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During the stationary growth phase, *Yarrowia lipolytica* prevents the overproduction of reactive oxygen species by activating an uncoupled mitochondrial respiratory pathway

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ABSTRACT

In the branched mitochondrial respiratory chain from *Yarrowia lipolytica* there are two alternative oxido-reductases that do not pump protons, namely an external type II NADH dehydrogenase (NDH2e) and the alternative oxidase (AOX). Direct electron transfer between these proteins is not coupled to ATP synthesis and should be avoided in most physiological conditions. However, under low energy-requiring conditions an uncoupled high rate of oxygen consumption would be beneficial, as it would prevent overproduction of reactive oxygen species (ROS). In mitochondria from high energy-requiring, logarithmic-growth phase cells, most NDH2e was associated to cytochrome *c* oxidase and electrons from NADH were channeled to the cytochromic pathway. In contrast, in the low energy requiring, late stationary-growth phase, complex IV concentration decreased, the cells overexpressed NDH2e and thus a large fraction of this enzyme was found in a non-associated form. Also, the NDH2e–AOX uncoupled pathway was activated and the state IV external NADH-dependent production of ROS decreased. Association/dissociation of NDH2e to/from complex IV is proposed to be the switch that channels electrons from external NADH to the coupled cytochrome pathway or allows them to reach an uncoupled, alternative, ΔΨ-independent pathway.

1. Introduction

Mitochondria from fungi, plants and parasites often contain branched respiratory chains, constituted by orthodox and "alternative" redox enzymes present in different stoichiometries [1–3]. In mitochondria from *Yarrowia lipolytica* the respiratory chain is composed by the four multi-subunit complexes (I to IV) found in animals and plants, plus an external type II NADH dehydrogenase (NDH2e) and an alternative oxidase (AOX) [4]. Both NDH2e and AOX are single-subunit peripheral oxido-reductases that lack proton-pumping activity [5,6].

Branched respiratory chains may include different alternative dehydrogenases that reduce ubiquinone without contributing to the proton gradient. Thus, ubiquinone may be reduced by complex I, succinate-dehydrogenase, glycerol-phosphate dehydrogenase, dihydroorotate-dehydrogenase or by internal or external NDH2s [7,8]. All these enzymes use flavin in the redox reaction [9–11]. From ubiquinol, electrons can reach either complexes III/IV (cytochromic pathway) or an AOX [12]. The many pathways available open the possibility that electrons may reach oxygen with different proton-pumping stoichiometries, even passing only through enzymes that do not translocate protons at all [13], i.e. the ADP:O ratio can vary widely in branched respiratory chains [14].

Structural models of AOX, whether monomeric or dimeric [15–17], suggest it has regulatory sites for nucleotides and/or for α-ketoacids [18,19]. Some yeast species contain only one AOX, which is activated under stress. Other species contain two isoforms of AOX, one constitutive but expressed at low levels and another one inducible under stress [16]. Remarkably, no species is known where the absence of complex-I and the presence of AOX coexist; it has been proposed that such a combination would lead to uncontrolled uncoupling [1,4].

Unless uncoupled respiration is desired, energy-requiring cells must avoid pairing non proton-pumping dehydrogenases with AOX [1]. Therefore, it is of interest to define the usefulness of the non-pumping enzymes. These enzymes might prove useful in medicine and biotechnology; alternative NDH2i (internal NDH2) has been expressed in mammalian cells to partially substitute for a non-functional complex I [20]. In addition, NDH2 has been expressed in

Abbreviations: NDH2e, alternative external NADH dehydrogenase; AOX, alternative oxidase; ΔΨ, transmembrane potential; CCCP, carbonylcyanide-3-chlorophenylhydrazone; ROS, reactive oxygen species

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aerobic fungi or yeast harboring complex I deficiencies with the aim to analyze mitochondrial function [21,22].

Alternative component activity must be tightly regulated in response to the energy requirements or to the redox state of the cell. In different species of yeast, AOX is over-expressed under stress [23] or in the stationary growth phase [24]. Channeling electrons between a proton-pumping complex and an alternative component would prevent excessive wastage of energy and thus functional associations such as complex I-AOX [25] or NDH2-complexes III/IV [26] have been proposed. In addition, some redox enzymes might alternate between a bound, electron-channeling state and a free, non-channeling state in order to regulate the proton pumping efficiency of the respiratory chain and thus the ADP:O.

Y. lipolytica mitochondria were isolated from cells grown to either the high-energy requiring logarithmic growth phase (log-phase) or the low-energy requiring stationary growth phase (stat-phase) [27–29]. It was observed that in the log-phase, NDH2e-derived electrons were channeled to the cytochromic pathway and NDH2e was bound to complex IV, probably in a complex III/complex IV super-complex. In contrast, in the stat-phase the NDH2e–AOX uncoupled-electron transfer pathway was activated, most likely as a result of the dissociation of NDH2e from complex IV. In these conditions ROS production was largely inhibited, suggesting a physiologic role for uncoupling.

2. Experimental procedures

2.1. Materials

NADH, Glycerol-phosphate, n-propylgallate, n-β-D-dodecylmaltoside, digitonin, mannitol, pyruvate, malate, cytochrome *c*, antimycin-A, rotenone and CCCP were from Sigma Chem. Co. (St. Louis, MO, USA). Coomassie blue G was from Serva (Heidelberg, Germany). The polyclonal antibody against *Y. lipolytica* NDH2e (anti-*Yl*NDH2e) was a kind gift from Dr. Stefan Kerscher, Zentrum der Biologischen Chemie, Frankfurt University, (Germany). Monoclonal antibodies against cytochrome *c* oxidase subunits II and III from *S. cerevisiae* were from Mitoscience (Eugene, OR, USA).

