redox energy to pump protons across the bioenergetic membrane is still not understood. Here we determined the pumping stoichiometry of mitochondrial complex I from the strictly aerobic yeast *Yarrowia lipolytica*. With intact mitochondria, the measured value of $3.8\overrightarrow{H}^{+}/2\bar{e}$ indicated that four protons are pumped per NADH oxidized. For purified complex I reconstituted into proteoliposomes we measured a very similar pumping stoichiometry of $3.6\overrightarrow{H}^{+}/2\bar{e}$. This is the first demonstration that the proton pump of complex I stayed fully functional after purification of the enzyme.

Keywords: Complex I; NADH:ubiquinone oxidoreductase; Proton pump; Energy transduction; Mitochondria; Proteoliposome

1. Introduction

Proton pumping NADH:ubiquinone oxidoreductase (complex I) is the largest and most complicated enzyme of the respiratory chain that resides in the inner membrane of mitochondria or the plasma membrane of bacteria [1]. It catalyzes reversible transfer of electrons from NADH to ubiquinone coupled to the translocation of *n* protons across the membrane:

$$\mathrm{NADH} + \mathrm{Q} + \mathrm{H}^{+} + n\mathrm{H}_{i}^{+} \longleftrightarrow \mathrm{NAD}^{+} + \mathrm{QH}_{2} + n\mathrm{H}_{o}^{+} \qquad (1)$$

where *n* (equal to $\overrightarrow{H}^{+}/2\bar{e}$) is the number of H^{+} translocated per two electrons passing through the redox centers of the enzyme from pyridine nucleotide to endogenous ubiquinone or, in vitro, to more hydrophilic ubiquinone analogues. In addition, one so-called scalar proton per oxidized NADH is consumed. A pumping stoichiometry of $4\overrightarrow{H}^{+}/2\bar{e}$ was reported for complex I in rat liver mitochondria [2] and submitochondrial particles [3]. Unlike cytochrome bc_1 complex and cytochrome *c* oxidase that contain spectrally distinct heme centers, complex I contains only flavin and iron–sulfur clusters i.e. redox centers that absorb at shorter wavelengths and have broad, overlapping spectra. This severely limits the experimental approaches available to follow the redox events during the catalytic turnover of complex I.

In mammals, complex I is composed of 45 protein subunits [4] and contains FMN and 8 iron–sulfur clusters as redox cofactors [5,6]. Two complex I associated, EPR detectable semiquinone species with different spin relaxation properties occur during catalysis and have been characterized [7,8]. Hypothetical schemes for the coupling mechanism of mammalian complex I are abundant in the literature (for a review see [9]). These mechanisms involve flavine [10,11], iron–sulfur cluster N2 [9,12] or ubiquinone [13–16] as key components of the pumping device. In spite of recent progress in the structural characterization of complex I [17–20], evidence on the molecular details of the proton translocation machinery is

Abbreviations: DBQ, *n*-decylubiquinone; DQA, 2-decyl-4-quinazolinyl amine; FCCP, carbonyl-cyanide-*p*-trifluoro-methoxy-phenylhydrazone; FMN, flavin mononucleotide; HAR, hexaammineruthenium (III)-chloride; Q_1, 2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone

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hardly available. The extreme complexity of complex I and the concomitant lack of conclusive experimental data still render testing of mechanistic models very difficult. Recent experimental data suggest that long range redox dependent conformational changes drive proton translocation across the bioenergetic membrane [21–25].

So far, 38 subunits have been characterized of complex I from *Yarrowia lipolytica* [26,27]. This enzyme contains one FMN and five EPR detectable iron–sulfur clusters [28]. As a model organism, *Yarrowia lipolytica* [29] offers a number of advantages including fast affinity purification of stable complex I of high purity and efficient site-directed mutagenesis. Electron transfer activity of the isolated enzyme can be fully restored by the addition of phospholipids [30].

For advanced functional studies, reconstituted systems that allow analysis of the proton pumping activity of complex I without interference from other components of the mitochondria should be used. Recently, we published an optimized protocol for the preparation of proteoliposomes containing *Y. lipolytica* complex I and demonstrated qualitatively that the reconstituted enzyme pumps protons [31]. Here we show that reconstituted complex I from *Y. lipolytica* pumps protons with the same $\overrightarrow{H}^{+}/2\overline{e}$ ratio as in intact mitochondria.

