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Alternative respiratory oxidases to study the animal electron transport chain

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ABSTRACT

Oxidative phosphorylation is a common process to most organisms in which the main function is to generate an electrochemical gradient across the inner mitochondrial membrane (IMM) and to make energy available to the cell. However, plants, many fungi and some animals maintain non-energy conserving oxidases which serve as a bypass to coupled respiration. Namely, the alternative NADH:ubiquinone oxidoreductase NDI1, present in the complex I (CI)-lacking *Saccharomyces cerevisiae*, and the alternative oxidase, ubiquinol:oxygen oxidoreductase AOX, present in many organisms across different kingdoms. In the last few years, these alternative oxidases have been used to dissect previously indivisible processes in bioenergetics and have helped to discover, understand, and corroborate important processes in mitochondria. Here, we review how the use of alternative oxidases have contributed to the knowledge in CI stability, bioenergetics, redox biology, and the implications of their use in current and future research.

1. Introduction

Mitochondria are the cellular organelles in charge of producing most of the adenosine triphosphate (ATP) in the cell. In contrast to other organelles, they are composed of two membranes, the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM), which allow the existence of several compartments in the mitochondria: the gap between inner and outer membranes, the intermembrane space (IMS), the space within the cristae (which is being increasingly recognized as a functionally different compartment than the IMS), and the interior of the organelle or mitochondrial matrix [1]. The IMM harbors the necessary components to produce ATP, which all together form the oxidative phosphorylation system (OXPHOS). The OXPHOS system is composed of five protein complexes, four complexes (I to IV) comprising the electron transport chain (ETC) and the complex V (CV) or H^+-ATP synthase. CI couples the oxidation of reduced nicotinamide adenine dinucleotide (NADH) into NAD^+, and the reduction of ubiquinone into ubiquinol, with the ejection of four H^+ from the matrix to the IMS. Complex II (CII) oxidizes succinate into fumarate and reduces ubiquinone into ubiquinol, without pumping H^+. Complex III (CIII) oxidizes ubiquinol into ubiquinone, reducing cyt c and pumping two H^+ per cyt c reduced. Finally, CIV oxidizes two reduced cyt c molecules and donates those electrons to reduce oxygen into water, using the released energy to eject four H^+ to the IMS. The activity of the ETC promotes the formation of a H^+ gradient across the IMM, the so-called H^+-motive force (μ or p.m.f.). μ is composed by an electrical component, the mitochondrial membrane potential (Δψmt) accounting for around 80 % of the μ, and a chemical component, the difference in pH (ΔpHmt), which accounts for the other 20 %.

Before the detailed description of the oxidative phosphorylation system, the main hypothesis on the system producing cellular ATP considered the existence of a macromolecular assembly, which physical association allowed electron transfer between complexes in the IMM. It was called the solid model [2]. Upon the discovery of the oxidative phosphorylation and the isolation of the separate complex activities, it became clear that the mitochondrial complexes were able to work individually. It was also discovered that there are electron carriers, ubiquinone and cyt c, which can diffuse along the IMM, allowing electron transfer between complexes. This enabled to put forward the hypothesis of the fluid model, in which the components of the ETC (including the mobile electron carriers) would act as individual lateral diffusants which, in turn, would allow the lateral mobility of electrons between individual complexes [3,4].

This hypothesis prevailed in the field until the development of blue-

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native polyacrylamide gel electrophoresis (BNGE) by Schägger and Pfeiffer [5], which allowed the separation of complexes in their native conformation and enabled the discovery of novel quaternary structures formed through the interaction between complexes, the supercomplexes. CI, $CIII_2$ and CIV form a single structure named the N-respirasome (as it consumes NADH) [6]. CIII and CIV supercomplex constituted the Q-respirasome (as it oxidizes ubiquinol) [7]. Also, other structures were discovered, such as the supercomplex CI + $CIII_2$ and several other forms of supercomplexes, including CIV and CV homodimers and homo-oligomers. All these were found in co-existence with individual CI, CII, $CIII_2$, CIV and CV. Interestingly, CI was predominantly found forming supercomplexes in mammals [5,7–9]. The BNGE observations questioned the fluid model and revived the solid one. After years of debate considering the fluid and solid models antagonize, a new model, the so-called plasticity model, propose that complexes and supercomplexes co-exist in the IMM and its proportion is adjusted to the cellular requirements [6,10–12]. Then the debate moves to answer new questions, such as what are the functional consequences of supercomplexes? How are they regulated?

To investigate these questions, the ectopic expression of the alternative oxidase (AOX) in cells and animals has been revealing [13–15]. AOX is an enzyme capable to oxidize ubiquinol into ubiquinone while reducing oxygen to water in a cyanide-insensitive reaction that does not pump H^+ across the mitochondrial inner membrane [16]. AOX are a family of enzymes present in animals, plants, and fungi, but has been lost in most of higher animals [16–21]. The reason for its disappearance in metazoans is unknown, but it has been speculated that it may be related to possible pathological effects in its presence. Other alternative oxidases, such as *Saccharomyces cerevisiae* derived NDI1 which performs NADH:ubiquinone oxidoreduction, have been ectopically express in animal cells [14,22–27]. It has been used to discern mitochondrial ROS pathways affecting physiological adaptations [28]. This review summarizes how the alternative oxidases AOX and NDI1 have been used to clarify the role of supercomplexes bioenergetics and in the stability of CI, and the role of ROS in aging, hypoxic adaptation, and pathophysiology [29].