2.2. Strains, culture and isolation of yeast mitochondria

The strains used in this work were the wild type: *Y. lipolytica* E150 (*MatB his1-1 ura3-302 leu2-270 xpr2-322*) and the *Δndh2e* mutant (GB5.2) [30]. All strains were a kind gift from Prof. Ulrich Brandt, ZBC, Frankfurt University (Germany). Cells were grown in YD (Yeast extract 1%, glucose 2%) [31] at 160 rpm, 30 °C for 15 or 96 h to be harvested at the logarithmic or late stationary growth phases, respectively. Cells were washed and re-suspended in 5 mM MES, 0.6 M mannitol, 0.1% BSA (pH 6.8, triethanolamine) and disrupted using a Bead Beater cell homogenizer (Biospec Products, OK, USA) with 0.45 mm glass beads (3×20 s pulses separated by 40 s resting periods). To isolate mitochondria, the homogenate was subjected to differential centrifugation [32] and protein concentration was determined by biuret [33]. Mitochondrial intactness was evaluated by measuring the respiratory controls which were between 2.0 and 3.0 [34]. The integrity of the external mitochondrial membrane was determined by reduction of diaminobenzidine in the presence of 10 μM antimycin A and cytochrome *c* either permeabilized with 0.1 mg/mg protein n-β-D-docecylmaltoside or not. Diaminobenzidine reduction was measured by absorbance changes at 490 nm. In non-permeabilized mitochondria the reaction was 9-fold less than in the presence of detergent. In addition, in the presence of external NADH as a substrate, rotenone inhibited less than 6% of the total oxygen consumption, indicating that external NADH was oxidized mainly by NDH2e.

2.3. Oxygen consumption measurements

The rate of oxygen uptake was measured in an oxygen meter model 782 (Warner/Strathkelvin Instruments) with a Clark type electrode in a 0.1 mL water-jacketed chamber at 30 °C [35] and data were analyzed using the 782 Oxygen System software (Warner/Strathkelvin Instruments). External NADH-dependent respiration was measured in the presence of rotenone in order to inhibit reverse electron transfer from ubiquinol to complex I; rotenone binds to the ubiquinone binding site of complex I [36]. Pyruvate plus malate was used to generate internal NADH which is oxidized by complex I. Cyanide or antimycin A was used to inhibit cytochrome *c* oxidase or the Qi site of complex III, respectively. Since both cyanide and antimycin A inhibit the cytochrome pathway, electrons are diverted towards AOX. The reaction mixture contained 0.6 M mannitol, 5 mM MES (pH 6.8), 20 mM KCl, 4 mM phosphate (the Tris salt was obtained using phosphoric acid and adjusting to pH 6.8 with Tris) and 1 mM $MgCl_2$. Mitochondria were added to a final concentration of 0.5 or 1.0 mg protein/mL. Respiratory controls using either external NADH or succinate were determined in the presence of 2.5 μM rotenone.

2.4. Protein separation by native electrophoresis and in-gel activities

BN- and CN-PAGE were performed as described [37,38]. Mitochondria were solubilized with 2 g n-dodecyl-β-D-maltoside (LM)/g protein, or 4 g digitonin (Dig)/g protein at 4 °C and centrifuged at 100,000 *g* at 4 °C for 25 min. Protein concentration of the supernatants was determined and 0.4 mg protein per well was loaded on 4–12% polyacrylamide gradient gels. For both BN and CN-PAGE digitonin (0.025%) was added to the gel preparation. The cathode buffer for CN-PAGE contained 0.01% LM and 0.05% deoxycholate as described in [39]. In-gel NADH:NBT oxido-reductase activity was determined by incubating the native gels in a mixture containing 10 mM Tris (pH 7.0), 0.5 mg nitro blue tetrazolium bromide (NBT)/mL and 1 mM NADH [40]. In-gel cytochrome *c* oxidase activity was determined using diaminobenzidine and cytochrome *c* as described in [41].

2.5. Ion exchange chromatography and enzymatic activities of the fractions

Mitochondria were solubilized with 0.8 g/g prot n-β-D-dodecylmaltoside (LM) in 1 mM Mg–SO_4, 1 mM PMSF, 50 mM HEPES, pH 8.0 plus 50 μg/mL TLCK (Tosyl-lysyl-chloromethyl ketone). The solubilizate was centrifuged at 100,000×*g* for 30 min and layered on top of a previously equilibrated DEAE-Sepharose column [42]. Once loaded, the column was washed with 3 volumes of 1 mM $MgSO_4$, 50 mM Tris, pH 8.0 and proteins were eluted with a 0–400 mM NaCl gradient. Fractions of 1 mL were collected. The protein concentration in each fraction was estimated spectrophotometrically at 280 nm. For NADH dehydrogenase or cytochrome *c* oxidase activities, 20 μL of each fraction was placed in a well within a micro plate in the presence of the substrate and an electron acceptor and the reaction was followed in a multimodal micro plate reader Synergy Mix, Biotek (VT, USA). Enzyme assays: a) NADH dehydrogenase, following the reduction of nitro blue tetrazolium bromide (NBT) at 570 nm. The reaction mixture was 10 mM Tris, pH 7.0, 1 mM NADH, 0.5 mg/mL NBT; b) Cytochrome *c* oxidase was measured in 50 mM phosphate buffer (sodium) pH 7.4, 2.5 mg/mL horse heart cytochrome *c*, 1 mg/mL diaminobenzidine and cytochrome *c* reduction was followed at 490 nm.

2.6. Immunoassays

Ion exchange chromatography fractions were loaded onto a nitrocellulose membrane for dot blot immunodetection with antibodies

against either NDH2e from *Y. lipolytica* or COXII from *S. cerevisiae*. Blots were quantified using the Image-J software. Also from SDS-Tricine-PAGE, proteins were electro-transferred onto a nitrocellulose membrane (BioRad, Hercules, CA, USA) for immunodetection. Membranes were washed, blocked, and incubated with anti-*Yl*NDH2e [30] (1:1000 for 1 h), using an alkaline phosphatase-conjugated anti-rabbit IgG (1:3000 for 2 h) as secondary antibody. A monoclonal antibody against subunit III of cytochrome *c* oxidase (COX III) from *S. cerevisiae* was used.

2.7. Cytochrome measurements using differential spectrophotometry

Mitochondria (2 mg prot/mL) were added to homogenization buffer without BSA. Absorbance spectra were recorded from 500 to 680 nm in a DW2000 Aminco Spectrophotometer in the presence of ferricyanide to obtain the oxidized spectra, which was assigned as base line. Then, the reduced spectrum was recorded after addition of sodium dithionite to the sample cuvette. Absorption coefficients were: cytochrome *b*, $\varepsilon_{562-575\ nm} = 28.5\ mM^{-1}\ cm^{-1}$ [43]; cytochrome $a + a_3$, $\varepsilon_{603-630\ nm} = 24\ mM^{-1}\ cm^{-1}$ [44].