2. Experimental procedures

2.1. Isolation of mitochondria

Intact mitochondria from *Y. lipolytica* were prepared essentially as described previously [32]: *Y. lipolytica* strain PIPO [29] was grown aerobically in 2 l Erlenmeyer flasks containing 250–300 ml of 1% yeast extract, 2% Bacto peptone and 1% glucose at 28 °C and 200 rpm. Cells (~4 l of final culture) were harvested at early logarithmic stage (OD ~3–4) by centrifugation and washed twice with ice cold water (3500×*g*, 10 min). The cells were then resuspended (0.1 g wet cells/ml) at room temperature in 50 mM Tris/HCl buffer (pH 8.6) supplemented with 5 mM dithiothreitol and incubated for 10 min, diluted with cold water and washed twice again. After the last centrifugation the weakened cells were resuspended (0.1 g wet cells/ml) in 1.2 M sorbitol and 10 mM Na/HEPES (pH 7.5) and 3–4 mg/ml zymolyase 20T (from *Arthrobacter luteus*, ICN Biomedicals) was added to digest the cell wall. The formation of spheroplasts was monitored spectrophotometrically; usually incubation for 10–15 min at 30 °C was sufficient for complete digestion. To stop zymolyase, 0.2 mM Pefablock SC was added, the spheroplast suspension was rapidly cooled in an ice-salt bath and centrifuged at 500–600×*g* for 10 min. The supernatant including a turbid fluffy upper layer was discarded and the pellet was resuspended and washed twice in the same buffer containing 4 mg/ml bovine serum albumine (fatty acid free). The pellet of the last centrifugation was resuspended in disruption buffer (0.4 M mannitol, 20 mM Tris/HCl (pH 7.3), 0.5 mM EDTA and 4 mg/ml bovine serum albumine) and spheroplasts were disrupted by 20 gentle strokes in a loosely fitted Dounce homogenizer. The suspension was diluted twice with isolation buffer (disruption buffer as given above but with 0.6 M mannitol) and centrifuged at 2000×*g* for 10 min. The supernatant was collected and centrifuged once more at 7000 ×*g* for 20 min; then the pellet was resuspended in a smaller volume of isolation buffer and centrifuged again. Eventually, the mitochondria were resuspended in 500–700 µl of the same buffer and used immediately. All operations were performed in the cold room and pre-cooled glassware and centrifuge tubes were used. The cytochrome content of the mitochondria was determined by difference redox spectra in the presence of detergent [33]. Intramitochondrial NAD(H) content was determined using the procedure by Estabrook [34].

2.2. Measurement of respiration and proton pumping of mitochondria

Polarographic measurements of mitochondrial activities (0.2–0.4 mg/ml protein) were performed in 2.5 ml of isolation buffer supplemented with 2 mM phosphate (pH 7.3) using an Oxygraph-2k system (Oroboros, Innsbruck, Austria) with DatLab software. All activity measurements were conducted at 25 °C and additions were made with Hamilton syringes.

Measurements of proton extrusion were performed in a Shimadzu UV-300 dual wave-length spectrophotometer specially designed for sensitive measurement of turbid samples with fast stirring. Proton uptake from the matrix of intact mitochondria was measured in isolation buffer except that 60 mM Tris/HCl as efficient buffer on the outside was added. This allowed using 80 µM of the membrane permeable indicator dye neutral red to monitor pH changes in the mitochondrial matrix at 529–475 nm. Mitochondria (2.7–3 mg/ml) were placed in a 2 ml cuvette at 30 °C and consecutively substrates, 40 mM KCl, 1 µM valinomycin and 2 mM cyanide were added and incubated for 4–6 min until a baseline drift had stabilized. 10 mM malate+10 mM pyruvate or 200 µM NADH in the presence of 5 µM DQA were used for reduction of intramitochondrial nucleotides or endogenous ubiquinone via NDH2, respectively. Pulses of redox dependent proton translocation from the matrix were initiated by the addition of defined amounts of ferricyanide in a small volume.