2. The role of AOX in CI stability

An early puzzling observation in patients was the description of cases of single CIII defects or combined defects in CI and CIII, associated with a single genetic alteration of CIII [30–32]. Mutations in the CIII subunit cytochrome *b* (Cyt b) in human and mouse that prevent the assembly of CIII also promote the degradation of CI [29,33]. Interestingly, this was not reproduced by pharmacological inhibition of CIII in wild type cells [29].

It was later proposed that CIII may act as a platform for CI assembly. This hypothesis was put forward as the absence of CIII would impede the incorporation of the NADH-oxidizing module (N-module) in CI which, in turn, would promote the destabilization of the rest of the complex [34,35]. However, the cells/animals mutants for N-module subunits, such as NDUFS4, NDUFS6 or NDUFA5, cannot assemble the N-module but maintain stable CI subassemblies, both alone or attached to CIII [36–39]. In addition, this model does not explain why CIV or cyt c depleted cells are unable to stabilize CI while having normal assembly of CIII [40–42]. Finally, it does neither agree with the fact that expression of AOX from *Emericella nidulans* in CIII, CIV and cyt c deficient cells allowed the assembly of functional CI [7,34,43]. In fact, the mechanism that promotes the degradation of CI in the three conditions (CIII, cyt c and CIV depleted cells) is triggered by a high ubiquinol/ubiquinone ratio that leads to a prominent over-oxidation of the CI N-module subunits by the occurrence of long lasting CI reverse electron transport (RET) in mutant cells [43]. Thus, preventing the increase in the ubiquinol/ubiquinone ratio by the overexpression of AOX, severe long-term hypoxia or prevent RET by the use of CI Q-site inhibitors (i.e. rotenone or piericidin) allow the stable assembly of fully functional CI. This new model of RET promoting CI instability successfully explains why CIV or cyt c mutants are neither able to stabilize CI, even in the presence of CIII; that CIII is not obligatorily required for CI assembly as cyt b mutant cells are able to stabilize CI in the absence of CIII and the presence of AOX; it may also explain why antimycin A (AA), a CIII inhibitor, fails to destabilize CI since, though AA is able to increase the ubiquinol/ubiquinone ratio [44], it is unable to produce CI RET as it completely depolarizes mitochondria, being its primary mechanism of ROS production the interference with the Q-cycle at CIII [6,45]. It is to mention that AA may not completely block CIII-electron transfer from $CoQH_2$ to cyt c since cells treated with this drug are still able to grow in the absence of uridine. This is in contrast to cells simultaneously treated with myxothiazol, a CIII-Qo site inhibitor, and AA, which still maintain full CI assembly and are uridine-dependent [29]. Again, this can be explained by the fact that CIII blockade promotes mitochondrial depolarization, which prevents RET and, thus, CI disassembly. This hypothesis supports observations in other OXPHOS mutant cells (i.e., cyt c^{KO} and $Cox10^{KO}$ cells), which can maintain polarized mitochondria probably through the reverse activity of CV [40,42,46], and are, thus, able to produce CI RET and disassemble CI [43]. To note, CI disassembly only occurs under long-lasting CI RET, as short bursts of ROS via RET are not even able to oxidize CI N-module subunits [47]. This work, together with others in parallel [28,48,49], posed the overexpression of AOX as a good methodology to inhibit RET in cells and, thus, to study the involvement of RET in a panoply of physiological and pathophysiological settings, such as aging, ischemia-reperfusion, or macrophage activation.

It should be noticed that the stability of CI in the absence of complexes III or IV is also dependent on its rate of synthesis and assembly and its rate of degradation by the mitochondrial protease machinery which, in turn, can also be modulated [50,51]. Particularly, human muscle with CIII assembly defects showed a greater impact on the amount of functional CI [29] than mouse muscle with CIV assembly defects [42]. By the same token, mouse cultured cells with CIV assembly defects [43] showed a more severe reduction in assembled CI than human cells lacking CIV [52]. Moreover, cells defective in CIII assembly may vary in its degree of CI degradation with time [29,43]. In any case, the expression of AOX always favors the accumulation of functional CI [34,43,52]. This indicates that the factors altering CI stability are complex and that the expression of AOX in vivo may be used to study the biochemical profile and phenotype of mouse models lacking CIII and/or CIV. Most of the work performed with AOX in OXPHOS mutants has been performed in cell lines expressing *Emericella nidulans* AOX. However, AOX from different species differ in relevant kinetic aspects, such as the affinity for ubiquinol capacity to compete with CIII, the dimerization capacity, etc. [20]. Therefore, the use of different sources of AOX will be of great value to complement previous studies.

3. The alternative electron transport chain

The use of alternative oxidases has helped to deeply study mitochondrial and cellular bioenergetics deeply. Not only AOX, but also the overexpression of the NADH:ubiquinone oxidoreductase NDI1 has been used to substitute mitochondrial respiratory complexes in mammalian cells [14,27].

Long-term treatment of animal cells with high amounts of ethidium bromide results in the depletion of mitochondrial DNA (ρ° cells; Fig. 1B) [53], which may be repopulated through mitochondrial transference (ρ^{+} cells) [54]. However, an alternative way to reconstitute the flow of electrons along the IMM, but without H^+ pumping, was to either overexpress AOX (ρ°AOX; Fig. 1C) or both NDI1 and AOX (ρ°NDI1/AOX; Fig. 1D) [14]. ρ°AOX mitochondria were only able to respire under succinate, whereas ρ^{0}NDI1/AOX were also able to respire under glutamate and malate [14]. In addition, both cell lines were able to survive and proliferate when the lactic acid fermentation was hindered by the presence of dichloroacetate (i.e. pyruvate dehydrogenase kinase inhibitor) or by the absence of pyruvate, indicating that the alternative

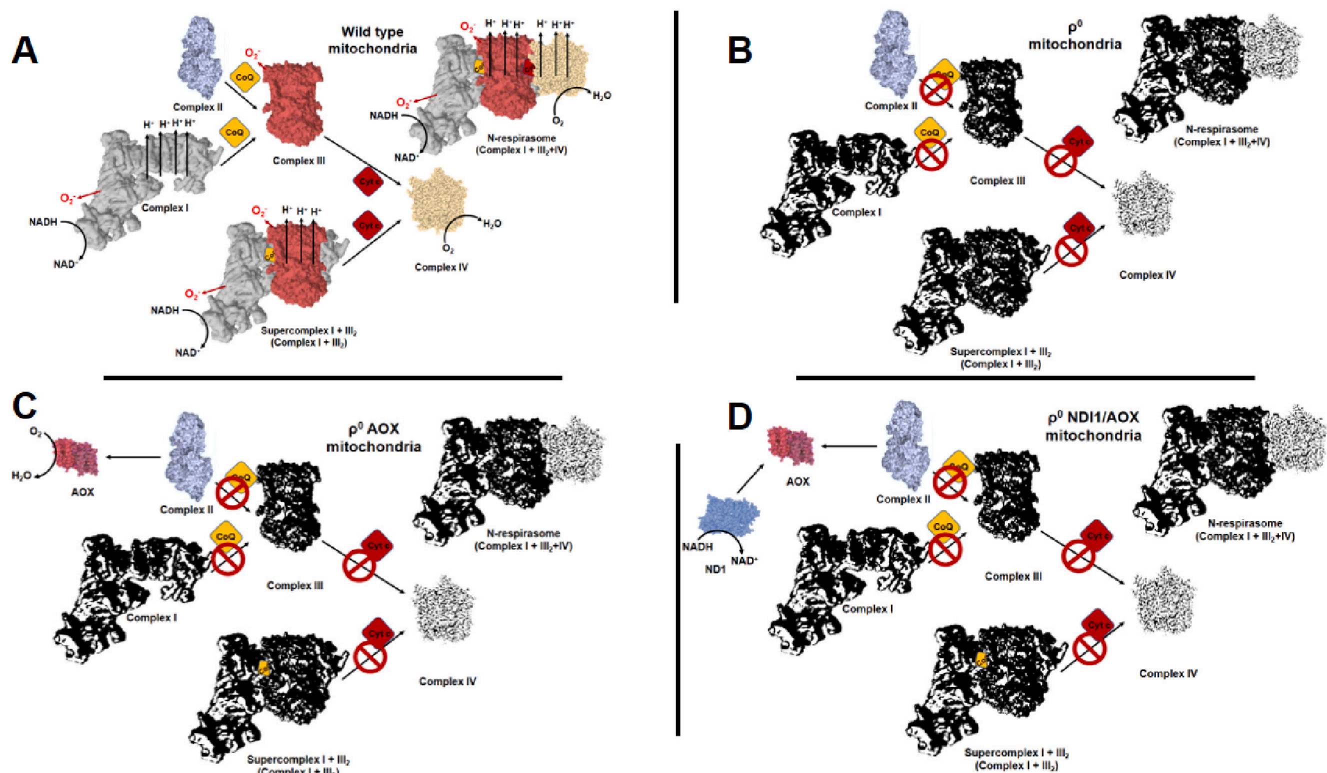

Fig. 1. Alternative oxidases enable the reconstruction of an alternative electron transport chain. (A) Scheme showing the ETC composition and function of a wild type mitochondria harboring CI, CII, CIII, CIV and several supercomplexes, according to the plasticity model. (B) Scheme showing the effect of depleting mitochondrial DNA (mtDNA) in ρ^0 cells regarding ETC components. (C) Scheme showing the effect of overexpression of AOX in ρ^0 mitochondria. (D) Scheme showing the effect of overexpression of NDI1 and AOX in ρ^0 mitochondria. Absent respiratory chain complexes I, III and IV are indicated in gray colors.

electron transport chain was metabolically and bioenergetically competent [14]. Very importantly, this concept of an alternative electron transport chain enables the splitting of several OXPHOS roles initially thought to be indivisible, such as electron transport, H^+ pumping, ATP synthesis, redox regulation, and mitochondrial ROS production. Indeed, this concept has been used in the last few years to elucidate the role of mitochondrial ROS in physiology and redox homeostasis (see below).