2.8. Reactive oxygen species measurements

Reactive oxygen species production rates in freshly purified mitochondria were measured using the Amplex Red Hydrogen peroxide/peroxidase method [45] in a Synergy HT multi-mode micro plate reader, Biotek (VT, USA). Hydrogen peroxide formation was determined following resorufin fluorescence as in [46]. Reaction mixture: 0.6 M mannitol, 5 mM MES (pH 6.8), 20 mM KCl, 10 mM phosphate, 1 mM $MgCl_2$, 10 μM Amplex Red (Invitrogen, Molecular Probes, Eugene, OR, USA), 0.1 units/mL horseradish peroxidase and 100 units/mL superoxide dismutase and 0.5 mM NADH or 10 mM pyruvate plus 10 mM malate as substrates. Mitochondria were added to a final concentration of 60 μg protein/well (0.3 mg prot/mL).

3. Results

3.1. In Y. lipolytica mitochondria, direct electron transfer between alternative respiratory chain components is observed only in the stationary phase

In yeast, the need for ATP is high in the logarithmic growth phase (log-phase) and decreases when the cells enter the stationary growth phase (stat-phase) [27]. Consequently, in the log-phase a high rate of oxygen consumption is needed to replenish the electrochemical proton gradient consumed by the synthesis of ATP. In contrast, in the stat-phase less ATP is needed, so a high rate of oxygen consumption would be possible only if a certain degree of uncoupling is promoted [13,14]. The branched mitochondrial respiratory chain from *Y. lipolytica* can probably be uncoupled to different extents, depending on the number of proton pumps participating in the electron pathway followed from different substrates to oxygen [13]. To test for possible electron routes, cells were harvested at two different growth phases and the respiratory activity of isolated mitochondria was evaluated (Fig. 1). The rate of oxygen consumption did not change with the growth phase, although it was always lower using pyruvate plus malate, which are complex I substrates (Fig. 1-A) than when using external NADH, which is oxidized by NDH2e (Fig. 1-B). In contrast, the electron pathway for NDH2e was different depending on the growth phase, as evidenced by the sensitivity to different respiratory inhibitors. In either stage, when using a substrate for complex I, the rate of oxygen consumption was partially inhibited by cyanide (complex IV inhibitor) or by propyl-gallate (AOX inhibitor) and only in the presence of both inhibitors did the rate of oxygen consumption approach complete inhibition. Thus, it seems that the electrons from complex I flow through both the cytochrome pathway and the AOX pathway (Fig. 1-A). In contrast, when NADH was used to feed electrons to NDH2e, the respiratory pathway was different depending on the growth stage of the cell (Fig. 1-B). In mitochondria from log-phase cells, cyanide inhibited most oxygen consumption, while propyl-gallate had negligible effects; the inhibition pattern changed in the stat-phase, where both cyanide and propyl-gallate exhibited partial effects. Again, the simultaneous addition of both inhibitors abolished oxygen consumption completely (Fig. 1-B). Thus, in the log phase the electrons from NDH2e are channeled to the cytochrome pathway and cannot reach AOX, while in the stationary phase the NDH2e to AOX pathway becomes active.

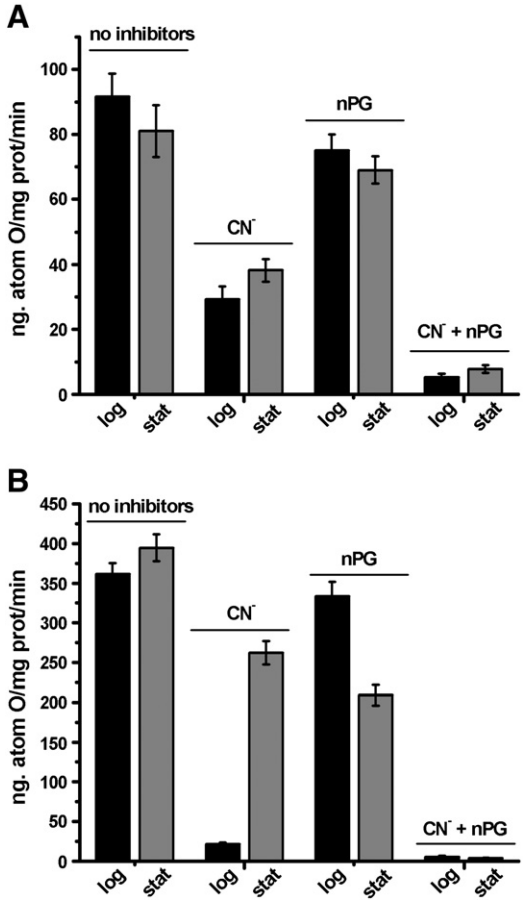
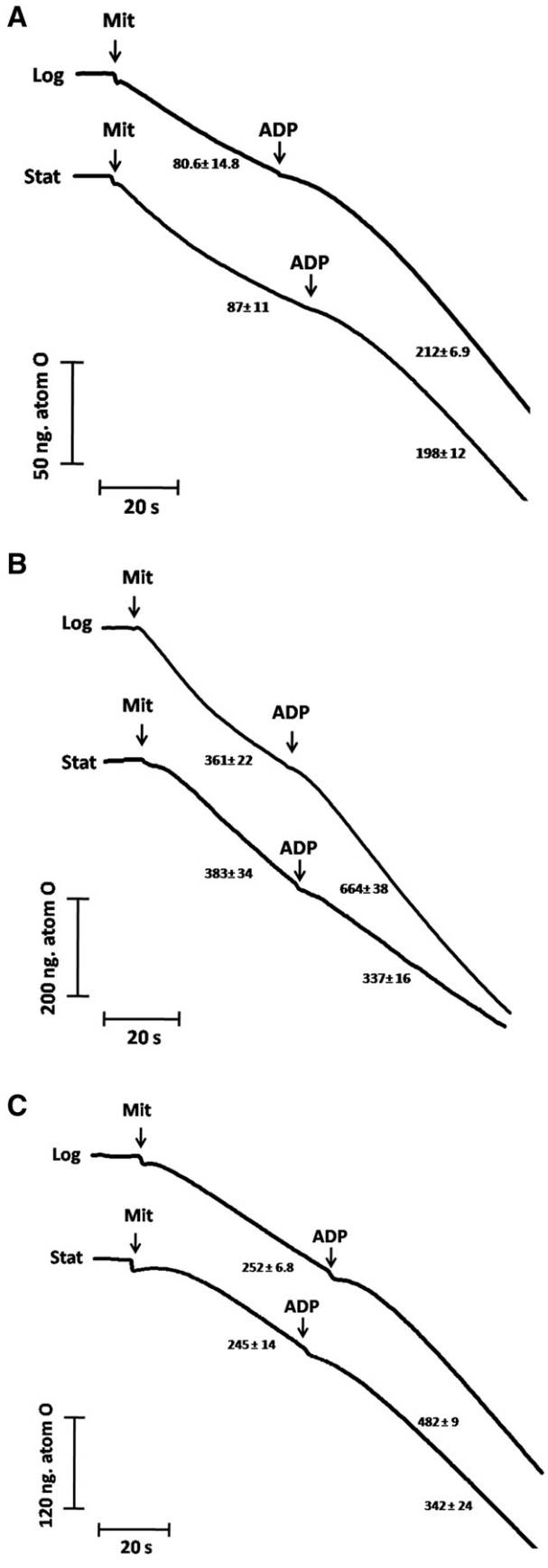


