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Alternative oxidase in the branched mitochondrial respiratory network: an overview on structure, function, regulation, and role

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Abstract

Plants and some other organisms including protists possess a complex branched respiratory network in their mitochondria. Some pathways of this network are not energy-conserving and allow sites of energy conservation to be bypassed, leading to a decrease of the energy yield in the cells. It is a challenge to understand the regulation of the partitioning of electrons between the various energy-dissipating and -conserving pathways. This review is focused on the oxidase side of the respiratory chain that presents a cyanide-resistant energy-dissipating alternative oxidase (AOX) besides the cytochrome pathway. The known structural properties of AOX are described including trans-membrane topology, dimerization, and active sites. Regulation of the alternative oxidase activity is presented in detail because of its complexity. The alternative oxidase activity is dependent on substrate availability: total ubiquinone concentration and its redox state in the membrane and O_2 concentration in the cell. The alternative oxidase activity can be long-term regulated (gene expression) or short-term (post-translational modification, allosteric activation) regulated. Electron distribution (partitioning) between the alternative and cytochrome pathways during steady-state respiration is a crucial measurement to quantitatively analyze the effects of the various levels of regulation of the alternative oxidase. Three approaches are described with their specific domain of application and limitations: kinetic approach, oxygen isotope differential discrimination, and ADP/O method (thermokinetic approach). Lastly, the role of the alternative oxidase in non-thermogenic tissues is discussed in relation to the energy metabolism balance of the cell (supply in reducing equivalents/demand in energy and carbon) and with harmful reactive oxygen species formation.

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- Alternative oxidase
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- Electron partitioning

Short historical introduction

Cyanide-insensitive respiration was first observed in plants in 1929 by Genevois in sweet pea seedlings (1). Most investigations made in the past were physiological, utilizing whole plants or intact tissues, and indicated the occurrence of cyanide resistance linked to heat production (2,3). When isolated mitochondria were used, studies were focused on the relative activities of both cyanide-insensitive and cytochrome pathways by measuring rates of respiration in the presence of an inhibitor of each pathway. In 1978, a cyanide-resistant quinol oxidase was solubilized from *Arum maculatum* mitochondria (4,5), and the alternative cyanide-resistant respiration was attributed to an enzyme called the alternative oxidase (AOX). Partial purification of the alternative oxidase was achieved (6,7) but its lability and limited activity have hampered enzymological approaches like kinetic and structural studies for a long time. In 1986, monoclonal antibodies to three induced proteins from *Sauromatum guttatum* responsible for cyanide-resistant respiration were obtained (8), strongly stimulating the field of the alternative oxidase research. These monoclonal antibodies were used to identify AOX proteins in a wide variety of plants (9,10), in fungi (11,12), in trypanosomes (13), and amoebae (14). Thus, the presence of the cyanide-resistant alternative oxidase is not limited to plant mitochondria and is quite widespread among other types of organisms. Isolation of cDNA and genes encoding the AOX protein has led to the first primary sequences (15), a transmembrane model (16,17) and structural modeling (9,10,18). In the last few years considerable progress has been made in the understanding of the regulation and role of AOX.

The aim of this paper is to provide an overview of the field and to stress the new achievements. For more detailed comprehensive reviews of the alternative oxidase field, the reader is referred to reviews by Moore and Siedow (16), McIntosh (19), Siedow and Umbach (10), Day and Wiskich (20), Day et al. (9), Wagner and Krab (21), Krab (22), and Vanlerberghe and McIntosh (23).

Branched respiratory network in mitochondria

The respiratory chain of plant mitochondria, taken as an example, differs in several ways from the mammalian mitochondrial respiratory chain (Figure 1). The dehydrogenase side presents two additional NAD(P)H dehydrogenases, which are not proton pumps

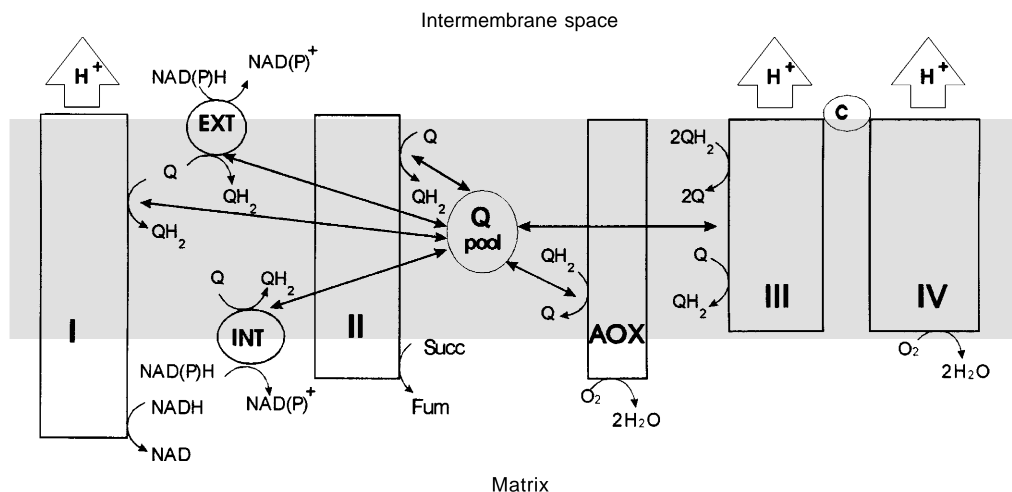

Figure 1 - Organization of the branched respiratory chain of plant mitochondria. Complex I, NADH dehydrogenase; complex II, succinate dehydrogenase; complex III, cytochrome bc_1; complex IV, cytochrome oxidase; AOX, alternative oxidase; c, cytochrome *c*; Q, ubiquinone; QH_2, ubiquinol; INT, internal NAD(P)H dehydrogenase; EXT, external NAD(P)H dehydrogenase.

(and therefore are not conserving energy), are not inhibited by inhibitors of the proton-pumping energy-conserving complex I (e.g. rotenone) and allow electrons originating from NADH to bypass complex I. Moreover, on the oxidase side a cyanide-resistant alternative oxidase is present besides the cytochrome pathway (complexes III and IV). This oxidase is not linked to proton gradient building and thus dissipates the free energy released during electron flow into heat (16). Ubiquinone (Q) has a central position in the respiratory chain network. This coenzyme links the different branches of the network, receiving electrons from the upstream dehydrogenases (reducing pathways) and giving electrons to the downstream oxidases (oxidizing pathways). Thus, during the oxidation of NADH formed by the Krebs cycle, one, two or three sites of energy conservation may be bypassed (i.e., when the rotenone-insensitive dehydrogenase and the cytochrome pathway are involved, when complex I and AOX are involved, or when the rotenone-insensitive dehydrogenase and AOX are involved, respectively), leading to a reduction in protonmotive force and finally in the level of ATP synthesis. In mitochondria with a branched network, the extent to which the dissipative pathways are used affects the energy yield in the cells. It is obviously of crucial interest for these cells to be able to control the relative contribution of each pathway of the network to the total oxygen uptake and it is a challenge for us to understand the mechanisms that regulate the partitioning of electron flow.

Electron flow through AOX can be specifically inhibited by various compounds like hydroxyamic acids (e.g. salicylhydroxamic acid (SHAM) and benzohydroxamic acid (BHAM)) (24), n-propyl gallate (25,26) and disulfiram (27). Then, the level of the cyanide-resistant oxygen uptake sensitive to one of these inhibitors is the diagnosis of AOX activity.

Characteristics of AOX protein

Primary structure of AOX

The amino acid sequence of a protein can be derived from the cDNA sequences. By analysis of the primary sequence of a protein first insights into its structure can be deduced. AOX primary sequences obtained in such a way contain a mitochondrial transit peptide at the N-terminus. The mature AOX protein from *S. guttatum*, as an example, has a calculated molecular mass of 32.2 kDa and contains 283 amino acids (15). Hydropathy plot analysis of several AOX amino acid sequences (15,16) has indicated important conserved features of the protein: a) two hydrophobic regions with a strong α-helical character and highly conserved amino acids have been proposed to be membrane-spanning helices, b) these two regions are separated by about 40 amino acids including an amphipathic helix probably exposed to the intermembrane surface, and c) two hydrophilic regions of about 100 amino acids on both terminal sides with highly conserved short regions at the C-terminus. A topologic model for the AOX protein has been proposed (9,10,16-19), with the amphipathic helix exposed on the cytosolic side and the hydrophilic domains extending into the matrix. Both cytosolic and matricial parts are linked by the two membrane-spanning helices.

Active sites of AOX

The ability of AOX to reduce oxygen to water involves a four-electron reduction reaction (16,28) to avoid production of damaging partially reduced oxygen species. Therefore, an active site containing transition metals has been predicted (16,29). Evidence that makes iron the leading metal candidate for the metal associated with the AOX active site comes from experiments with culture of the yeast *Hansenula anomala* in which the absence of iron leads to an inactive 36-kDa AOX protein (30). Its activity is

recovered by the addition of iron to the culture medium. All of the plant AOX amino acid sequences (in the C-terminal domain) reveal the presence of two copies of the conserved iron-binding motif (E-X-X-H), providing the additional argument that AOX contains iron. By comparison with coupled binuclear iron proteins, an iron-binding four-helical bundle model of the AOX active site has been proposed analogous to that of methane monooxygenase (10,18,31).

The reducing substrate (i.e. ubiquinol (QH_2)) binding site has been postulated to be situated on the matrix side of the mitochondrial membrane in a hydrophobic pocket formed by the two membrane-spanning helices (10,18). The three fully conserved residues (T, E, Y) in the pocket are potential ligands for ubiquinone. Then, in the proposed structural model of AOX, the binuclear iron center where oxygen is reduced to water should be close to the postulated binding site of reducing QH_2 which itself is in the vicinity of the binding site for the allosteric effector of the enzyme, pyruvate (see below).

Dimeric structure of AOX

The structural property of AOX has been revealed by SDS-PAGE electrophoresis and immunoblotting using chemical cross-linkers and oxidizing and reducing agents (32). These experiments have demonstrated that AOX exists as a dimer of 65 kDa in the inner mitochondrial membrane and that two distinct states of the dimer can be identified: an oxidized state in which the dimer is covalently cross-linked by a disulfide bridge (-S-S-) and a reduced state (-SH HS-) which is maintained through non-covalent interactions. The intermolecular disulfide bridge is formed at the level of a conserved cysteine residue (in the plant AOX) situated in the N-terminal domain exposed to the matrix of each monomer (Figure 2). A second conserved cysteine residue (in the plant AOX) implicated in pyruvate binding is situated in the same N-terminal domain close to the membrane surface of each subunit (33).

AOX genetics

The AOX protein is encoded in the nucleus. In many organisms the AOX monomer is revealed as multiple bands of approximately 36 kDa on immunoblots using a single monoclonal antibody. In the case of plant AOX protein, depending on the species and tissues, one, two or three bands were observed. Therefore, the question was if these

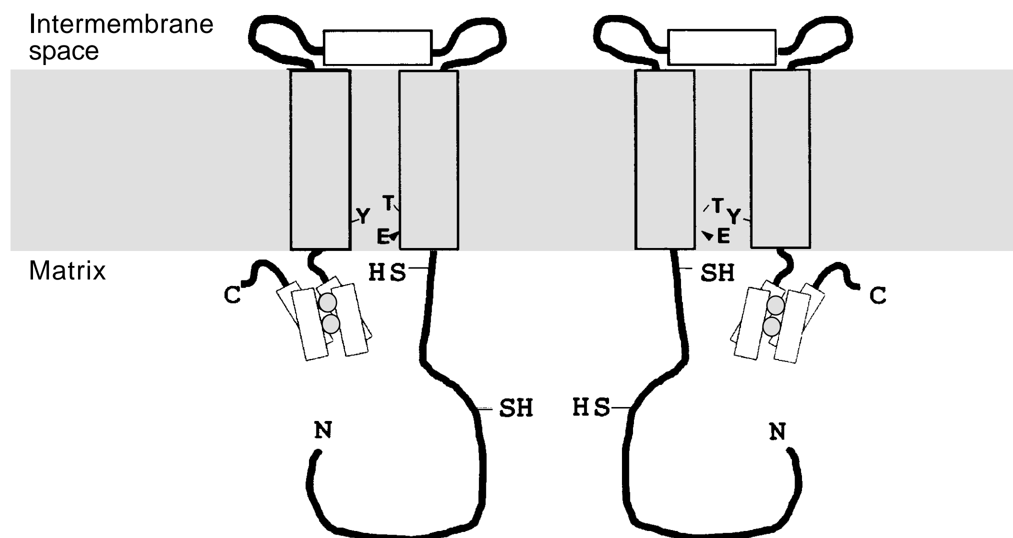

Figure 2 - Topologic model of the dimeric plant AOX in the inner mitochondrial membrane. Highly conserved residues T, Y, and E, located in the two transmembrane helices, are proposed to be a potential quinone-binding site. The four-helix bundle (postulated binuclear iron center) is located in the C-terminal hydrophilic domain of the protein on the matrix side of the inner mitochondrial membrane. On the same side of the membrane, the relative positions of the two conserved cysteine residues are shown. One, located in the N-terminal hydrophilic domain closest to the membrane (near the postulated diiron site and the postulated quinone-binding site), may be the binding site for pyruvate (allosteric effector). Second, the more N-terminal cysteine residue may be the redox-active group involved in conversion between the more active noncovalently associated reduced state of the enzyme and the less active oxidized state. Based on Siedow and Umbach (10), Moore et al. (18), and Umbach and Siedow (33).

bands originate from multiple genes (isoforms) or from a single gene by differential processing or by post-translational modification of the protein. The existence of three AOX genes has been recently demonstrated in soybean using a polymerase chain reaction approach (34,35). Differential expression of these genes has been shown in soybean tissues, explaining the presence of multiple bands on immunoblots. Presently, it is suggested that AOX multigene families may be a common feature in plants (34,35).

AOX activity

The activity of AOX is controlled by several parameters among which we can distinguish regulatory events and substrate availability (Figure 3).

Regulation of AOX activity can occur at different levels: i) gene expression that affects the amount of the protein in the membrane and differential gene expression that modifies the ratio between isoforms; ii) post-translational modifications of the protein (i.e. its redox status that affects the nature of the dimer); iii) the action of allosteric effectors like pyruvate.

The substrate availability is linked to the concentration of total quinone in the inner mitochondrial membrane, the redox state of quinone in the membrane, and O_2 concentration.

Control of AOX synthesis

Various events can influence the amount of AOX protein present in a cell. The developmental state plays a role in plants, as in thermogenic Araceae, where the amount of AOX increases during flowering (36). In non-thermogenic soybean cotyledons, AOX activity increases immediately after germination and during senescence (20). During the phase of growth in amoeba *A. castellanii* cell culture, a parallel exists between AOX activity and protein expression that decreases with the age of the amoeba culture, reaching a stationary phase (14). Under a large variety of stress treatments like chilling in tobacco, wounding in potato tubers, osmotic stress, etc. the expression of the AOX protein is increased (9,19,37). In cell cultures AOX can be induced when the cytochrome pathway activity is decreased, either with specific inhibitors or by disruption of mitochondrial protein synthesis (9,19). These results indicate that nuclear gene expression is sensitive to the activity of the cytochrome pathway and that a communication must exist between mitochondria and the nucleus. In yeast, it has been shown that the ability of an inhibitor of the cytochrome pathway to induce AOX synthesis (38) is linked to its ability to cause superoxide formation during mitochondrial respiration. Superoxide anion itself could induce synthesis of AOX protein

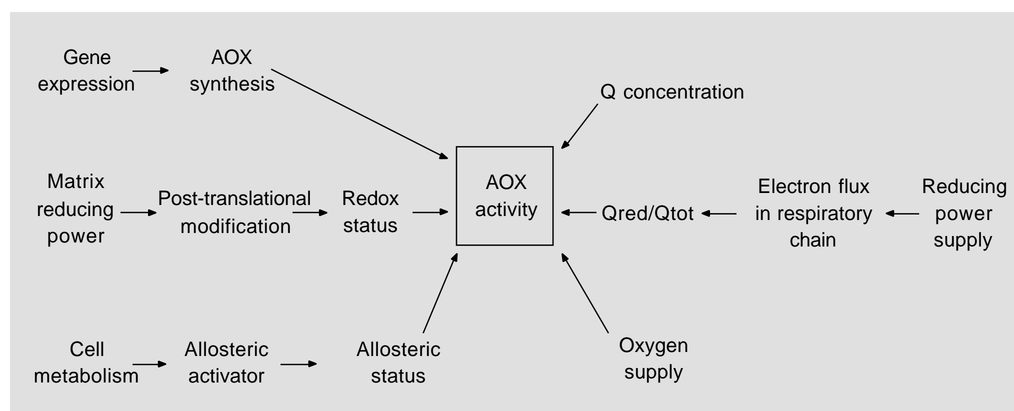

Figure 3 - Mechanism of regulation of the alternative oxidase activity.

(39) and be the messenger between mitochondria and the nucleus. In tobacco cell cultures, H_2O_2 increased levels of AOX mRNA and AOX activity (40). Salicylic acid, a cellular signal required for the induction of disease resistance in plants, can also be responsible for AOX synthesis (41,42). It has been suggested that salicylic acid-inhibiting catalase activity increases the level of H_2O_2 which in turn would stimulate gene expression including AOX genes (21).

Differential expression of three AOX genes in soybean has been shown to be tissue-dependent and physiological state-dependent (during cotyledon greening) (35). Variation in relative abundance of AOX transcripts is generally correlated with the amount of protein isoforms detected by immunoblotting in roots and cotyledons. The three isoforms (AOX1-3) are found in cotyledons whereas in roots only AOX3 is present. A difference in the proportion of AOX isoforms leads to variation in the AOX properties between tissues (basal activity, pyruvate stimulation, $^{18}O_2$ discrimination; see below). Moreover, the presence of three types of AOX subunits in the same tissue produces heterodimers (32,35) and could provide a very flexible mechanism of regulation of AOX activity in response to cellular metabolic states. AOX isoforms and their heterodimerism may be a general feature in plants.

Regulation by redox states

It has been demonstrated that two types of dimeric structure of AOX exist, an oxidized form and a reduced form (32). The reduced form can be four- to five-fold more active than the oxidized form (18,32). The ratio of oxidized to reduced protein varies considerably between species and tissues. In cotyledon soybean mitochondria AOX is mainly reduced, while in root soybean mitochondria it is 50% oxidized (43). In tobacco leaf mitochondria, AOX is largely oxidized and its activity is unmasked by reductants like dithiothreitol (DTT) (20). Reduction of the AOX protein can also occur during oxidation of specific Krebs cycle substrates like isocitrate and malate that are able to reduce $NADP^+$ in plant mitochondria (44). Thus, it has been proposed that the AOX reduction state depends on NADPH generation and involves NADPH-reduced glutathione or the thioredoxin coupling system (10,20,32,44). It has recently been shown that the redox state of AOX *in vivo* is different from that determined in isolated mitochondria due to spontaneous oxidation of the reduced species of the protein during organelle isolation (45). Control of the redox status of AOX could be a powerful mechanism of regulation of its activity *in vivo* that may link AOX activity to the general redox state of the cell (i.e., an increase in the reducing power (NADH, NADPH) induces activation of AOX).

Regulation by allosteric effectors

In plant mitochondria, short ketocarboxylic acids such as pyruvate, hydroxypyruvate and glyoxylate can activate AOX (9,46,47). Activation by these short ketoacids does not involve their metabolism as is the case for succinate and malate that are not effectors of AOX (46,47). Pyruvate is the most effective activator of plant AOX with a half maximal stimulation at 0.1 mM with intact mitochondria and at less than 5 µM when used with inverted submitochondrial particles (47). It has been concluded that pyruvate acts from the matrix side of mitochondria (47,48). Recent results indicate that activation of AOX by pyruvate involves formation of a thiohemiacetal with the sulfhydryl group of the cysteine residue just upstream of the first membrane helix of the protein (Figure 2). This is a potential allosteric effector binding site of the AOX protein (33). Pyruvate stimulation is reversible and is observed in mitochondria where AOX is in its reduced form, as in soybean cotyledons (20). In tobacco

leaf mitochondria, with a very low AOX activity largely present in the membrane in the oxidized form, no effect of pyruvate is observed until reduction of the protein by DTT occurs. This observation has led to the conclusion that the two systems of AOX regulation (i.e., redox state of the protein and pyruvate binding) interact with each other modulating AOX activity. The AOX dimer must be in its reduced form to be activatable by pyruvate, and the reduced form of the enzyme reveals little activity in the absence of pyruvate.

The effect of pyruvate consists in a lowering of the range of the ubiquinone redox state over which the AOX is active in isolated mitochondria. It has been attributed to lowering the so-called "effective" K_m of AOX for its reduced substrate, ubiquinol (49). This should be understood as an increase in the reactivity of the enzyme towards QH_2 which does not exclude the effect of pyruvate on the catalytic constant of the enzyme. The link between the two regulatory mechanisms (i.e., redox state of the protein and allosteric effect of pyruvate) is such that the active reduced AOX dimer will slowly oxidize ubiquinol until pyruvate is present on its binding site.

In microorganisms such as *Acanthamoeba castellanii* (50-52), *Euglena gracilis* (53), *Moniliella tomentosa* (54,55), *Paramecium tetraurelia* (56), *Neurospora crassa* (57), and *Hansenula anomala* (58) the alternative oxidase is stimulated by purine nucleotides that probably act from outside the inner mitochondrial membrane (56,59). In mitochondria of the amoeba *A. castellanii*, stimulation of AOX activity by GMP resembles the pyruvate effect on plant mitochondria (affecting AOX activity *vs* quinone redox state) (60). In amoeba mitochondria, the AOX activity which is not sensitive to pyruvate also seems not to be regulated by the redox status of the protein (14). It may be a general feature of AOX in microorganisms, especially because comparison of known amino acid sequences of AOX protein from *N. crassa* (12) and *H. anomala* (61) indicates lack or displacement of the two regulatory cysteine residues conserved in all plant AOX proteins.

Quinone concentration dependence

As every enzyme, AOX is controlled by its substrate-product concentrations. Thus, the actual concentration of Q and QH_2 in the inner mitochondrial membrane is also important. The Q content in soybean cotyledon mitochondria is 3.5 times higher than the Q content in soybean root mitochondria (35,62). For instance, even if succinate can reduce ubiquinone by 90% in both tissues, the concentration of ubiquinol present in root mitochondria will be only 30% of that found in cotyledon mitochondria. This difference in QH_2 concentration may be responsible for a markedly lower AOX activity in root mitochondria. This may indicate that ubiquinone concentration can be a limiting factor of electron transfer between a dehydrogenase and AOX. However, stimulation by pyruvate (see above) is more important in root mitochondria whereas it is rather limited in cotyledon mitochondria in the presence of the AOX reductant, DTT (35,43). Therefore, it seems that reduced quinone concentration is virtually sufficient to saturate AOX in cotyledon mitochondria while in root mitochondria, pyruvate, which lowers the amount of ubiquinol required for AOX activity (see above), acts in such a way that ubiquinol also saturates AOX. These results illustrate the interplay between substrate (QH_2) availability and allosteric effector (pyruvate) presence indicating some interactions between their binding sites.

Quinone redox state dependence

Early studies using inhibitors (63,64) of both oxidase pathways indicated that the AOX pathway was active only when the cytochrome pathway activity was low, i.e., in

the absence of ADP (state 4) or in the presence of cytochrome chain inhibitors. On the other hand, inhibition of AOX apparently did not modify the activity of the cytochrome pathway. Thus, AOX activity appeared to be controlled by the activity of the cytochrome pathway, giving rise to the overflow paradigm: AOX is only active when the cytochrome pathway is saturated or close to being saturated (63-66). Measurements of Q reduction state during succinate oxidation by soybean cotyledon mitochondria have shown that AOX becomes active only when the Q pool is 40-50% reduced, and then its activity increases sharply and non-linearly with higher Q reduction level (67,68). In contrast, the rate of state 3 respiration is linearly related to the degree of Q reduction (reduced Q (Qred) versus amount of total Q in the membrane (Qtot); Qred/Qtot) and reaches its largest measured values at a low Qred/Qtot ratio at which the AOX activity in the presence of a cytochrome pathway inhibitor is not detectable (68). In the absence of inhibitors, the rate of state 4 respiration versus Qred/Qtot is linear until AOX becomes active for a Qred/Qtot ratio higher than 50%, and after this point AOX increases its activity dramatically. The relationship between respiratory rate and Qred/Qtot in the cytochrome pathway (in state 4 respiration, with BHAM) is linear (69). To our knowledge, no results concerning the dependence of the cytochrome pathway activity in state 3 respiration (in the presence of BHAM) on ubiquinone reduction level have been published. This general behavior described above is not identical in mitochondria originating from every plant species or tissue. Indeed, different responses of AOX activity to the Q redox state may occur depending on the redox state of the AOX protein and on the presence of allosteric activators. Hence, the variety of AOX activity widely described with different substrates in different mitochondria cannot only be explained by changes in Q redox state. For instance, the difference between external NADH and succinate in the kinetic relationship between AOX activity and Qred/Qtot disappears in the presence of pyruvate (70).

O_2 concentration dependence

Because of the O_2 concentration in the incubation medium widely used to study plant mitochondria (about 200-250 µM at air saturation), oxygen reduction by AOX should be independent of the O_2 concentrations. Indeed, the apparent affinity of AOX for O_2 is high (apparent oxygen K_m around 1-2 µm) (71-73) and it is lower than the cytochrome oxidase affinity (apparent oxygen K_m around 0.1-0.15 µM) (72,74,75). At variance, much higher values of apparent K_m of AOX for O_2 have been reported (76), ranging from 10 to 20 µM depending on species, tissues and age of the plants investigated. It has also been shown that the apparent K_m of AOX for O_2 varied with quinone reduction level: the more reduced the quinone pool the lower is the affinity for O_2. A kinetic model fitting these data implies two sequential two-electron reduction steps of AOX (four-electron-reduced enzyme) and an irreversible activation step of AOX occurs before the reduction of O_2 to water (76).

In summary, the regulation of electron flow through the mitochondrial alternative pathway is very complex: on the one hand, the maximum level of AOX activity is a function of both the amount of AOX protein present in the membrane and its redox status (post-translational modification), and on the other hand, the level of engagement of the pathway is dependent on the amount of ubiquinone and on its redox state together with the protein's allosteric status as determined by pyruvate concentration. Moreover, protein redox status and allosteric status are not independent. A precise understanding of the interplay and quantitative analysis of these various levels of regulation can be reached only by measurements of the actual activities of the two branching oxidases dur-

ing steady-state respiration (i.e. electron partitioning).

Electron partitioning

The interplay of the various levels of regulation of AOX will influence not only AOX activity but also the way the electrons are distributed between the alternative and cytochrome pathways (i.e. electron partitioning). Indeed, it is obvious that an increase in AOX activity at a constant quinone-reducing pathway rate due to an increase either in the affinity of AOX for ubiquinol or in the AOX maximal rate will modify the flux of electrons through the cytochrome pathway. Thus, regulation of AOX activity also means regulation of electron partitioning. The only way to describe quantitatively electron partitioning is to measure the actual contribution of each branching oxidase. This determination is hampered by the fact that both oxidative pathways have the same substrates (QH_2, O_2) and the same products (Q, H_2O). Previous estimations of partitioning were based on the use of inhibitors of both pathways (63,64). As mentioned before, the usefulness of inhibitor studies was based on the concept that the alternative pathway behaves as an electron “sink” (overflow) (65,66) and that it is kinetically unfavored, being unable to compete with the cytochrome pathway for QH_2. This overflow paradigm used to be such a strong dogma that it hid the obvious fact that inhibition of one pathway inevitably affects the Qred/Qtot ratio and then modifies electron flux in the other pathway. Fortunately, the recent information on regulation of AOX has led to a reexamination of the overflow assumptions (69,77,78). Numerous studies have demonstrated that several factors enhance AOX activity (see above) and then enable it to compete with an unsaturated cytochrome pathway. Thus, measurement of O_2 consumption rates in the presence of inhibitors to ascertain the electron partitioning between branched quinol-oxidizing pathways is no longer considered valid. Consequently, most of the electron partitioning determinations made in the past using only rate measurements in the presence or absence of inhibitors must be considered as misestimated. In order to determine electron partitioning between AOX and the cytochrome pathway it is necessary to measure the true contributions of both pathways to total respiration when both are active. These measurements must be performed using well-defined experimental conditions in order to control all the factors that are known to potentially affect both oxidizing pathway activities. Only in this way will an accurate quantitative description of the respiratory network be obtained. Several strategies have been developed to measure the true contributions of branching pathways not only *in vitro* but also *in vivo*.

Kinetic approach

A kinetic approach has been developed (69) taking into account the interplay between quinol-oxidizing and quinone-reducing electron fluxes in the total steady-state respiratory rate and the redox state of quinone. This method assumes a homogenous redox state of the quinone pool in the inner mitochondrial membrane as the common link between the reducing and oxidizing branches of the network and allows a prediction of the true contribution of each pathway. Practically, the method implies separate inhibitor titrations of the dehydrogenase, the cytochrome pathway and AOX together with the determination of the redox state of ubiquinone in each of these situations (for a graphic representation see Figure 6C in Van den Bergen et al. (69)). The dehydrogenase kinetic curve versus Qred/Qtot ratio is obtained by inhibiting overall electron flux with an oxidase inhibitor (e.g. AOX by SHAM or BHAM, and the cytochrome pathway by KCN or myxothiazol) that increases the Qred/Qtot ratio and consequently de-

creases the rate of dehydrogenase activity (product inhibition). The cytochrome pathway curve is obtained in the presence of SHAM by decreasing the overall electron flux with a dehydrogenase inhibitor (e.g. malonate for the succinate dehydrogenase) that decreases the Qred/Qtot ratio and consequently the activity of the cytochrome pathway (decrease in substrate availability). The AOX curve is obtained in the presence of KCN by decreasing the overall electron flux with a dehydrogenase inhibitor. The sum of the cytochrome pathway curve and the AOX curve gives the total oxidizing pathway curve when Qred/Qtot decreases. The total non-inhibited steady-state respiration is given by the cross-point of the dehydrogenase curve and the total oxidizing pathway curve. This intersection gives the Qred/Qtot ratio in the absence of inhibitor when both pathways are active. The steady-state when AOX is blocked is given by the intersection of the dehydrogenase curve and the cytochrome pathway curve, while the steady-state when the cytochrome pathway is blocked is given by the intersection of the dehydrogenase curve and the AOX curve. The true contribution of the cytochrome pathway to non-inhibited steady-state respiration is the rate obtained on the cytochrome pathway curve for Qred/Qtot without inhibitors, when both oxidizing pathways are active, while the true contribution of the alternative pathway is the rate obtained on the AOX curve for the same Qred/Qtot ratio. Thus, the kinetic approach described above allows a prediction of the real contribution of each quinol-oxidizing pathway at any given uninhibited total steady-state rate.

However, although this method is powerful, a new set of curves must be rebuilt for every chosen or encountered experimental condition. Apart from this heavy work, the main default of this method is its basic hypothesis of a quinone homogenous pool that has never been demonstrated and never disproved. Nevertheless, the kinetic approach has been useful to emphasize the inadequacy of the classic inhibitor approaches (21,69).

Oxygen isotope differential discrimination

Another method based on the fractionation of oxygen isotopes which can occur during plant mitochondrial respiration has been developed (79-83). Fractionation takes place because molecules containing the lighter isotope react a little more readily than molecules that contain the heavier isotope. Respiratory discrimination is determined by observing progressive changes in isotopic composition of oxygen within a closed system (i.e. the build-up of $^{18}O^{16}O$ relative to $^{16}O^{16}O$ after consumption of a substantial proportion of the available oxygen). A substantial difference found in the oxygen isotope discrimination of AOX and cytochrome oxidases, with AOX showing a higher fractionation factor (79), has led to development of a mass spectrophotometric technique that is used to estimate directly steady-state partitioning of electrons between the two oxidases. This noninvasive method allows the measurement of the contribution of both oxidizing pathways to total respiration (in the absence of inhibitors) in isolated mitochondria (43,79,80,83) as well as in intact tissues (81,82).

In order to determine the degree of electron partitioning to the AOX and cytochrome pathways, the discrimination (D) factors for the cytochrome pathway (Dc) and for AOX (Da) must be measured separately in the presence of an inhibitor of the other pathway (Dc measured in the presence of SHAM, Da measured in the presence of KCN) (79). These factors represent the limits within which the overall respiratory discrimination factor (in the absence of inhibitors) (Dn) must be found. Various D factors are calculated from the isotopic ratios according to the equation:

$$D\ (‰) = \frac{\ln R/Ro}{-\ln f} \times 1000$$

where R is the isotopic ratio of oxygen at the sampling time, Ro is the initial isotopic ratio, and f is the fraction of remaining oxygen in the reaction cell (79). Knowing the values of Dn, Da, and Dc, the partitioning coefficient A can be calculated according to the equation:

$$A\ (\%) = \frac{(Dn - Dc)}{(Da - Dc)} \times 100$$

Measurements in material such as *Chlamydomonas reinhardtii* which lacks cytochrome oxidase by mutation confirm that the uninhibited discrimination factor (Dn = 25‰) represents the AOX discrimination factor (Da = 24.2‰), indicating that respiratory flux is solely through AOX and that the obtained values are no artifacts caused by inhibitors (82).

The oxygen isotope fractionation technique showed that AOX can be engaged in respiration under state 3 conditions when the cytochrome pathway is not saturated (83), and it quantified the effect of several regulatory factors on electron partitioning into AOX. Moreover, this method allows the illustration of tissue dependence of this partitioning and the comparison of intact tissue and isolated mitochondria partitioning coefficients (43,83).

This very elegant method is, at present, the only one that can be used with intact tissues or with whole plants. However, the calibration used in this method requires the use of inhibitors for each respiratory pathway and assumes that change in the total respiration rate does not change the discrimination factor. Moreover, the narrowness between the end points (i.e., Dc and Da) limits the sensitivity of the method and the time-consuming measurements will prevent its use for kinetic studies of electron partitioning in various conditions.

ADP/O method

Recently, a method based on the non-phosphorylating property of AOX has been successfully developed and used to determine contributions of the two quinol-oxidizing pathways to total respiration in isolated amoeba mitochondria (84,85). This method involves pair measurements of the ADP/O ratio in the presence or in the absence of an AOX inhibitor (e.g. BHAM) and measurements of the overall state 3 respiration in the presence of GMP (an activator of AOX in amoebae) and succinate as oxidizable substrate (plus rotenone). This method was first proposed some time ago (63,86) but never used with success although it avoids the use of the rates of electron transport of both pathways in the presence of inhibitors and moreover it is not based on the homogenous quinone pool hypothesis. If

$$V_3 = V_{cyt} + V_{alt}$$

where V_3 is steady-state 3 respiration, V_{cyt} is contribution of the cytochrome pathway, V_{alt} is contribution of AOX and if

$$\alpha = \frac{(ADP/O)_{overall}}{(ADP/O)_{cyt\ (+BHAM)}} = \frac{\frac{ADP}{O_{cyt} + O_{alt}}}{\frac{ADP}{O_{cyt}}} = \frac{O_{cyt}}{O_{cyt} + O_{alt}} = \frac{V_{cyt}}{V_{cyt} + V_{alt}}$$

where O_{cyt}, O_{alt} = amount of oxygen taken up related to the activity of the cytochrome and AOX pathways, respectively; then,

$$V_{cyt} = V_3 \times \alpha$$
$$V_{alt} = V_3 - V_{cyt}$$

The ADP/O method is valid only if: i) the ADP/O ratio in the presence of cyanide is equal to zero, ii) BHAM does not induce a proton leak (has no uncoupling effect), and thereby does not affect mitochondrial transmembrane potential, iii) the ADP/O ratio in the presence of BHAM is independent of the state 3 respiratory rates (within the applied range), and iv) isolated mitochondria are well coupled and stable during the experi-

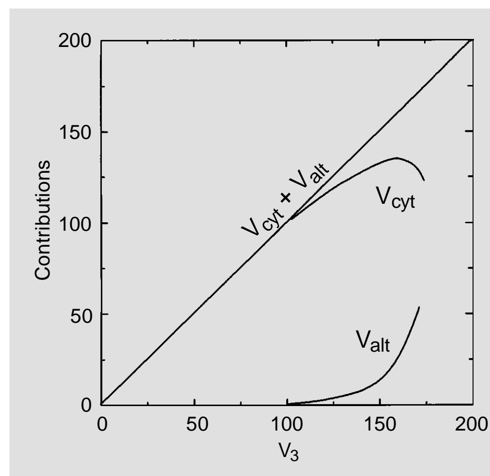

Figure 4 - Contributions of the cytochrome pathway (V_{cyt}) and of the alternative pathway (V_{alt}) to state 3 respiration (V_3).

mental procedure. All these requirements are positively verified for amoeba mitochondria (84,85). The ADP/O method allows the determination of the contributions of both pathways to overall state 3 respiration when it is decreased by increasing the concentration of n-butyl malonate, a non-penetrating inhibitor of succinate uptake by mitochondria. When overall state 3 respiration declines the AOX contribution decreases sharply and becomes negligible (when state 3 is lower than 100 nmol O min^{-1} mg protein^{-1}) while the cytochrome pathway contribution first increases, then passes through a maximum (at state 3 rate around 160 nmol O min^{-1} mg protein^{-1}) and sharply decreases for lower state 3 respiration (Figure 4). These results show that AOX is significantly engaged before saturation of the cytochrome pathway, and consequently can compete for electrons with the unsaturated cytochrome pathway. This represents the first attempt to examine in detail the steady-state kinetics of the two quinol-oxidizing pathways when both are active and to describe electron partitioning between them when the steady-state rate of the quinone-reducing pathway is varied.

The ADP/O method, perhaps the best for detailed kinetic and regulation studies of branching oxidases since it is readily applicable, cannot, however, be applied to state 4 respiration and could hardly be used with intact tissues. It has been used with isolated phosphorylating mitochondria and could be applied to permeabilized cells.

In summary, at present, three different methods are available to study electron partitioning between branching oxidizing pathways. Each of the described methods has its own domain of application because of its own limitation or restraining basic hypothesis. Nevertheless, together they offer the best possibilities to study in depth the mechanism of regulation of electron partitioning.

Role of the alternative oxidase

The only obvious physiological function of AOX can be recognized in specialized plant thermogenic tissues (spadices of Araceae) as heat generation related to increase in temperature taking part in the reproductive processes (87). In non-thermogenic tissues or cells the role of AOX is now beginning to be better understood thanks to new information concerning its mechanisms of regulation. Indeed, in these cells AOX must play a more fundamental role at the level of metabolism, given the biochemical controls to which the activity of this enzyme is submitted (described above). Metabolic conditions that lead to an increase of the reducing power in the cell will increase the Qred/Qtot ratio, the level of mitochondrial NADPH (therefore the level of reduced AOX diner) and pyruvate (allosteric activator) and thus will increase electron partitioning to AOX. Situations where the phosphate potential is high (high ATP/ADP ratio) will lead to the decrease of electron flux into the cytochrome pathway due to proton-electrochemical potential back pressure and consequently increase the partitioning to AOX. Such conditions (high reducing power and ATP/ADP ratio) are consequences of imbalances between supply of reducing substrates and energy-carbon demand for biosynthesis, both being coupled by the respiratory chain activity. Operation of AOX may counteract

these imbalances because it is not directly controlled by the energy status of the cell and may prevent fermentation and favor biosynthesis. These imbalances could be counteracted by fast regulation of AOX activities (allosteric and redox status of the protein) or as a result of slow changes in the environment (various stresses), by modification of the amount of AOX protein (gene expression). The activity of AOX has been shown to prevent the formation of reactive oxygen species by mitochondria at the level of ubiquinol when the cytochrome pathway is slowed down (21,88,89). Thus, AOX may play a protective role in mitochondria by preventing over-reduction that leads to harmful reactive oxygen species production. AOX could also play a role in mitochondrial-chloroplastic interactions during photophosphorylation which increases the reducing power (NADPH) and phosphate potential (ATP) in the cell (90). In conclusion, AOX appears to have a central role in the balance of cell energy metabolism.

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References

1. Genevois ML (1929). Sur la fermentation et sur la respiration chez les végétaux chlorophylliens. *Revue Génétique Botanique*, 41: 252-271.
2. Lance C (1972). La respiration de l'*Arum maculatum* au cours du développement de l'inflorescence. *Annales des Sciences Naturelles Botaniques*, 12: 477-495.
3. Meeuse BJD (1975). Thermogenic respiration in Aroids. *Annual Review of Plant Physiology*, 26: 117-126.
4. Huq S & Palmer JM (1978). Isolation of a cyanide-resistant duroquinol oxidase from *Arum maculatum* mitochondria. *FEBS Letters*, 95: 217-220.
5. Rich PR (1978). Quinol oxidation in *Arum maculatum* mitochondria and its application to the assay, solubilisation and partial purification of the alternative oxidase. *FEBS Letters*, 96: 252-256.
6. Bonner Jr WD, Clarke SD & Rich PR (1986). Partial purification and characterization of the quinol oxidase activity of *Arum maculatum* mitochondria. *Plant Physiology*, 80: 838-842.
7. Elthon TE & McIntosh L (1986). Characterization and solubilization of the alternative oxidase of *Sauromatum guttatum* mitochondria. *Plant Physiology*, 82: 1-6.
8. Elthon TE, Nickels RL & McIntosh L (1989). Monoclonal antibodies to the alternative oxidase of higher plant mitochondria. *Plant Physiology*, 89: 1311-1317.
9. Day DA, Whelan J, Millar AH, Siedow JN & Wiskich JT (1995). Regulation of the alternative oxidase in plants and fungi. *Australian Journal of Plant Physiology*, 22: 497-509.
10. Siedow JN & Umbach AL (1995). Plant mitochondrial electron transfer and molecular biology. *Plant Cell*, 7: 821-831.
11. Lambowitz AM, Sabourin JR, Bertrand H, Nickels R & McIntosh L (1989). Immunological identification of the alternative oxidase of *Neurospora crassa* mitochondria. *Molecular Cell Biology*, 9: 1362-1364.
12. Sakajo S, Minagawa N, Komiyama T & Yoshimoto A (1991). Molecular cloning of cDNA for antimycin A-inducible mRNA and its role in cyanide-resistant respiration in *Hansenula anomala*. *Biochimica et Biophysica Acta*, 1090: 102-108.
13. Clarkson AB, Bienen EJ, Pollakis G & Grady RJ (1989). Respiration of bloodstream forms of the parasite *Trypanosoma brucei brucei* is dependent on a plant-like alternative oxidase. *Journal of Biological Chemistry*, 264: 17770-17776.
14. Jarmuszkiewicz W, Wagner AM, