Respiratory chain network in mitochondria of *Candida parapsilosis*: ADP/O appraisal of the multiple electron pathways

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Abstract In this study we demonstrated that mitochondria of *Candida parapsilosis* contain a constitutive ubiquinol alternative oxidase (AOX) in addition to a classical respiratory chain (CRC) and a parallel respiratory chain (PAR) both terminating by two different cytochrome *c* oxidases. The *C. parapsilosis* AOX is characterized by a fungi-type regulation by GMP (as a stimulator) and linoleic acid (as an inhibitor). Inhibitor screening of the respiratory network by the ADP/O ratio and state 3 respiration determinations showed that (i) oxygen can be reduced by the three terminal oxidases through four paths implying one bypass between CRC and PAR and (ii) the sum of CRC, AOX and PAR capacities is higher than the overall respiration (no additivity) and that their engagement could be progressive according to the redox state of ubiquinone, i.e. first cytochrome pathway, then AOX and finally PAR. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Mitochondrion; Electron transport route; Alternative oxidase; *Candida parapsilosis*

1. Introduction

It has been recently shown that a cyanide-resistant respiration occurs frequently in yeasts, essentially in those incapable of aerobic fermentation [1]. In order to rationalize this finding, it has been proposed that the aerobic fermentation and the cyanide-resistant respiration are two strategies developed by yeasts as alternatives to the cytochrome pathway respiration, when the activity of this pathway is restricted, in order to allow catabolism to proceed. The cyanide-resistant ubiquinol alternative oxidase (AOX) is widespread in eukaryotes as higher plants, many fungi and some protozoa. It branches at the ubiquinone (Q) level bypassing two sites of energy conservation in the cytochrome pathway. The regulatory features of AOX differ among organisms [2–4]. In plant mitochondria, the activity of AOX is stimulated by α-keto acids and regulated by the redox state of the enzyme (oxidation/reduction of dimeric form). The fungal and protozoan AOX generally exists as monomer and is independent of organic acid stimulation but can be stimulated by purine nucleotides. Several functions have been assigned to AOX as the free redox energy-dissipating enzyme: participation in thermogenesis in spadices of Araceae, regulatory function when excess of reducing power occurs, response to various stress conditions in plants, and decreasing mitochondrial reactive oxygen species generation [2,5–8].

Candida parapsilosis is a parasitic non-fermentative yeast presenting a huge natural resistance to a large spectrum of antibiotics [9]. This resistance to drugs, acting on different mitochondrial functions, has been assigned to the appearance of two types of alternative electron flux pathways: (i) an inducible cyanide-resistant AOX branched at the level of Q, similar to the plant AOX, and (ii) a secondary parallel respiratory chain (PAR) involving alternative quinone (Qx), cytochrome *b* (cytb_{PAR}), cytochrome *c* (cytc_{PAR}), and terminal oxidase (oxc_{PAR}), insensitive to antimycin A (AA) and inhibited by amytal and a high concentration of salicylohydroxamate and cyanide [10–12]. It has been proposed that AOX and PAR drive electrons to oxygen from Krebs' cycle intermediates and from cytosolic NADH, respectively. It has been also hypothesized that partitioning of electron flux between the classical respiratory chain (CRC) and PAR could occur at the level of Q–Qx and cyt*c*–cytc_{PAR} (electron transfer would be from cytc_{PAR} to cyt*c* according to their redox potential) and that CRC and PAR activities could be additive, i.e. the sum of their capacities would be equal to the total measured respiration.

The present study demonstrates that in mitochondria of *C. parapsilosis*, AOX is constitutive and regulated in a fungi-type manner. We show that the AOX, PAR and CRC activities are not additive and that one bypass exists between PAR and CRC in the Q–oxygen span. We also determine the electron flux capacities and phosphorylation yield of various electron pathways.

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Abbreviations: AA, antimycin A; AOX, alternative oxidase; BHAM, benzohydroxamate; BSA, bovine serum albumin; CRC, classical respiratory chain; DTT, dithiothreitol; PAR, parallel respiratory chain; cytb_{PAR}, cytochrome *b* of PAR; cyt*c*, cytc_{PAR}, cytochromes *c* of CRC and PAR, respectively; oxc_{PAR}, terminal cyt*c* oxidase of PAR; Q, ubiquinone; Qx, alternative quinone

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2. Materials and methods

2.1. Cell culture and mitochondrial isolation

C. parapsilosis CCT 3834 (ATCC 22019) was grown at 37°C under vigorous aeration in complete liquid medium (2% glycerol, 2% Bactopeptone (Difco), 1% Bacto-yeast extract (Difco)) until middle stationary phase. Mitochondria were isolated and purified as described before [13]. Mitochondrial protein concentration was determined by the biuret method [14].

2.2. SDS–PAGE and immunoblotting of AOX

Mitochondrial protein was solubilized in the sample buffer (1% (w/v) SDS, 60 mM Tris–HCl, pH 6.8, 10% glycerol, 0.004% bromophenol blue, with or without reducing agent, 1 mM dithiothreitol (DTT)) and boiled for 5 min. Electrophoresis was carried out in a manner similar to that of Laemmli [15] using 5% polyacrylamide stacking gel and 12% polyacrylamide resolving gel, followed by Western blotting. Bio-Rad prestained low molecular mass markers were used. Antibodies against AOX proteins of *Sauromatum guttatum* (generously supplied by Dr. T.E. Elthon) were used at a dilution of 1:1000. AOX bands were visualized using the Amersham ECL system.

2.3. Oxygen uptake

Oxygen uptake was measured polarographically using a Clark-type electrode in 1.3 ml of standard incubation medium (28°C) containing: 125 mM sucrose, 65 mM KCl, 10 mM HEPES pH 7.2, 2.5 mM KH_2PO_4, 1 mM $MgCl_2$, plus or minus 0.2% bovine serum albumin (BSA) with 0.4–0.5 mg of mitochondrial protein. Concentrations of respiratory chain inhibitors: 4 μM AA (to block complex III of CRC), 1 or 10 mM KCN (to block cyt*c* oxidases, complex IV or complex IV and oxc_{PAR}, respectively), 2 or 10 mM benzohydroxamate (BHAM) (to block AOX or AOX and cytb_{PAR}, respectively). Details of measurements are included in the legends of the figures. The ADP/O ratio was determined by the ADP pulse (0.17 mM) method. The total amount of oxygen consumed during state 3 respiration was used to calculate the ADP/O ratio.

3. Results and discussion

3.1. Regulation and constitutive character of AOX in C. parapsilosis mitochondria

The huge resistance to drugs of *C. parapsilosis* was attributed to activity of an inducible AOX and development of PAR in mitochondria. As in our study of *C. parapsilopsis* cells grown in the absence of drugs, any evidenced mitochondrial electron transport pathway is constitutive. As shown in Fig. 1A, respiration with external NADH was inhibited partly by 4 μM AA that blocks complex III of CRC. Oxygen consumption was further inhibited by 10 mM KCN that blocks the two terminal cyt*c* oxidases of CRC and PAR. The remaining cyanide-resistant respiration was significantly stimulated by 0.6 mM GMP and fully inhibited by 2 mM BHAM, an inhibitor of AOX. These results indicate that the GMP-stimulated AOX activity is present constitutively in *C. parapsilosis* mitochondria. Moreover, the AOX protein of *C. parapsilosis* was immunodetected with antibodies raised against AOX protein of *S. guttatum* as shown in Fig. 1B. A single protein band, with a molecular mass around 38 kDa, was revealed both in the absence and presence of reducing agent (1 mM DTT) suggesting that AOX of *C. parapsilosis* exists as monomer.

In isolated mitochondria of *C. parapsilosis*, the AOX activity, measured as cyanide (10 mM)-resistant respiration, was not sensitive to DTT and pyruvate (not shown), while it was stimulated by GMP in concentration-dependent manner (Fig. 2A). The results presented in Figs. 1 and 2 clearly indicate that *C. parapsilosis* AOX does not reveal regulatory features of the plant-type oxidase that is stimulated by DTT and pyruvate inducing, respectively, a change in the redox state and a chemical modification (thiohemiacetal formation) of the enzyme at the level of a single cysteine [3–4]. Similarly to other tested fungal (*Neurospora crassa* and *Pichia stipitis*) and protozoan (*Acanthamoeba castellanii*) AOXs [16,17], monomeric AOX of *C. parapsilosis* is insensitive to organic acid stimulation but is stimulated by GMP. Stimulation of *C. parapsilosis* AOX by GMP reveals lower affinity ($S_{0.5} = 70$ μM) (Fig. 2A) compared to *A. castellanii* AOX ($S_{0.5} = 30$ μM) (Hryniewiecka, unpublished data).

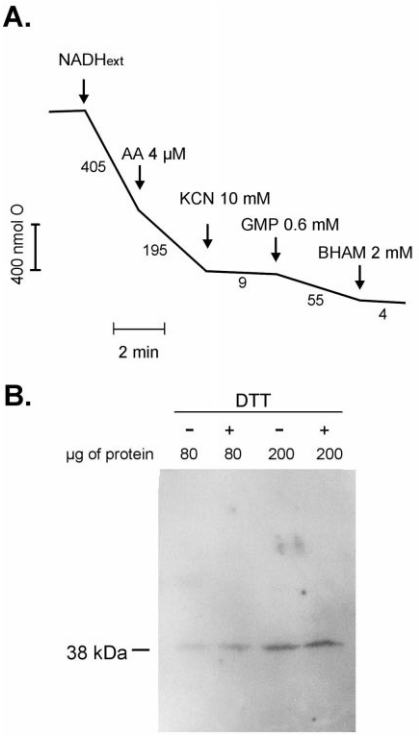

Fig. 1. Presence of constitutive AOX in *C. parapsilosis* mitochondria. A: Oxygen uptake was measured with external NADH (1 mM) as reducing substrate. Inhibitors: 4 μM AA, 10 mM KCN, and 2 mM BHAM were used to block complex III, the two terminal cyt*c* oxidases (of CRC and PAR), and AOX, respectively. GMP (0.6 mM) was used to activate the AOX activity. Numbers on the trace refer to O_2 consumption rates in nmol O min^{-1} mg^{-1} protein. B: Immunodetection. Where indicated, 1 mM DTT was added to the sample buffer. Amount of protein loaded on lane is indicated.

On the other hand, as shown in Fig. 2B, the *C. parapsilosis* AOX activity was negatively regulated by linoleic acid, an abundant fatty acid. It has been shown recently that as in mitochondria of mammal brown adipose and some non-thermogenic tissues, many plants, and some protozoa [18,19], mitochondria of *C. parapsilosis* possess an uncoupling protein that enables free fatty acid-activated H^+ re-entry into matrix, dissipating the proton motive force in mitochondria [13]. The inhibitory effect of linoleic acid on cyanide-resistant respiration was found in some plants and *Hansenula anomala* [20–22]. However, the plant-type AOX is inhibited by a low concentration of free fatty acids [22]. Thus the sensitivity to linoleic acid of *C. parapsilosis* AOX ($I_{0.5} = 33$ μM) (Fig. 2B) is lower than that of plant AOX (50% inhibition by 10 μM linoleic acid) [22], while protozoan *A. castellanii* AOX is insensitive to linoleic acid [23]. Different sensitivities of AOX activity to fatty acids among these organisms could reflect

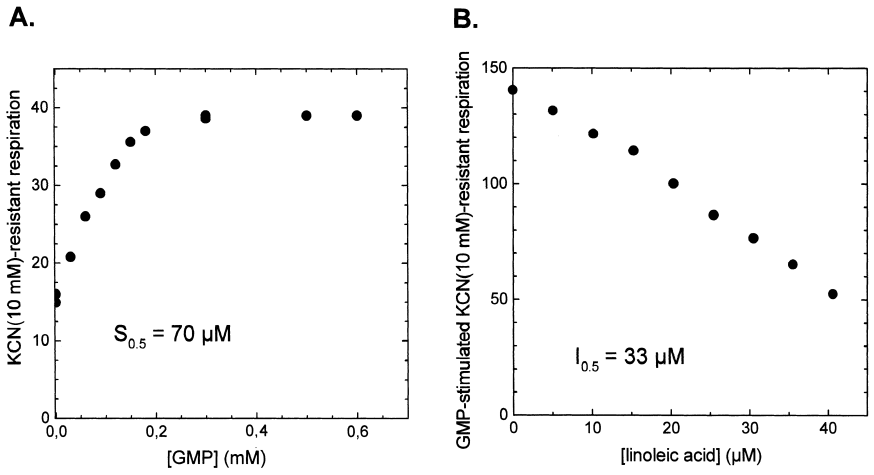

Fig. 2. Concentration dependence of GMP stimulation (A) and linoleic acid inhibition (B) of cyanide (10 mM)-resistant respiration. Oxygen uptake in the presence of 10 mM KCN (to exclude respiration through CRC and PAR) was measured (A) with 1 mM external NADH or (B) with cocktail of internal NADH substrates (5 mM 2-oxoglutarate+5 mM malate+5 mM pyruvate). Increasing concentrations of GMP and linoleic acid were obtained by successive additions when steady state respiration was achieved. When the sensitivity of the cyanide-resistant respiration to linoleic acid was measured (B), BSA (that chelates free fatty acids) was omitted in incubation medium. Respiratory rates are in nmol O min^{-1} mg^{-1} protein. The half maximum stimulation ($S_{0.5}$) of 10 mM cyanide-resistant respiration by GMP was 70 μM GMP and the half maximum inhibition ($I_{0.5}$) of 10 mM cyanide-resistant respiration by linoleic acid was 33 μM.

different functional connection between the two energy-dissipating systems, AOX and uncoupling protein.

3.2. Respiratory chain network in mitochondria of *C. parapsilosis*

3.2.1. The three terminal oxidases and the four electron transport pathways. As shown above, in mitochondria of *C. parapsilosis* grown in the absence of any drugs, AOX is constitutive. Thus, three terminal oxidases are present constitutively in these mitochondria, namely, AOX which is inhibited by 2 mM BHAM, the cyt*c* oxidase (complex IV) which is inhibited by 1 mM KCN, and the parallel cytc_{PAR} oxidase which was claimed to be inhibited by high KCN and hydroxamate (BHAM) concentrations (10 mM) [11,12]. According to the electron partitioning between CRC and PAR previously proposed by Guérin et al. [14], four different electron pathways were possible from ubiquinol to oxygen: CRC, AOX, PAR, and the fourth pathway: cytb_{PAR} → cytc_{PAR} → cyt*c* → complex IV. In this study, we verified activity of electron transport pathways in the mitochondrial respiratory network of *C. parapsilosis* using different inhibitors (and their concentrations). We evidenced that the fourth pathway is the following: complex III → cyt*c* → cytc_{PAR} oxidase (oxc_{PAR}) (Fig. 3). Indeed, as shown in Table 1, the difference between the respiration in the presence of 2 mM BHAM+4 μM AA with and without 1 mM KCN was negligible (9 nmol O min^{-1} mg^{-1} protein), while the respiration in the presence of 10 mM BHAM and 1 mM KCN was significant (84 ± 10, S.E.M., nmol O min^{-1} mg^{-1} protein). This last result also indicates that 10 mM BHAM acts at the level of cyt*b* of PAR and not

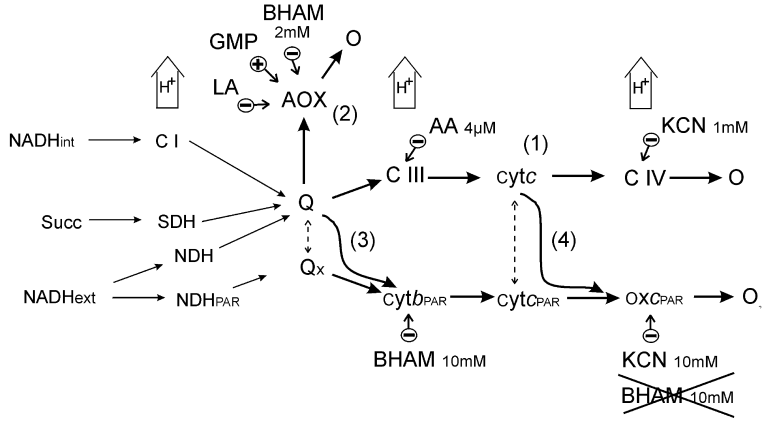

Fig. 3. Respiratory network of *C. parapsilosis* mitochondria: three terminal oxidases and four electron transport paths. Complex I (C I), complex III (C III) and complex IV (C IV, cyt*c* oxidase) are the three complexes of CRC. Respiratory substrates: succinate, Succ; $NADH_{int}$, internal NADH; $NADH_{ext}$, external NADH. SDH, NDH, NDH_{PAR} are succinate and external NADH dehydrogenases. Components of the PAR: NDH_{PAR}, Qx, cytb_{PAR}, cytc_{PAR} (cytochrome *c*) and oxc_{PAR} (second terminal cyt*c* oxidase). Numbers between brackets are four electron transport paths, namely: (1) cytochrome pathway of CRC (C III → cyt*c* → C IV), (2) AOX, (3) PAR (cytb_{PAR} → cytc_{PAR} → oxc_{PAR}) working when Q is very reduced, and (4) fourth pathway (C III → cyt*c* → cytc_{PAR} → oxc_{PAR}) working when complex IV is blocked. Targets of inhibitors and activator are indicated by arrows. Dotted lines indicate putative interactions. Sites of H^+ pumping are shown.

Table 1
Quantitative analysis of electron routes to oxygen in *C. parapsilosis* mitochondria: ADP/O and respiratory capacity determinations

Conditions	ADP/O (±S.E.M.)	Electron routes to O_2 (according to numbering in Fig. 3)		Respiratory rates (±S.E.M.) (nmol O min^{-1} mg^{-1} protein)
		possible	effective	
No inhibitors+GMP 0.6 mM	2.06±0.08	1, 2, 3, 4	1, 2 (CRC+AOX)	466±47
BHAM 10 mM	2.49±0.05	1, 4	1 (CRC)	347±30
BHAM 10 mM+AA 4 μM (or +KCN 10 mM)	0	no	no	15±2
BHAM 2 mM+KCN 1 mM	1.49±0.06	3, 4	4 (CRC→oxc_{PAR})	84±8
BHAM 10 mM+KCN 1 mM	1.69±0.08	4	4 (CRC→oxc_{PAR})	84±10
KCN 10 mM+GMP 0.6 mM	0.68±0.03	2	2 (AOX)	166±15
KCN 10 mM+AA 4 μM+GMP 0.6 mM	0.74±0.05	2	2 (AOX)	155±21
BHAM 2 mM+AA 4 μM	0.90±0.07	3	3 (PAR)	52±2
BHAM 2 mM+AA 4 μM+KCN 1 mM	0.77±0.08	3	3 (PAR)	43±1
KCN 1 mM+AA 4 μM+GMP 0.6 mM	0.92±0.02	2, 3	2 (AOX)	147±8
BHAM 2 mM	2.33±0.10	1, 3, 4	1 (CRC)	365±42

Oxygen uptake was measured as described in Section 2 in the presence of three substrates feeding the respiratory chain at the level of complex I (5 mM 2-oxoglutarate+5 mM malate+5 mM pyruvate). Conditions of respiration measurements (i.e. presence of inhibitors and activator) are given in the first column. ADP/O was measured by ADP pulse (170 mM) method. Electron transport routes are numbered according to the scheme described in Fig. 3. Possible and effective electron routes to O_2 are given in the third and fourth columns. Data are average of 3–4 measurements (±S.E.M.).

at the level of the terminal oxidase of PAR as suggested by Guérin et al. [10–12]. Fig. 3 shows the current scheme of respiratory network of *C. parapsilosis* mitochondria.

3.2.2. Quantitative description of the four electron transport pathways. On the basis of electron transport pathway network and sensitivity to inhibitors (Fig. 3) we performed a quantitative description of electron flux capacities and phosphorylation yield of the different pathways in *C. parapsilosis* mitochondria. Using 11 conditions of incubation (Table 1), with substrates of complex I (2-oxoglutarate+malate+pyruvate), we determined ADP/O ratio values, respiratory rates, and effective main electron routes to oxygen. Table 1 presents results of experiments where the total respiratory rate (no inhibitors, +GMP) was 466±47 (S.E.M.) nmol O min^{-1} mg^{-1} protein and ADP/O was 2.06±0.08 (S.E.M.). In the presence of 10 mM BHAM, the ADP/O was 2.49±0.05 (S.E.M.) indicating that in these conditions the three coupling sites of CRC were used but not the fourth pathway. Thus, in the presence of 10 mM BHAM, the main pathway was CRC with a capacity of 347±30 (S.E.M.) nmol O×min^{-1}×mg^{-1} protein. When complex III was blocked by AA in the presence of 10 mM BHAM, the respiratory rate was very low and ADP/O not measurable. Similarly, the residual respiration was measured in the presence of 10 mM BHAM and 10 mM KCN. When complex IV was inhibited by 1 mM KCN and AOX by 2 mM BHAM (or when AOX and cytb_{PAR} were blocked by 10 mM BHAM), the fourth pathway (CRC→cyt$c$→oxc_{PAR}) was forced to work (because of a high level of cyt$c$ reduction) as can be concluded from the ADP/O (1.49±0.06 (S.E.M.) or 1.69±0.08 (S.E.M.)) implicating two coupling sites. The capacity of this fourth pathway was 84±10 (S.E.M.) nmol O min^{-1} mg^{-1} protein. When AOX was the main effective pathway (in the presence of 0.6 mM GMP and 10 mM KCN with or without 4 μM AA), ADP/O was 0.68±0.03 (S.E.M.) or 0.74±0.05 (S.E.M.) corresponding to the engagement of the first phosphorylation site and the capacity was 166±15 (S.E.M.) or 155±21 (S.E.M.) nmol O min^{-1} mg^{-1} protein. Under conditions when AOX was blocked by 2 mM BHAM, complex III by 4 μM AA, the respiratory capacities and ADP/O in the absence or presence of additionally added 1 mM KCN (that blocks complex IV) were similar (52±2 (S.E.M.) and 43±1 (S.E.M.) nmol O min^{-1} mg^{-1} protein, and 0.9±0.07 (S.E.M.) and 0.77±0.08 (S.E.M.), respectively) indicating that electron flux from PAR to complex IV (i.e. the fourth pathway of Guérin et al. [10–12]) was not efficient. Thus under these conditions, electron flux took place entirely through PAR reaching oxc_{PAR}. When complexes III and IV were blocked (by 4 μM AA and 1 mM KCN), ADP/O and capacity were characteristic of AOX acting with complex I (0.92±0.02 (S.E.M.) and 147±8 (S.E.M.) nmol O min^{-1} mg^{-1} protein) thereby indicating that PAR was not engaged yet, likely because of too low Q reduction level when AOX is engaged. Finally, in the absence of any inhibitor, the ADP/O ratio was 2.06±0.08 (S.E.M.) and in the presence of 2 mM BHAM the ADP/O ratio was 2.33±0.10 (S.E.M.). When compared to the control ADP/O (i.e. ADP/O measured when the main non-proton pumping pathways, AOX and PAR, were excluded), 2.49±0.05 (S.E.M.), these results mean that PAR is poorly engaged when complex III is active, even when AOX is blocked, and suggest that in the absence of inhibitors electrons are mainly shared out between AOX and the cytochrome pathway.

3.2.3. Additivity of the various electron transport pathways. Our results on the respiratory pathway capacities measured with isolated mitochondria are restricted to situations in which one of the activities is functioning while the other are blocked. Even if these measurements do not reflect the true contributions of respective electron pathways into overall respiration (because any change in one inevitably effects the others), they do allow relative comparisons. Thus, according to the results described in Table 1, the capacities of the three electron pathways are not additive. Indeed, the sum of the calculated general average capacities of CRC (359±19 (S.E.M.) nmol O min^{-1} mg^{-1} protein), AOX (156±8 (S.E.M.) nmol O min^{-1} mg^{-1} protein) and PAR (47±3 (S.E.M.) nmol O min^{-1} mg^{-1} protein) is 562±30 (S.E.M.) nmol O min^{-1} mg^{-1} protein and is higher than the overall measured respiration (466±47 (S.E.M.) nmol O min^{-1} mg^{-1} protein). Thus, at least with the tested respiratory substrates (i.e. substrates of complex I) and in our conditions of cell growing, the additivity of electron pathways is not observed.

Our results on respiratory network demonstrate that in mi-

tochondria of *C. parapsilosis*, grown without respiratory chain inhibitors, three terminal oxidases and four electron pathways leading to oxygen exist. Screening of the respiratory network by ADP/O determination with substrates of complex I indicates that the cytochrome pathway and AOX share the electrons of the Q pool. The capacity of the cytochrome pathway was more than twice the AOX capacity. On the other hand, engagement of PAR was only effective when both the cytochrome pathway and AOX were blocked, thus when the reduced state of quinone was very high. Moreover, the PAR capacity was only one tenth of the total respiration. We have also shown that the bypass between the cytochrome pathway and PAR occurred from cyt*c* to the terminal oxidase of PAR when complex IV is blocked, thus when cyt*c* is very reduced. Finally, it can be concluded that the capacities of three pathways (AOX, PAR and cytochrome pathway) are not additive, as their sum is higher than the total measured respiration, and that their engagement in the overall respiration could be progressive according to the redox state of Q: first cytochrome pathway, then AOX, and then PAR.

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