Fig. 1. Effect of oxygen consumption inhibitors on mitochondria isolated from cells grown to the logarithmic or stationary phase. Experimental conditions: 0.6 M mannitol, 5 mM MES, 20 mM KCl, 4 mM phosphate, 1 mM $MgCl_2$, pH 6.8. Oxygen consumption was measured with a Clark electrode in a closed water-jacketed chamber at 30 °C. Rates of respiration were calculated in the presence of 5 mM pyruvate plus malate (A) or 1 mM NADH in the presence of 2.5 μM rotenone (B). Additions as indicated: without inhibitors, in the presence of 100 μM cyanide, 50 μM n-propylgallate or both. Columns indicate mean ± SD (n = 5).

3.2. NDH2e-dependent oxygen consumption is uncoupled in mitochondria from Y. lipolytica in the stationary growth-phase

In mitochondria from *Y. lipolytica* cells grown to either the log- or the stat-phase, the channeling of electrons through NDH2e into the cytochrome pathway was further analyzed by evaluating coupling (Fig. 2). That is, measuring the rate of oxygen consumption in state III and in state IV, as well as the resulting respiratory controls (RC = state III/state IV). In the presence of pyruvate–malate, both the rates of oxygen consumption and the RCs remained high, both in the log-phase and the stat-phase (i.e., $RC_{log} = 2.3 \pm 0.1$ and $RC_{stat} = 2.13 \pm 0.2$) (Fig. 2-A). In contrast, when the NADH-



supported respiration was measured, coupling was dependent on the growth phase as follows: in the log-phase, $RC_{log} = 1.82 \pm 0.06$, while in the stat-phase there was no increase in the respiration rate after the addition of ADP (Fig. 2-B). Therefore, it may be concluded that in the stat-phase an uncoupled pathway where electrons enter the respiratory chain at NDH2e, was activated. The NADH-dependent uncoupling was not due to an increase in permeability of the inner mitochondrial membrane, since the response to pyruvate–malate remained intact.

The above results indicate that an NDH2e-dependent uncoupled pathway is activated in the stationary phase, opening the question on whether other non-coupled pathways become active. In this regard, it is known that complex II is not a proton pump, and thus a complex II to AOX pathway would also be fully uncoupled. Thus, we also explored the behavior of electrons coming from succinate (Fig. 2-C). In contrast to the results with NADH, in the presence of succinate the response to ADP was lost only partially in mitochondria from the stationary cells, i.e., $RC_{log} = 1.9 \pm 0.1$, $RC_{stat} = 1.4 \pm 0.1$. That is, even though the RC decreased, it was not abolished, suggesting that even in the stat-phase the electrons from complex II are partially reaching the cytochrome pathway. If there is a physical association between complex II and the III–IV super-complex, this association must be very labile, as it has not been observed in native gel electrophoresis from neither *Y. lipolytica* nor from other mitochondria [26,47]. Altogether the above data indicate that in *Y. lipolytica* a specific NDH2e–ubiquinone–AOX pathway is active in the stat-phase but not in the log-phase.

3.3. In the stationary growth-phase NDH2e is found either free of associated to complex IV

It was previously reported that in spite of the differences in isoelectric points, NDH2e and complex IV co-elute from an ion-exchange chromatography column and also, association of NDH2e with a super-complex III/IV was proposed by native gel experiments [26]. This association was proposed to be the structural basis for the electron channeling between NDH2e and the cytochrome pathway observed in mitochondria isolated from log-phase grown cells [26].

In order to determine the mechanism for the loss of electron channeling detected in the stationary growth phase (Figs. 1 and 2), we tested whether the association between NDH2e and complex IV varied depending on the growth phase of the cells (Fig. 3). Mitochondria from cells in the log (Fig. 3-A) or in the stat-phase (Fig. 3-B) were solubilized and loaded into two independent DEAE-Biogel-A ion-exchange chromatography columns. The concentration of LM used to solubilize was 0.8 mg/mg protein where, contrary to what happens at 2 mg LM/mg protein, supramolecular interactions are partially preserved (Fig. 3). The NADH dehydrogenase activities did vary slightly between the log (Fig. 3-A) and the stationary (Fig. 3-B) growth phase. However, it was difficult to evaluate the change in the specific NDH2e activity due to interference caused by complex I and other enzymes capable of oxidizing NADH. In order to subtract all NADH dehydrogenase activities that were not due to NDH2e it was decided to run a homogenate from a Δndh2e strain through the same column as the WT samples (Fig. 3-C). The Δndh2e sample expressed all NADH activities except NDH2e, and thus it was subtracted from the activities of the WT strain, aiming to obtain a good estimate of the

Fig. 2. Respiratory controls of mitochondria isolated from cells grown to the logarithmic or stationary phase. Reaction mixture: as in Fig. 1 except for the inhibitors. Mitochondria were added to a final concentration of 1 mg prot/mL from cells harvested in the log- (Log) or the stat-phase (Stat). Respiratory substrates were: A) 5 mM pyruvate plus malate; B) 1 mM NADH; C) 10 mM succinate. Where indicated, 1 mM ADP was added. When NADH or succinate was used, 2.5 μM rotenone was added to the reaction mixture. Representative traces are shown (n = 5).

NDH2e activity in the log and the stationary growth phases respectively (Fig. 3-D). Subtraction resulted in 3 peaks of NDH2e which eluted at 150, 190 and 230 mM NaCl. Peaks at 150 and 230 mM closely correspond to two of the peaks observed for cytochrome *c* oxidase activity in Fig. 3-A. In stat-phase, 4 peaks of NDH2e resulted after subtraction of the ΔNDH2e (Fig. 3-D). The first one, eluted at 80 mM NaCl, corresponds to the overexpressed, free-fraction of NDH2e, while the other 3 seem to be the same as in the log-phase. These results were confirmed when the activity profile from the wild type strain in the log-phase (Fig. 3-A) was subtracted from

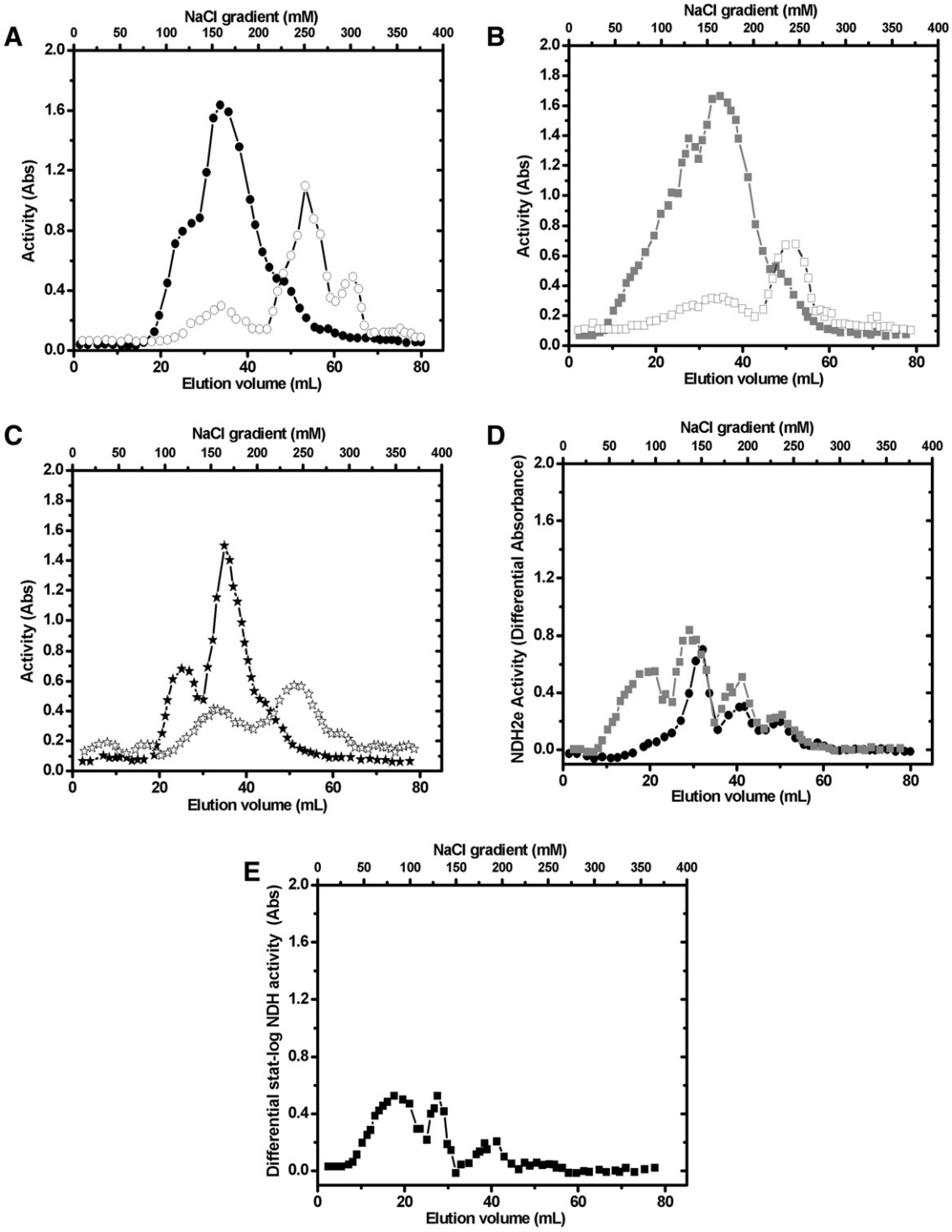


Fig. 3. Ion-exchange chromatographic elution activity and protein profiles of mitochondria from cells harvested at different growth phases. Mitochondrial extracts were loaded on a DEAE-Biogel A gel ion-exchange chromatography column previously equilibrated, washed and eluted using a 0–400 mM NaCl gradient. 1 mL fractions were collected and enzyme activities were measured as described in [26]. NADH dehydrogenase activity (filled symbols); cytochrome *c* oxidase activity (empty symbols). Samples were mitochondria from (A) Log-phase grown cells; (B) Stat-phase grown cells; (C) ΔNDH2e cells. (D) Differential trace for NADH dehydrogenase activity, A minus C (●) and B minus C (■). (E) Differential of stat-phase minus log-phase NADH dehydrogenase activity; both activities were measured from the wild type preparation. (F) Dot blot analysis was conducted on the fractions and results were plotted for NDH2e (G) and for cytochrome *c* oxidase (H). A representative experiment is shown (n = 2).