4. The role of AOX in mitochondrial bioenergetics

The functional role of respiratory supercomplexes has been strongly debated for years. A seminal paper on the role of supercomplexes used flux control analysis to estimate the functional association between CI, CIII and CIV in isolated mitochondria and submitochondrial particles (SMPs) [55]. Flux control analysis is performed by titrating an inhibitor on its specific enzymatic activity and, in parallel, on the whole metabolic pathway to which the enzyme belongs. The ratio between both, at the initial concentrations of inhibitor, will be far from unity if the metabolic pathway is composed by several, independent enzymes; however, if such enzymes are associated in a supercomplex, the metabolic pathway would behave as a single unit and the inhibition of any of the enzymes would reach a flux control value of nearly the unity. Using this rationale, Lenaz and co-workers observed that both CI and CIII were highly rate-controlling over NADH oxidation, in contrast to CIV. In addition, they found that CII was the only rate-limiting step on succinate oxidation, suggesting that CI and CIII are functionally associated, while CIV and CII appear to be more independent [55].

A few years later, a genetic approach showed that this functional association, not only at the level of CI and CIII, but also at the level of CIV, was a consequence of the physical association mediated by super assembly of the respiratory complexes into supercomplexes. Mouse fibroblasts with abnormal low expression of CIII, which virtually displayed it all in supercomplexes without a free form of dimer CIII, showed lower CII + CIII enzymatic activity, but similar CI + CIII activity [56]. In addition, a new role for a CIV subunit isoform was described, as it served as an internal bridge between CIII and CIV inside to form supercomplexes, the supercomplex assembly factor-1 (SCAF1) also named Cox7a2l. In the absence of functional SCAF1 in cell lines and liver mitochondria, the super assembly between CIII and CIV is lost and the supercomplexes I + III_2 + IV (N-respirasome) and III_2 + IV (Q-respirasome) are lost [56–59]. In heart, the N-respirasome can be generated either with SCAF1 or with Cox7a2 being structurally different in the way that CIII and CIV interact and functionally different in the kinetic of electron transfer from NADH to O_2 and the production of ROS [7]. Under normal conditions, independent CI and CII-based respiration are lower than the respiration obtained if both substrates are added simultaneously [7,56]; however, in the absence of SCAF1 CII- based respiration equal to CI + CII-based respiration, meaning that in the presence of CIII+CIV interaction CI and CII-respiration are independent to some extent and that if SCAF1 is removed, such partial independency disappears [7,56,60]. Altogether, these data indicated the existence of separated pools of both ubiquinone and cyt c, which would work partially independent from the bulk ubiquinone/cyt c within the rest of the IMM. These results are in accordance with the proposed model of plasticity, as they imply the partially independent function of supercomplexes (i.e. substrate channeling) and free complexes in the same environment, depending on the metabolic requirement of the cell, and are in contrast to the fluid model. To note, the latter supports the existence of a single ubiquinone/cyt c pool, which necessarily follows the Loss of Memory (LOM) principle [6,61]. This implies that when a very differentiated, upstream metabolic pathway (NADH vs $FADH_2$ accumulation of electrons) flow into an undifferentiated and unique pool of ubiquinone/cyt c the information of the donor is lost.

The existence of segmentation of the CoQ and cyt c pools was controversial [60,62]. An ingenious approach using AOX from *Trypanosoma brucei* was designed to address the existence of ubiquinone channeling in mammals [63]. The authors measure the oxidation of NADH in a buffer containing sub mitochondrial particles (SMPs), after CIV was inhibited with cyanide, recombinant AOX was added into the reaction mixture. The authors showed that AOX elicited a more prominent oxidation of NADH than the native SMPs. This was interpreted as evidence that supercomplexes do not retain a partially differentiated ubiquinone pool. However, several caveats may compromise this interpretation: 1) AOX was not added under normal respiring conditions, but only after inhibiting CIV; thus, AOX could not exert any competence with CIII+CIV and competence for ubiquinone under flux control could not be tested. 2) The specificity for CI-dependent NADH oxidation was not tested (e.g., rotenone or piericidin A-dependent NADH oxidation), which is extremely important given that there are several NADH-oxidases in the IMM, besides CI. 3) Only NADH oxidation was tested, not respiration; in other words, whether the increase in NADH oxidation by AOX caused an increase in respiration or other forms of oxygen consumption (e.g., ROS production) was not tested. 4) Given that AOX was dropped into the reaction mixture, an appropriate incorporation into the membrane (e.g., to prevent AOX protein aggregation) to yield proper function and activity could not be warranted; this, in turn, could increase ROS production by unspecific interacting with the ETC components, concomitantly increasing NADH oxidation in a respiration-independent manner.

All these potential confounding factors have been recently solved using a panoply of tools, all including the use of AOX from *Emericella nidulans*. As mentioned above, *cyt b* mutant cells expressing AOX can assemble functional CI (*cyt b* mutant+AOX). Equally *cox10* KO cells expressing AOX assemble CI, which mostly super assembles with to CIII (*cox10* KO + AOX). These cell lines allowed studying cells in which CI was either alone or super assembled with CIII, neither of them capable of doing CIV-dependent respiration. Intact mitochondria displaying normal supercomplex pattern and *cox10* KO + AOX were able to show additive partial additive respiration when respiring under both CI and CII substrates, in contrast to *cyt b* mutant + AOX which could not increase oxygen consumption when both substrates were present (Fig. 2). In addition, *cyt b* mutant + AOX decreased rotenone dependent NADH oxidation in the presence of succinate, indicative of competition between both substrates. However, wild type cells expressing AOX, and CIV deficient cells expressing AOX, were able to maintain the rate of NADH oxidation, even in the presence of succinate, further supporting the notion of an independent ubiquinone pool when both CI and CIII are super assembled. Importantly, neither respiration nor NADH oxidation was fully inhibited upon CIV inhibition, suggesting the existence of a CIV-insensitive NADH oxidation and respiration, due to the presence of AOX. The results in wild type mitochondria from cultured cells were confirmed in heart and skeletal muscle mitochondria from AOX-overexpressing mice [7] (indicative of that ubiquinol from supercomplexes can be used, at some extent, by AOX in a non-competitive manner). It is to mention that *cox10* KO + AOX and *cyt b* mutant+AOX possess different nuclear DNA (nDNA) and mtDNA background, which could promote small differences in their bioenergetics, Also, a possible factor influencing these results is the $\Delta\psi$mt, which was not measured, nor the flux control by AOX. However, these caveats were overcome by a study using heart mitochondria from mice overexpressing *Ciona intestinalis* AOX showed that coupled respiration under CI substrates was only dependent on the native ETC, and not on AOX. These experiments were carried out calculating the respiratory control index (RCI) after titrating non-coupled mitochondria (+/− AOX) with low amounts of ADP. If AOX was competing for ubiquinol with CIII, the increase in respiration after coupling (i.e. RCI) in AOX-overexpressing mitochondria would be lower; however, if AOX did not participate, the peak in respiration would be the same. Indeed, the RCI was similar in both types of mitochondria, indicating the existence of substrate channeling between CI and CIII [64]. A difference between *Emericella nidulans* AOX and *Ciona intestinalis* AOX results arose from the fact that Szibor et al. [64] observed no changes in respiration after either CIII or CIV inhibition, whereas Calvo et al. [7] showed partial inhibition of respiration upon CIV inhibition. Given that both AOX can use the CoQ from CII, especially when CIII or CIV are absent, it is possible that this difference arises from the type of AOX used. In the absence of oxygen wild type mitochondria CIII pool becomes fully reduced only in the presence of both NADH and succinate, indicating that single CIII becomes less reduced by electrons coming from NADH, even in the absence of functional CIV [60]. Thus, it is possible that AOX from *Ciona intestinalis* and *Trypanosoma brucei* can interact with the CoQ in the supercomplex and that AOX from *Emericella nidulans* is not, as occurs with single CIII in wild type mitochondria. This reveals a very important issue in the study of mitochondrial bioenergetics with AOX, the species and type of AOX used in each study. Thus, it is important to note that Fedor

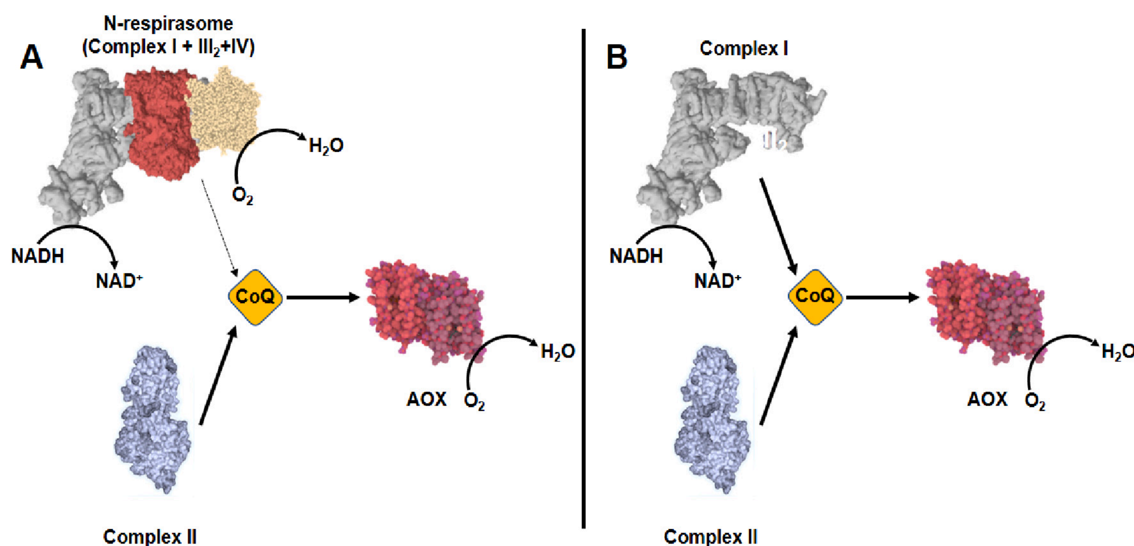

Fig. 2. AOX allows the study of the partial segmentation of the ubiquinone pools. (A) Scheme showing that AOX overexpression is unable to outcompete for ubiquinol against the functioning CI-containing supercomplexes, but it can use the ubiquinol produced by CII. (B) Scheme showing that free CI, stabilized upon AOX overexpression, provides ubiquinol to a common ubiquinone pool.

et al. [63] used AOX from *Trypanosoma brucei*, whereas Calvo et al. [7] used AOX from *Emericella nidulans* and Szibor et al. [64] used AOX from *Ciona intestinalis*.

AOX from different species harbor different kinetic properties, different ubiquinol affinities, different types of regulation and different rates of oxygen consumption [16,21]. For instance, the AOX-dependent respiration in *Emericella nidulans* is 21.2 %, whereas in *Trypanosoma brucei*, it can reach up to 35 % [65,66]. *Ciona intestinalis* AOX is known to form homo-oligomers when expressed in mammalian cells [64], whereas they were not detected in *Emericella nidulans* AOX [43]. Fungal AOX has a *Km* for ubiquinone-1 of 68.5 μM, whereas the AOX from *Trypanosoma brucei* has a *Km*, also for ubiquinone-1 under similar reaction conditions, of 338 μM. To note, the *Km* of CIII for ubiquinol-1 is 13 μM [67,68]. *Trypanosoma brucei* AOX is allosterically inhibited by ATP [69]. All these features call for caution when interpreting and comparing the results from the usage of different AOX, even in similar cellular systems or reaction buffers. There is a need to compare the bioenergetic properties with AOX from different species to rule out the potential alterations introduced by AOX from different species. In addition, this feature of different AOX having a spectrum of properties opens a panoply of possibilities to study different parameters in mitochondrial function, bioenergetics and homeostasis.

5. Alternative oxidases in the study of ROS

The study of reactive oxygen species (ROS) is an extremely challenging field in Biochemistry, mostly because the short duration of their different species, the panoply of mechanisms producing them and the fact that the levels of ROS are governed both by production and quenching of every species [70,71]. ROS are sequential one-electron reduction products derived from oxygen. The first species formed, after one electron donation to oxygen, is the superoxide anion. A subsequent one-electron reduction of superoxide produces hydrogen peroxide and, a further one-electron reduction generates hydroxyl radical [45]. There are several mechanisms involved in the production of ROS by the mitochondria and all of them involve the OXPHOS complexes [44,45,72]. Under specific circumstances, such as succinate accumulation, ubiquinol accumulates and, if mitochondria are sufficiently polarized, CI can catalyze its reverse reaction, consuming $\Delta\psi mt$, carrying electrons backwards which react with oxygen producing superoxide anion. Such mechanism, the so-called RET, can be inhibited by ubiquinone-site inhibitors of CI (e.g. rotenone) and uncouplers (e.g. carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone, FCCP), and it is one of the best characterized mechanism of ROS production by mitochondria which has been involved in a spectrum of physiological and pathophysiological situations, such as macrophage maturation or ischemia-reperfusion injury [28,48,49].

It has been described that mitochondrial ROS production increases with age and that lack of detoxification is detrimental and shortens lifespan in *Drosophila*. In the same study, overexpression of NDI1 promoted the accumulation of ubiquinol which, in turn, was found to increase mitochondrial ROS production through CI. Strikingly, the increase in lifespan through NDI1 overexpression was reversed by overexpression of AOX in the IMM or catalase (i.e. H_2O_2-removing enzyme) in the mitochondrial matrix. Importantly, NDI1 mediated raise in ROS levels could be decreased by treatment with rotenone or FCCP, indicating that the mechanism implied was CI RET. To further study the involvement of ROS, the authors knocked down the expression of either the mitochondrial superoxide dismutase (SOD), SOD2, or the PTEN Induced Kinase 1 (PINK1) and observed that both were deleterious for the flies as it lowered CI and aconitase 2 activities, and dramatically decreased lifespan. Strikingly, increase in RET-dependent ROS by overexpression of NDI1 restored CI activity and lifespan in both models. These results indicate that ubiquinol accumulation and ROS produced via RET are necessary for homeostasis and that preventing ubiquinone reduction is associated with deleterious phenotypes inducing aging and age-related phenotypes [28].

As mentioned above, CIV deficiency in cells promotes increased production of ROS through RET which could be rescued by AOX overexpression. This was further tested in CIV deficient-mouse overexpressing *Ciona intestinalis* AOX. Notably, these mice rather decreased their life-span in comparison to KO mice, which was explained by the fact that ROS acted as a trigger for mitochondria biogenesis [73]. In parallel, overexpression of *Ciona intestinalis* AOX in wild type mouse confirmed the existence of separate ubiquinone pools (see above) and allowed the investigation of its role in CI RET [64]. Non-phosphorylating heart mitochondria respiring on succinate showed large production of ROS due to RET. The presence of AOX did not increase the respiratory rate under these conditions and ADP had the same effect on wild type and AOX-overexpressing mitochondria. Interestingly, succinate promoted the accumulation of ubiquinol independently of the presence of ADP or AOX. In contrast, AOX overexpression led to a substantially lower production of ROS by RET in isolated mitochondria [64]. Later, another study from the same group showed that isolated mitochondria produced less ROS under succinate respiration both in normal conditions and during reperfusion after a period of ischemia (20 or 30 min) [74]. However, the effect of AOX on ROS production in isolated mitochondria did not correlate with the lack of effect of its overexpression on ischemia-reperfusion injury, which has been shown to rely on RET [48]. The authors discussed that mito-hormesis may lie behind the lack of effect of AOX on ischemia-reperfusion injury. AOX may lower the basal production of ROS in cells, which conform a necessary signal towards proper cardiac remodeling [75]. Interestingly, overexpression of NDI1 improved myocardial injury in rat models [76,77]. Together with the results in *Drosophila* (see above), and despite the fact that NDI1 increases RET-induced ROS, it is possible that AOX lowers the hypoxic ROS signal as it may scavenge some of the ubiquinol which would normally promote superoxide production at the level of CIII during the first minutes of hypoxia [44,78,79]. In this way, as the hypoxic ROS signal is necessary for the adaptation of tissues through the stabilization of the hypoxia-inducible factors α-subunit (HIF1-α) and HIF1-α activity is beneficial for myocardial ischemia-reperfusion injury [80–82], decreasing the ROS signal during hypoxia may result detrimental for ischemia-reperfusion injury. On the contrary, overexpression of NDI1, as it increases the levels of ubiquinol (Fig. 3), would boost the ROS signal during hypoxia and promote larger HIF1-α activity during hypoxia, protecting the tissue during reperfusion [28]. To note, the comparison between hypoxia and ischemia models should be taken carefully, as the hallmarks of ischemia are quantitatively lower in hypoxic systems, making the latter a limited platform for the study of the former [83]. It is to mention that as the ROS signal requires the activity of the Na^+/Ca^{2+} exchanger (NCLX) and the buffers for experiments with isolated mitochondria do not normally contain Ca^{2+} (or sometimes Na^+) [44,84], hypoxic ROS are not generally detected in these type of experiments; however, RET conditions are easily induced with isolated mitochondria [85,86]. Thus, it is also conceivable that this hypothesis has passed unnoticed since the hypoxic ROS signal cannot be detected under normal experimental conditions with isolated mitochondria.

Alternative oxidases have been also used to study hypoxic redox signaling and adaptation through HIFs. In this case the authors used a similar approach as the alternative ETC. They stimulated the stable expression of a dominant negative form of the mitochondrial DNA polymerase (DN-POLG) in human cells which allowed the removal of the mtDNA and, thus, the elimination of the whole OXPHOS machinery. Overexpression of *Ciona intestinalis* AOX and NDI1 allowed the reconstruction of a functional ETC without two of its otherwise indivisible features, H^+ pumping and ROS production. By using these tools, the authors showed that metabolite levels were maintained in NDI1/AOX cells in comparison to DN-POLG. Also, acetylation marks, which had disappeared in DN-POLG cells, were restored in NDI1/AOX cells, in contrast to proliferation. But, in addition, the restoration of respiration though a functional ETC, without H^+ pumping or ROS production, was

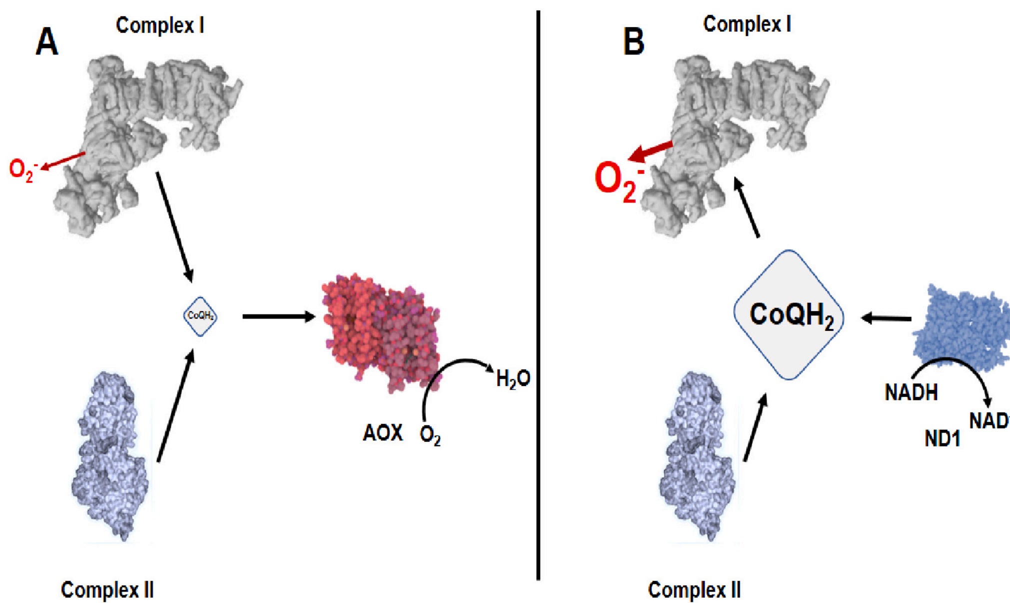

Fig. 3. Alternative oxidases allow the manipulation of ubiquinone redox state and ROS production by mitochondria. (A) Scheme showing the oxidizing effect of AOX on ubiquinol in the IMM. (B) Scheme showing the reducing effect of NDI1 on the ubiquinone pool in the IMM.

not able to restore the ability to stabilize HIF-1α in hypoxia [87]. The latter is of critical importance as during the last few decades, the ROS dependency vs independency of HIF-1α stabilization has hardly debated. Until the use of alternative oxidases [87], the only approach to validate the dependency of HIF-1α stabilization with the ETC was using ρ° cells or ETC mutants [78,79,88]. Such approaches were counter-argued by the fact that those mutants were unable to respire which modifies the oxygen gradient, increasing the cellular oxygen content and allowing the degradation of HIF-1α even at low oxygen tensions [89]. Thus, the use of alternative oxidases for the study of hypoxic redox signaling has been of critical importance as they strongly support the notion of that hypoxia adaptation, through the stabilization of HIF-1α subunits, occurs in an ETC and a ROS-dependent process.

In the same line, *Ciona intestinalis* AOX has been used to study hypoxic pulmonary vasoconstriction (HPV) in mouse pulmonary aortic smooth muscle cells (PASMCs) [90]. Previously, it was proposed that CIII was responsible for the production of ROS during hypoxia which, in turn, were dependent on the particularly high expression of COX4I2 in CIV in PASMCs, and responsible for the HPV [91,92]. COX4I2 reduces the affinity of CIV for oxygen and has been shown to confer sensitivity to slight reductions in oxygen tension in specialized tissues [93–96]. Overexpression of AOX in wild type mouse was able to prevent production of superoxide anion and HPV in PASMCs, in this way bypassing CIII-dependent ROS production. In contrast, it was unable to prevent HIF-1α stabilization. These results highlight two important aspects in hypoxic redox signaling: 1) as mentioned above, AOX from *Ciona intestinalis* diminishes the amount of ubiquinol, a key molecule in hypoxic ROS production as it is the electron donor to oxygen to form superoxide [44], and, as a consequence, expression of AOX drives the levels of ubiquinol insufficient to promote a relevant amount of superoxide to trigger a redox signal [90]. 2) As mentioned above hypoxic ROS production and HIF-1α stabilization is blunted in NDI1/AOX expressing ETC-null cells after 4 h in 1 % oxygen (DN-POLG) [87]; in contrast, AOX in wild type PASMCs was able to prevent ROS production, but not HIF-1α stabilization after 36 h in 1 % oxygen (PASMCs) [90]. This means that either the exceptionally high levels of COX4I2 in PASMCs renders HIF-1α stabilization independent of ROS production (i.e. due to differential metabolite profile or basal HIF-1α stabilization) or that simply shorter hypoxic incubations (i.e. 4 h) render HIF-1α stabilization dependent on ROS, in contrast to longer incubations (i.e. 36 h). In this line, it would be interesting to test the effect of NDI1 in HPV and hypoxic ROS production, given its effect on increasing ubiquinol levels [28] and, in addition, the effect of COX4I2 removal in ROS production and HIF-1α stabilization in non-specialized cells. Nevertheless, the use of AOX has been critical to dissect the mechanism of oxygen sensing in HPV and the future use of this and other alternative oxidases may help to further clarify the mechanism of ROS production in specialized cells.

6. Alternative oxidases in future research

In this review we have summarized how the use of alternative oxidases has helped to the understanding of mitochondrial physiology in vitro and in vivo. Nevertheless, alternative oxidases can still be of much help in elucidating and understanding critical mitochondrial processes. It would be extremely helpful to perform the same kind of experiments in human cells as: 1) In human cells free CI is less abundant than in mouse being more prone to super assembly with CIII. 2) Equally to mouse cells, CI stability is very dependent of the presence of CIII [29,31]. 3) The recovery of CI assembly by AOX would provide a completely lonely CI which can be compared to a fully super assembled CI. 4) It would enable the study of the partially segmented ubiquinone pools in human cells, though some experiments supporting their existence have already been published [34].

The expression of alternative oxidases in flies has been of extreme utility to dissect the molecular mechanism leading to mitochondrial ROS production in relation to lifespan. In this regard, the use of alternative oxidases in mouse would enable the confirmation of the results seen in flies and the contribution of the alternative oxidases and the reduction status of ubiquinone to overall redox signals, such as during Na^+-dependent hypoxic signaling, reperfusion, or macrophage maturation, among others.

Ubiquinone has been shown to be necessary for the function of many proteins in mitochondria, from enzymes involved in cell proliferation [97] to thermogenesis [98]. Thus, the modulation of ubiquinone redox state by alternative oxidases may be helpful to elucidate relationships between the ETC redox state and mitochondrial physiology. As NDI1 can promote RET in flies, and this may also happen in mammals, it would be interesting to study whether the consumption of Δψmt by RET may impact other critical processes in the mitochondria, such as mitochondrial Ca^{2+} and Na^+ homeostasis, mitochondrial permeability transition

pore (mPTP) opening, ATP production by CV, protein import or mitophagy.

In summary, alternative oxidases provide a powerful set of tools with which important aspects of mitochondrial biology can be studied. These molecules, which can separate indivisible processes in mitochondria, conform the nucleus of a very attractive field which will most probably help to close current debates and contribute to further discussions in mitochondrial research.

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Conceptualization and writing P.H.-A. and J.A.E. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interests.

Data availability

No data was used for the research described in the article.

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