2.3. Preparation of complex I proteoliposomes

Mitochondrial membranes for complex I isolation were prepared according to published protocols [35]. The enzyme was affinity purified from isolated mitochondrial membranes that were solubilized with n-dodecyl-β-D-maltoside as described [36] and was stored as small aliqouts in liquid nitrogen. Complex I proteoliposomes were prepared essentially following published procedures [31,36]: A mixture of asolectin (10 mg/ml) and octylglucoside (1.6%) in 50 mM Na/Mops (pH 7.2), 40 mM NaCl, 0.1 mM EDTA, 2 mM KCl was sonicated on ice under a flow of argon until the solution became transparent. Purified complex I (0.3–0.5 mg/ml) was added to the mixture and was incubated at 0 °C for 30 min. 50 mg/ml washed Biobeads SM-2 were added to absorb the detergent. After gentle agitation on ice for 1 h, another 70 mg/ml of Biobeads were added three times in 1 h intervals. Prior to use, the Biobeads were washed with methanol and water several times and finally put into reconstitution buffer. The beads were taken from the buffer, dried rapidly on filter paper, weighed and added to the detergent/lipid/enzyme mixture. After 4 h total incubation time, the beads were removed, the proteoliposomes were diluted 8–10 times in the same buffer (except that 3 mM Na/Mops was used) and centrifuged for 40 min at 85,000×*g* (4 °C). The pellet was resuspended by very gentle pipetting in the same weak buffer and stored on ice.

2.4. Measurement of catalytic activities and proton pumping of proteoliposomes

NADH-dependent activities of proteoliposomes (340–400 nm, 2–5 µg protein/ml) and oxonol response (623–604 nm, 30–45 µg protein/ml) measurements were carried out in reconstitution buffer with a Shimadzu Multispec 1501 diode array spectrophotometer in a 2 ml cuvette with permanent stirring at 25 °C. Routinely, freshly prepared proteoliposomes were tested for specific activities and respiratory control. The concentration of the substrates and other additions were NADH, 100 µM; HAR, 2 mM; DBQ, 70 µM; Q_1, 100 µM and FCCP, 0.25–0.5 µM; oxonol, 2 µM. The oxonol response was calibrated for each batch of proteoliposomes with potassium diffusion potentials as described by Cooper et al. [37] and was linear up to 130 mV.

Measurements of the $\overrightarrow{H}^{+}/2\overline{e}$ stoichiometry with complex I proteoliposomes were carried out in a Shimadzu UV-300 spectrophotometer with a head-on photomultiplier setup for turbid samples and fast stirring. For optical registration of pH changes in the medium during the redox reaction, phenol red (559–600 nm) was used as external indicator. The permanent basal drift due to CO_2 absorption from the air was eliminated by applying a gentle argon flow over the sample. Usually, 0.13–0.25 mg protein in liposomes in 2 ml of 3 mM Na/Mops, pH 8.0, 40 mM NaCl, 0.1 mM EDTA, 60 µM phenol red was used. After addition of 150 µM Q_1 (or 100 µM DBQ), 0.5 µM valinomycin and 20 mM KCl,

the reaction was started by defined amounts of NADH freshly prepared in the same buffer. It should be noted that the first pulse was not used for stoichiometry calculations, as it was necessary to fully activate complex I [38,39]. All measurements were conducted at 30 °C and additions were made with Hamilton syringes. The phenol red response was calibrated using HCl additions as a standard. The time constant for instrument response including stirring was less than 0.5 s. The measurements were recorded on paper and converted to digital format using WinDig software. Data analysis and fitting was performed using the Origin 6.0 software package.

2.5. Chemicals

Asolectin (= total soy bean phospholipids extract with 20% lecithin) was purchased from Avanti Polar Lipids (Alabaster, Alabama). n-dodecyl-β-D-maltoside was obtained from Glycon (Luckenwalde, Germany) and octyl-β-D-glucopyranoside from Biomol. Oxonol VI (bis-(3-propyl-5-oxoisoxazol-4-yl) pentamethine oxonol) was purchased from Molecular Probes Europe (Leiden, The Netherlands). Chelating Sepharose was from Pharmacia. The inhibitors, ionophores and all other detergents, dyes and other chemicals were from Sigma. All hydrophobic compounds were dissolved in dimethylsulfoxide.

3. Results and discussion

3.1. Characterization of intact mitochondria

For reliable measurements of $\overrightarrow{H}^{+}/2\bar{e}$ stoichiometries, the quality of the mitochondria prepared from *Y. lipolytica* was critical. Therefore we first assessed the intactness and homogeneity of our mitochondrial preparation. The cytochrome content per mg protein was determined in a representative preparation at 0.66 nmol/mg cyt $c+c_1$, 0.41 nmol/mg cyt *b* and 0.11 nmol/mg cyt aa_3. The NADH content was in the range of 5–7 nmol/mg protein and seemed to depend somewhat on the growth phase of the *Y. lipolytica* cells at harvest time. For different substrates *Y. lipolytica* mitochondria exhibited good respiratory control and ADP:O ratios that were close to commonly observed values (Table 1). Activities with substrates of NAD^+ dependent matrix dehydrogenases were completely inhibited by the specific complex I inhibitors rotenone and DQA (not shown). Since *Y. lipolytica* mitochondria contain an alternative NADH dehydrogenase (NDH2) at the outside of the inner mitochondrial membrane [35] our preparation oxidized external NADH at high rates in an rotenone insensitive manner, resulting in a correspondingly lower ADP:O ratio. All measured activities were completely blocked by addition of cyanide or antimycin (not shown), indicating the absence of alternative oxidase [40,41]. The rate of uncoupled succinate oxidation by the mitochondria increased gradually over time, probably due to decreasing inactivation of complex II by oxaloacetate (not shown). State III respiration rates were not stimulated by the addition of 10 μM cytochrome *c* (horse heart), indicating that most of the outer mitochondrial membrane was intact. Uncoupled oxidation of externally added NADH was insensitive to specific complex I inhibitors and oxidation of substrates of NAD^+ dependent dehydrogenases was greatly diminished (around 90%) upon addition of 70 μg/ml alamethicin due to the loss of matrix pyridine nucleotides. This decrease in activity could be partially restored by adding back excess NAD^+ (not shown). From these characteristics we concluded that our preparation was composed mainly of intact mitochondria.

Table 1
Oxidative phosphorylation by intact mitochondria from *Y. lipolytica*

Substrate [a]	State III respiration	Respiratory control Ratio [b]	ADP:O Ratio [c]
	$\mu mol\ O \cdot min^{-1} \cdot mg^{-1}$		
malate+glutamate	0.18	1.8	2.6
malate+pyruvate	0.62	4.7	2.6
2-oxoglutarate	0.32	5.1	3.2
NADH (+DQA)	0.98	2.9	1.7

[a] Concentration of substrates were 15 mM for malate, glutamate, pyruvate, oxoglutarate and 1 mM NADH (+2 μM DQA).
[b] Respiratory control ratios (ratio of state III to state IV respiration) were determined by successive additions of limited amounts of ADP (0.1–0.5 mM).
[c] The ADP:O ratio was determined from the actual recordings using "total O" as in [53].

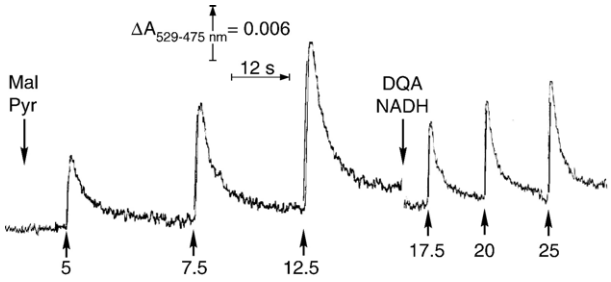

Fig. 1. Oxidant pulses with intact *Y. lipolytica* mitochondria. Representative recordings of the matrix alkalization due to proton translocation induced by oxidation of intra- (left) and extramitochondrial (right) NADH by small pulses of ferricyanide (upward arrows) with intact mitochondria (3 mg/ml) are shown. The numbers give the final concentration of ferricyanide (μM) added for each pulse. See text for further details.

3.2. Proton pumping stoichiometry of intact mitochondria

Fig. 1 shows a typical experiment to measure $\overrightarrow{H}^{+}/2\bar{e}$ stoichiometries with intact *Y. lipolytica* mitochondria. Intramitochondrial pyridine nucleotides were kept reduced by the addition of pyruvate and malate in the presence of potassium cyanide. Each peak reflects a pulse of matrix alkalization monitored as neutral red absorption change upon addition of a small amount of ferricyanide. Rapid proton uptake by complexes I and III was followed by a slower return of the matrix pH essentially to the initial level. The transient pH changes were abolished by prior addition of the cytochrome bc_1 complex inhibitors antimycin or stigmatellin (not shown). In the representative experiment shown in Fig. 1, 5 μM of DQA were added after several successive pulses of ferricyanide to inhibit complex I and external NADH was used for the reduction of the respiratory chain via the external NADH dehydrogenase NDH2. Now the observed changes of matrix pH that were induced by the addition of pulses of ferricyanide were exclusively due to the proton pumping activity of the cytochrome bc_1 complex and were inhibited by antimycin or stigmatellin, but also by the NDH2 inhibitor HDQ [42]. The addition of 2 mM phosphate

greatly decreased the apparent pH changes in the matrix due to the activity of the phosphate carrier [43]. In the presence of 2.5 μM of FCCP very short pH jumps of much smaller amplitude were observed that disappeared with higher (>5 μM) concentrations of the uncoupler (not shown). The initial neutral red response was obtained by extrapolating the exponential decay of the pH gradient to zero time in a semilogarithmic plot ([44]; Fig. 2).

The neutral red response was found to be proportional to the amount of ferricyanide added over the concentration range used and the slope of this linear dependence is a measure for the number of protons translocated per oxidant consumed (Fig. 3). It is difficult to obtain an absolute calibration for the absorption change of the indicator dye to calculate the number of protons extruded from the mitochondrial matrix per ferricyanide reduced. However, it is well established that $2\overrightarrow{H}^{+}/2\bar{e}$ are pumped out by the cytochrome bc_1 complex [45,46]. Therefore, the neutral red response with external NADH as a substrate that was only due to the proton pumping activity of cytochrome bc_1 complex could be used as an internal standard (by analogy with [2]).

In the representative data set shown in Fig. 3, the slope of the plot was 2.8 times higher with complex I substrates than with external NADH. On average the ratio between the slopes derived from all measurements with seven different batches of mitochondria (including datasets from [25]) was 2.9, which corresponds to a proton translocation stoichiometry of $5.8\overrightarrow{H}^{+}/2\bar{e}$ for complex I and cytochrome bc_1 complex together or $3.8\overrightarrow{H}^{+}/2\bar{e}$ for complex I alone. As oxidant pulse measurements tend to underestimate the pumping stoichiometry because of proton back leakage (see for example [43] and also [47]) we concluded that *Y. lipolytica* complex I pumps $4\overrightarrow{H}^{+}/2\bar{e}$.

3.3. Characterization of complex I containing proteoliposomes

As for intact mitochondria, we first determined the functional properties of the complex I proteoliposomes (Table 2). Addition of 0.25 μM FCCP as an uncoupler

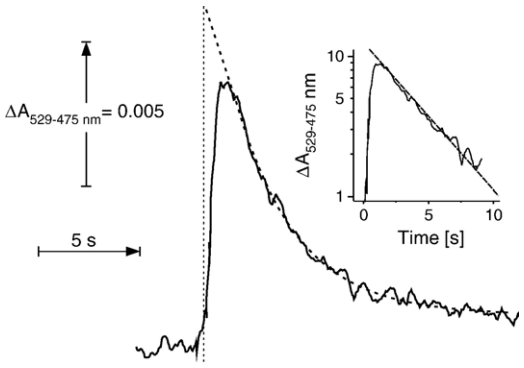

Fig. 2. Calibration of the neutral red response with intact mitochondria. Extrapolation of the absorption change for a representative oxidative pulse of 20 μM ferricyanide in the presence of external NADH is shown. All other conditions were as in Fig. 1. Insert, semilogarithmic plot of the pulse and linear fit of the decay.

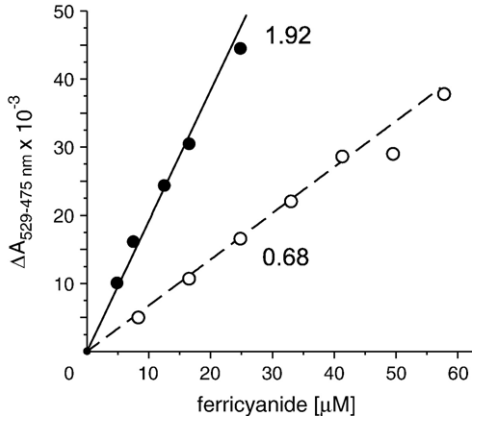

Fig. 3. Determination of pumping stoichiometries with intact mitochondria. The extrapolated neutral red response for complex I+cytochrome bc_1 complex (matrix NADH, closed symbols) and cytochrome bc_1 complex+non-pumping alternative NADH-dehydrogenase (external NADH, open symbols) as a function of the amount of ferricyanide added to initiate the reaction. The conditions were as in Fig. 1. The slopes are given next to the fitted lines.

increased the NADH:DBQ oxidoreductase activity 2.2- to 4.5-fold. This respiratory control ratio varied from preparation to preparation and tended to be higher when reconstitution was carried out at a smaller scale (0.3–0.5 mg of total protein). In some cases this value was below 2.0 and we considered these batches to be not of sufficient quality for reliable stoichiometry measurements. When Q_1 rather than DBQ was used as electron acceptor, the respiratory control ratios and the activities in the presence of uncoupler were somewhat lower (Table 2). The specific complex I inhibitor DQA inhibited NADH:DBQ (>95%) and NADH:Q_1 (>90%) oxidoreductase activities with an efficiency comparable to mitochondrial membranes (not shown). Since neither solubilization of the proteoliposomes with octylglucoside nor permeabilization with alamethicin stimulated NADH:HAR oxidoreductase activity or uncoupled NADH:ubiquinone oxidoreductase activity (not shown), we concluded that the orientation of the active site of complex I was predominantly to the outside in our proteoliposomes.

Using pulses of ubiquinone in the presence of excess NADH was not feasible for several reasons to determine pumping stoichiometries with complex I reconstituted into proteoliposomes. The problems using this approach included difficulties to deliver small aliquots of ubiquinone reliably to the enzyme, high K_m values and inactivation of reduced complex I during extended incubation in the presence of high concentrations of NADH. Therefore, reductant rather

Table 2
NADH-dependent activities of complex I containing proteoliposomes

NADH:HAR	NADH:DBQ		NADH:Q_1	
Activity [a]	Activity	RCR [b]	Activity	RCR
25.0	6.5	2.2–4.5	4.3	2.0–3.0

[a] All activities (μmol NADH · min^{-1} · mg^{-1}) are in the presence of FCCP.
[b] Respiratory control ratio ±FCCP; NADH:HAR activity was not changed upon addition of uncoupler.

than oxidant pulses had to be used to initiate redox linked proton translocation The K_m of complex I from *Y. lipolytica* for NADH is 15 μM [35]. This is significantly higher than for the bovine enzyme [48] and required the use of pulses in the same concentration range as the K_m value, which may appear problematic on first sight. However, assuming a complex I concentration of about 100 nM in the cuvette and a turnover number of at least 100 s^{-1} at 30 °C, it can be estimated that 90% of a pulse of 15 μM will be consumed in about 3 s, which should be sufficiently fast for the observed half-time of pH relaxation and extrapolation procedure used (see below).

We monitored the generation of a membrane potential in proteoliposomes as spectral changes resulting from the redistribution of the lipophilic dye oxonol VI [49]. Positively charging the membrane on the inside results in the accumulation of the dye in the lipid phase and a prominent red shift of its spectrum. Fig. 4 shows that in the presence of excess Q_1 small additions of NADH to the proteoliposomes caused an uncoupler and ionophor sensitive oxonol response. Similar results were obtained with DBQ as electron acceptor (not shown). The amplitude of the oxonol response is a measure for the electric potential across the membrane and was essentially proportional to the concentration of added NADH (1–5 μM) for $\Delta\Psi$=20–120 mV (Fig. 4, insert). It should be noted that these transient optical changes were small as compared to the response obtained during the steady state reaction of complex I under otherwise similar conditions [31]. The oxonol response was completely abolished in the presence of uncoupler (FCCP or gramicidin) or if the enzyme was inhibited by DQA (not shown).

3.4. Proton translocation by complex I containing proteoliposomes

Fig. 5A shows a typical recording of pH changes monitored by phenol red in a weakly buffered solution of complex I containing proteoliposomes. In the presence of

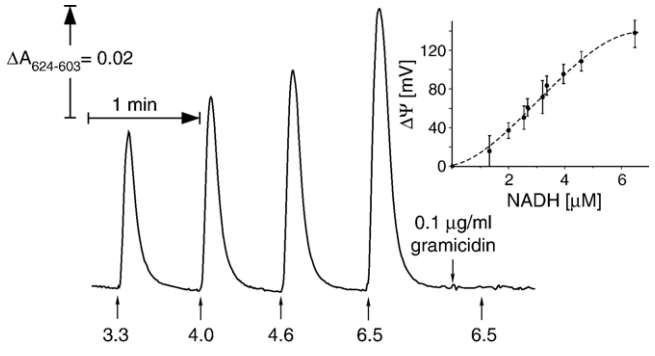

Fig. 4. Membrane potential formation with complex I proteoliposomes. NADH induced pulses of oxonol response with complex I proteoliposomes (0.05 mg protein/ml) in the presence of 120 μM Q_1. Additions were made as indicated by arrows and the numbers give the final concentration of NADH (μM) added. Insert, $\Delta\Psi$ formed as a function of the NADH concentration used for each pulse. The error bars give the standard deviations from 3 to 5 pulses at the same NADH concentration that were measured with different samples.

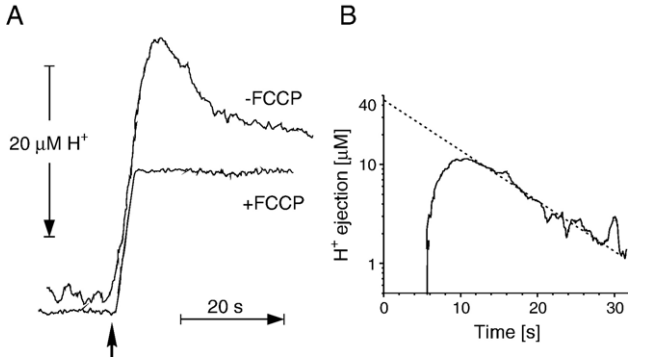

Fig. 5. Calibration of the phenol red response with complex I proteoliposomes. (A) Representative trace of proton uptake by proteoliposomes (0.13 mg protein/ml) following a pulse of 14 μM NADH (arrow) in the presence of 120 μM Q_1 as monitored with phenol red. The overshoot of the response was completely abolished by the addition of 10 μM FCCP. (B) Semilogarithmic plot of pulse shown in A in the absence of uncoupler. The pH shift after re-equilibration that was equimolar to the amount of added NADH was subtracted.

120 μM Q_1, rapid alkalization occurred after a pulse of 14 μM NADH that was maximal within 2–3 s. Subsequently, slow equilibration of inner and outer pH could be observed. K^+/valinomycin was always present during these experiments, but we found that this addition increased proton uptake into the vesicles only slightly (not shown). When all added NADH had been consumed, the alkalization phase was followed by slow exponential reacidification of the outside buffer towards a certain pH level. The half time for this equilibration process was about 8–10 s. The overshoot of vectorially translocated protons was totally abolished in the presence of gramicidin or FCCP (Fig. 5A) and the more alkaline pH attained in the medium corresponded to the final pH value reached in the absence of uncoupler. Thus the uncoupler insensitive pH change corresponded to the protons consumed upon NADH oxidation and ubiquinone reduction (see Eq. (1)). As one of these so-called scalar protons is formed per NADH consumed, the irreversible pH change can also be used to calibrate the neutral red response. The total amount of protons pumped in the first seconds after the pulse can then be assessed by extrapolating back the exponential decay of the pH gradient (Fig. 5B; [50]). This number divided by the amount of NADH added represents the proton pumping stoichiometry $\vec{H}^+/2\bar{e}$. It should be noted that different approaches have been used to extrapolate the amount of pumped protons for the pulse method that have been discussed controversially in the literature. These include back extrapolation to zero time [50], extrapolation to a point half-way between start, and end of the reaction [44] or two exponential models [51]. We have tried to use the latter model to analyze or data with some adjustments that were necessary because it does not take into account the scalar alkalinization associated with complex I turnover. However, it was difficult to obtain a unique solution for the two-exponential fits and the resulting stoichiometry values tended to be only slightly lower (<10%) than those reported here. Thus, we considered back extrapolation to zero time as giving the most reasonable estimate of the pumped protons as it takes into consideration

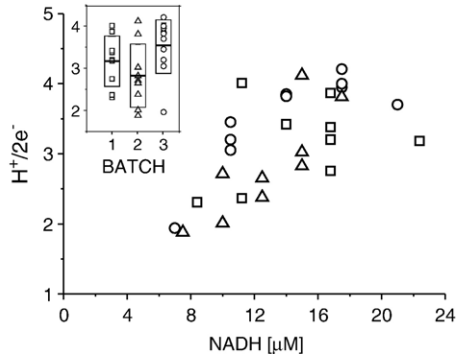

Fig. 6. $\overrightarrow{H}^{+}/2\bar{e}$ stoichiometry of purified complex I after reconstitution into proteoliposomes. Stoichiometry values of individual measurement with three different batches of proteoliposomes are plotted as a function of the NADH concentration used for each pulse. Symbols correspond to measurements with different batches. Insert: Distribution of $\overrightarrow{H}^{+}/2\bar{e}$ values for three batches of complex I containing proteoliposomes. The standard deviations and mean values are shown as boxes with a horizontal line in the middle.

proton back leak from any origin and not only the proton released after the end of the reaction.

When we calculated the average stoichiometries for three different batches of proteoliposomes, we obtained mean values spread over a rather wide range from 2.8 to $3.6\overrightarrow{H}^{+}/2\bar{e}$ (Fig. 6, insert). To analyze the reason for this high variability and to assess the feasibility of the NADH pulse approach we plotted all $\overrightarrow{H}^{+}/2\bar{e}$ values calculated from the individual pulses as a function of the amount of added NADH (Fig. 6): Up to about 14 μM NADH there was a trend that larger pulses gave higher stoichiometries. Independent of the batch of liposomes used, the lowest stoichiometries were obtained with pulses smaller than 10 μM NADH and in all three batches maximal values of around $4\overrightarrow{H}^{+}/2\bar{e}$ were measured at higher concentrations of the reductant. We concluded that at NADH concentrations significantly below the K_m value, the buildup of the proton gradient became too slow and resulted in an underestimation of the extrapolated phenol red response. The mean stoichiometry value calculated for all pulses above 14 μM NADH was 3.6 ± 0.4 $\overrightarrow{H}^{+}/2\bar{e}$ ($n=16$). Thus we concluded that the pumping stoichiometry of purified and reconstituted complex I was the same as in intact mitochondria.

4. Conclusions

Our results demonstrate that complex I from the strictly aerobic yeast *Y. lipolytica* pumps $4\overrightarrow{H}^{+}/2\bar{e}$ in intact mitochondria. The same stoichiometry has been measured with rat heart mitochondria [2] and submitochondrial particles [3]. However, in the only study on the pumping stoichiometry of purified complex I after reconstitution into proteoliposomes a value of only $1.4\overrightarrow{H}^{+}/2\bar{e}$ was reported for the bovine heart enzyme [52]. This low value may have resulted from technical problems with proton translocation measurements, but could also have reflected a functional defect of the purified complex. Here we show that after reconstitution into proteoliposomes yeast complex I pumps protons at the same stoichiometry as in the native mitochondrial environment. This is the first demonstration that the proton pump by a purified complex I when the enzyme is fully functional.

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