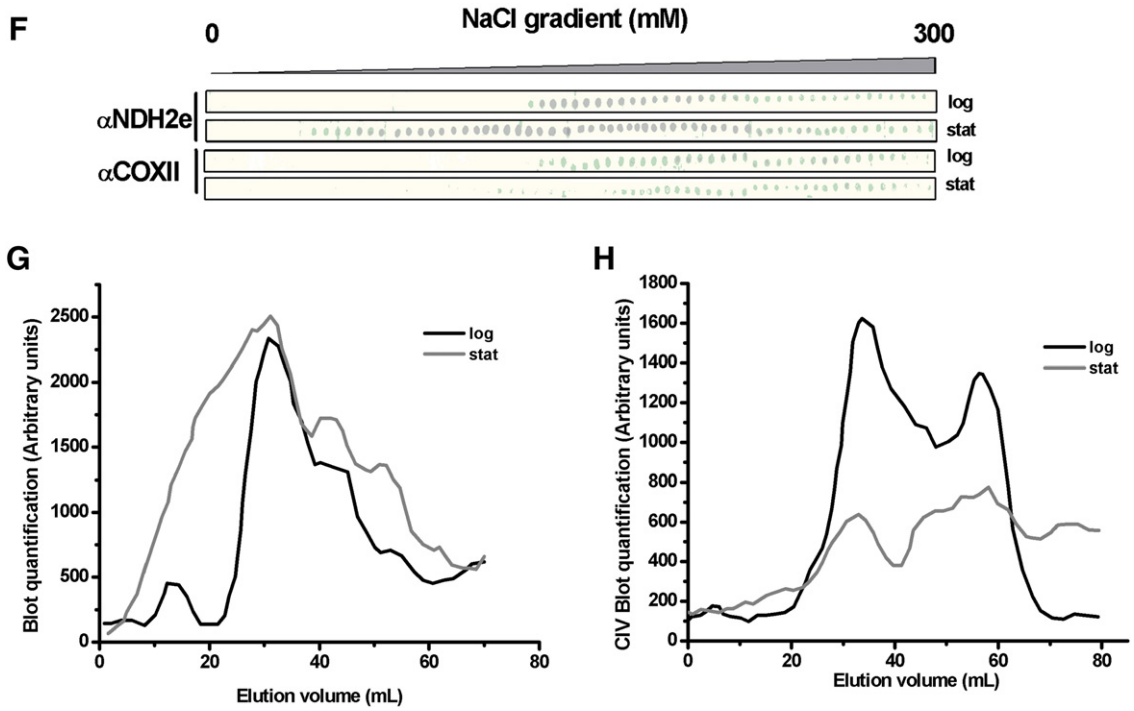


Fig. 3 (*continued*).

the profile from the wild-type strain in stat phase (Fig. 3-B) and in the subtraction (Fig. 3-E) a peak corresponding to a free NDH2e was observed at 80 mM NaCl, i.e. at the same ionic strength as in Fig. 3-D. The results suggest that upon reaching the late stationary phase, a large proportion of NDH2e is detached from complex IV.

In regard to complex IV, it was observed that it eluted in two peaks regardless of the growth phase, suggesting that it eluted as a super-complex in the first peak while the second peak represented the monomeric form of cytochrome *c* oxidase (Fig. 3-A and B). This elution pattern of cytochrome *c* oxidase was not detected in an earlier report using a higher detergent: protein ratio in the chromatography column [26]. Nonetheless, the total activity of cytochrome *c* oxidase seemed to decrease in the stat-phase (Fig. 3-B) as compared to the log-phase (Fig. 3-A). Thus, the release of NDH2e resulting from the increase in this protein combined with a decrease in complex IV might be the mechanism that turns off electron channeling to the cytochromic pathway.

To further characterize the putative growth phase-related redistribution of NDH2e, we performed a dot-blot assay for each of the fractions from the chromatography columns using an anti-*Y. lipolytica* NDH2e antibody and an anti-*S. cerevisiae* COXII subunit antibody to detect the distribution of NDH2e and complex IV in the elution profiles (Fig. 3-F). The immunochemical results confirmed the data obtained from the activity measurements, i.e. in the log-phase most NDH2e co-elutes with complex IV, while in the stat-phase a large proportion of NDH2e dehydrogenase runs free, exiting the column earlier, while only a fraction seems to co-elute with complex IV (Fig. 3-G). In addition, in stat-phase there seems to be an increase in the total amount of NDH2e (Fig. 3-G). When the complex IV immunoblots were quantitated, the presence of two populations of complex IV was observed in both growth phases, suggesting that in addition to the monomeric form of complex IV, a supercomplex containing complex IV was preserved in the column (Fig. 3-H). In addition, in the stat-phase the amount of complex IV seems to decrease (Fig. 3-H).

3.4. In gel activities and spectrophotometric assays confirm that the concentration of NDH2e increases while complex IV decreases

The NDH2e activity seemed to be much higher in the stat-phase than in the log-phase. An increase in the expression of this enzyme would explain the presence of free NDH2e. In addition, an increase in the NDH2e/COX ratio would result from either an increase in NDH2e or a decrease in complex IV. In order to test whether the NDH2/complex IV ratio was increased in the stationary growth phase, in-gel NADH dehydrogenase and cytochrome *c* oxidase activities of digitonin-solubilized mitochondria were measured after hrCN-PAGE (Fig. 4-A). In-gel activity of NADH dehydrogenase stained four bands: the higher molecular mass band was complex I, while the next two bands, marked as NDH2e and NDH2e*, were stained due to the activity of the alternative NADH dehydrogenase, since they were absent in the electrophoretic pattern of the NDH2e deletion mutant. These two bands probably represent bound and free populations of NDH2e. The fourth, lower band, present in all gels, might correspond to the activity of an NADPH dehydrogenase, as it was previously identified by LC-MS/MS analysis [26] and it also appeared in the ΔNDH2e mutant. As expected, cytochrome *c* oxidase in-gel activity revealed several bands probably corresponding to diverse complex IV-containing super-complexes. The lower band corresponds to monomeric complex IV and the upper bands to different complex IV-containing super-complexes.

In the stat-phase an increase in the band marked as NDH2e was observed, which probably corresponds to the free form of the alternative dehydrogenase, while the band marked as NDH2e* did not seem to change as compared with that observed in the log-phase (Fig. 4-A). In parallel, a decrease in cytochrome *c* oxidase activity was detected in the stat-phase, suggesting that complex IV may be down-regulated at the same time as NDH2e expression increases (Fig. 4-A). In addition, western blots of subunit III of cytochrome *c* oxidase indicated that complex IV was found at lower concentrations in the stat- than in the log-phase (Fig. 4-B). To confirm whether complex IV decreased in the stat-phase, the absorption

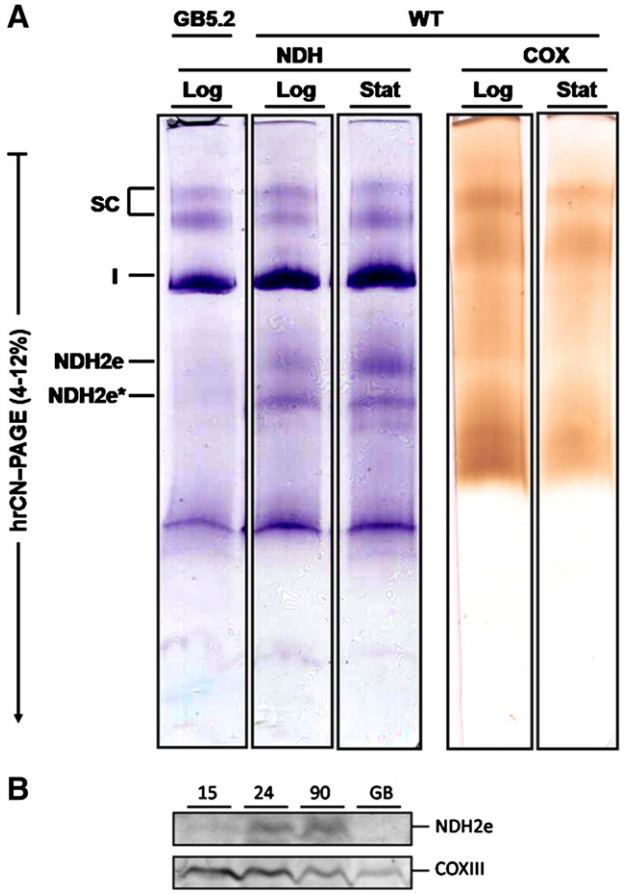


Fig. 4. Activity and protein amounts of NDH2e and complex IV. (A) In-gel NADH dehydrogenase (NDH) or cytochrome *c* oxidase (COX) activities were qualitatively determined after hrCN-PAGE of mitochondria isolated from cells grown to the log-phase (15 h) or to the stat-phase (90 h). In addition to complex-I and NDH2e, other dehydrogenases were detected. The most intensely colored band corresponds to complex I. NDH2e activity could be assigned due to the lack of staining in a *Δndh2e* mutant strain (GB5.2). Two bands corresponding to the alternative enzyme marked as NDH2e and NDH2e* probably correspond to a cytochromic pathway-interacting form and to a free form. I, complex I; IV, complex IV; SC, super-complexes; (B) Western blot of isolated mitochondria for detection of NDH2 and the complex IV subunit COXIII in different growth phases. Numbers indicate time of growth (hours) from the wild type strain; GB, *Δndh2e* mutant strain. A representative gel and blot are shown (n = 3).

spectra of cytochromes *b* and aa_3 and the activity of cytochrome *c* oxidase were measured (Fig. 5). The concentration of cytochrome *b* remained almost constant, while cytochrome $a + a_3$ decreased from 0.07 nmol/mg protein in the log-phase to 0.03 nmol/mg protein in the stat-phase (Fig. 5-A). In these same conditions the rate of oxygen consumption using ascorbate and TMPD also decreased during the stat-phase (Fig. 5-B). Altogether, the differences in the in-gel activities (Fig. 4-A), the decreased complex IV contents detected by western blot (Fig. 4-B) and the absorbance spectra (Fig. 5), are consistent with an increase in the NDH2e:complex IV ratio that in turn would lead to accumulation of non-associated NDH2e that would be free to reduce AOX.

3.5. In mitochondria from cells in the stationary phase, NADH oxidation produces less reactive oxygen species

The uncoupled (NDH2e–AOX) respiratory pathway increased during the stat-phase, although a fraction of NDH2e was still interacting with the cytochrome pathway (Fig. 3). Thus, in the stat-phase the electrons from NDH2e are probably fed to both the cytochromes and the AOX instead of being channeled to the cytochrome bc_1 complex. At high $\Delta\Psi$, complex III has been reported to be an important source of reactive oxygen species (ROS) [48]; therefore, the activation of the alternative pathway would be a most desirable mechanism to decrease the rate of ROS production. To test this hypothesis, the rate of hydrogen peroxide formation was measured in mitochondria isolated from cells either in the log- or the stat-growth phase using NADH or pyruvate–malate as substrates (Fig. 6). In the presence of NADH, mitochondria from log-grown cells exhibited a large rate of ROS production, while those from cells in the stat-phase decreased ROS production, as expected from the activation of the NDH2e–AOX pathway. In contrast it was observed that the pyruvate–malate-supported rate of H_2O_2 production was lower and not modified by the growth phase (Fig. 6). In addition, rotenone blocked electron transfer at the level of complex I, promoting an increase in ROS production from this complex (Fig. 6) as expected from data in the literature [49].

4. Discussion

Depending on the growth phase and metabolic requirements, yeast cells undergo large variations in energy needs [27]. In contrast to multiplying cells, quiescent cells need less ATP, and in this situation a fully coupled, slow respiratory chain would overproduce ROS [50]. In order to increase the rate of oxygen uptake in the stat-phase, the respiratory chain must be partially uncoupled from ATP synthesis. Indeed, uncoupling devices such as the mitochondrial permeability transition and the action of uncoupling proteins have been described [51,52]. Here, the possible existence of a third uncoupling mechanism was explored, namely the activation of an alternate electron transfer pathway in which NADH is oxidized by an alternative dehydrogenase and oxygen is reduced by an alternative oxidase. This alternate route does not pump protons, and therefore does not contribute to $\Delta\Psi$ build-up. In mitochondria from *Arabidopsis thaliana*, the activation of the alternative pathway seems to depend on the pyruvate-mediated stimulation of AOX [19]. Pyruvate or other ketoacids activate AOX at a cysteine residue (C127) located just upstream of the membrane-embedded region [53]. This residue is not present in the *Y. lipolytica* AOX and thus ketoacids do not activate this enzyme. Lack of ketoacid-activation is also observed in the AOX from *Acanthamoeba castellanii* [54].

The energy requirements in a cell should vary widely from the early log-phase, where anabolism is very active and supports rapid rates of cell division and growth, to the stat-phase, a low-energy mode when cells do not grow or reproduce [27]. In quiescent cells fully coupled mitochondria would maintain a high transmembrane potential and low rate of oxygen consumption, overproducing ROS [55]. In these conditions, an increase in the $NADH/NAD^+$ ratio would down-regulate catabolic pathways both in the cytoplasm and within the mitochondrial matrix. Thus, in the stat-phase the combined activities of the alternative components or the respiratory chain would be most advantageous, as an uncoupled electron flux would be rapid and independent of $\Delta\Psi$, decreasing the rate of ROS production and reestablishing the balance in the $NADH/NAD^+$ couple needed for an active metabolism.

In the log-phase NADH-derived electrons are channeled to the cytochromic pathway and not to the non-productive AOX. This was demonstrated by the complete inhibition of oxygen consumption mediated by cyanide and by the presence of a high respiratory control. Electron channeling to cytochromes is probably due to the physical interaction of NDH2e with complex IV [26]. By contrast, in the low energy-requiring stationary growth phase, NADH was allowed to reach the alternative electron pathway, as indicated by the partial resistance to cyanide and by the loss of respiratory control. Activation of the alternative pathway was probably due to the combination of the increased expression of NDH2e and the down-regulation of complex IV, which led to the accumulation of high levels

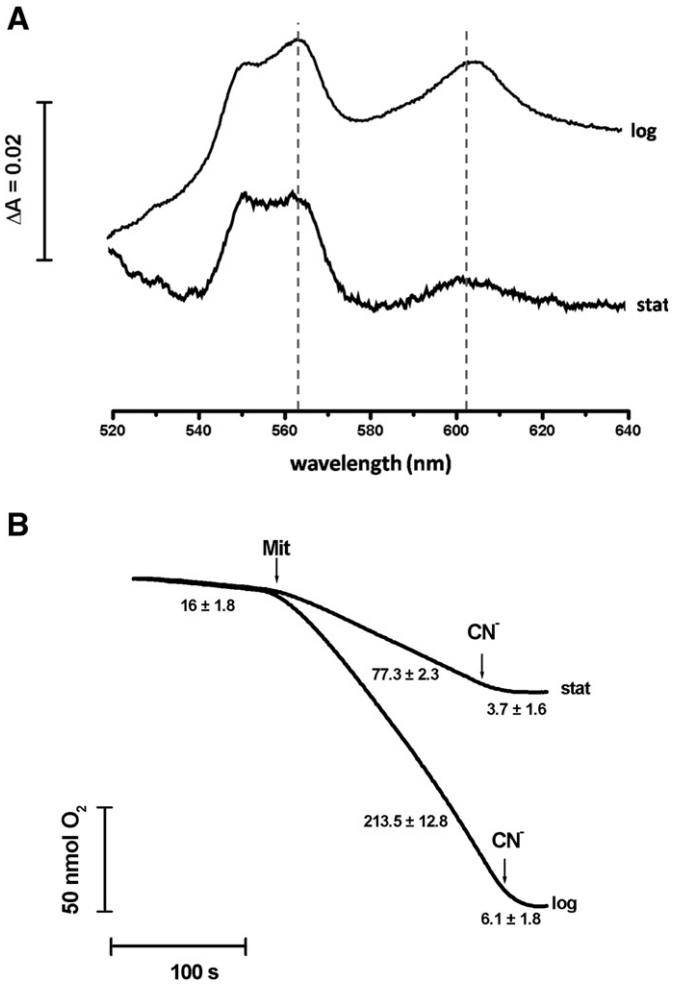


Fig. 5. Differential spectra and cytochrome *c* oxidase activity. (A) Dithionite-reduced minus ferricyanide-oxidized absorbance spectra of mitochondria from cells grown to the logarithmic or to the stationary growth phase. 2 mg/mL mitochondria were added to the reaction mixture described in Fig. 1 and spectra were recorded in an Aminco/Olis DW2000 spectrophotometer. Wavelength limits were 500 and 680 nm. The baseline was recorded after ferrycyanide addition to the reference cuvette. Then, two or three grains of dithionite were added to the sample cuvette and the reduced differential spectrum was recorded. Dashed lines indicate the cytochrome *b* and cytochromes $a+a_3$ peaks. Representative spectra are shown (n = 5). (B) cytochrome c oxidase activity was measured by cyanide-sensitive oxygen consumption in the presence of 5 mM ascorbate, 1 μM, TMPD (Tethrametyl-phenylene-diamine) and 10 μM antimycin A in the same reaction mixture as for Fig. 1.

of free NDH2e. Once released, NDH2e may reduce a non-localized ubiquinone pool which in turn, could be oxidized by AOX.

In bacteria, the contribution of different terminal oxidases to respiration depends heavily on the carbon/energy source [56,57]. By contrast, in mitochondria different respiratory pathways may be switched on and off through dynamic interactions between respiratory enzymes [25]. Through this mechanism, proton pumping would be adjusted to best fit the energy-needs of the cell. In the log-phase, the ability of NDH2e to replenish the cytoplasmic NAD^+ pool would be dependent on the utilization of ATP. However, it may be speculated that in the stat-phase a non-attached NDH2e is needed to oxidize cytoplasmic NADH regardless of a constantly high proton-motive force or the lack of ATP. In other organisms there is ample evidence indicating that AOX is activated in the stat-phase, releasing respiration from ATP production and preventing ROS overproduction [58,59].

Different supercomplexes were detected in mitochondria from *Podospora anserina* at different life stages, allowing to switch on and off selective respiratory pathways that involved either AOX or complexes III/IV [25]. In *Y. lipolytica* grown to the log-phase, NDH2e is bound to super-complex III/IV. In contrast, in the stationary phase overexpressed NDH2e would saturate the binding sites in complex IV, which is present in lower concentrations. The free fraction of NDH2e would not channel electrons to the cytochrome pathway,

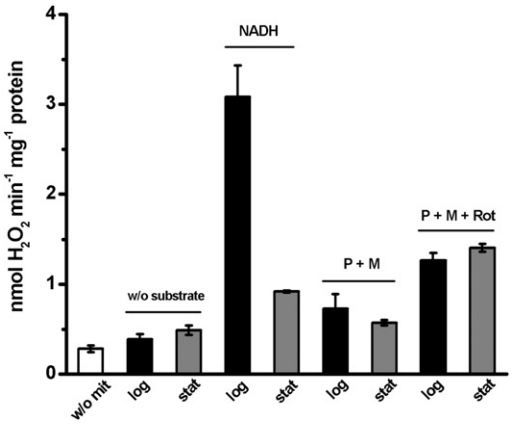


Fig. 6. Reactive oxygen species produced in isolated mitochondria. The production of hydrogen peroxide was measured following resorufin fluorescence in a multiwell microplate reader (Biotek) and the fluorescence increase slope is reported. Reaction mixture: 0.6 M mannitol, 5 mM MES, 20 mM KCl, 0.5 mM $MgCl_2$, 4 mM Pi, 10 μM Amplex Red, 0.1 units/mL horseradish peroxidase, 100 units/mL superoxide dismutase in the presence of 10 mM pyruvate and 10 mM malate or 0.25 mM NADH. Where indicated rotenone was 25 μM. Mean fluorescence ± SD (n = 5).

but instead, reach a non-localized ubiquinone-pool, thus activating the AOX pathway. This alternate electron pathway results in uncoupling of oxidative phosphorylation preventing the overproduction of ROS.

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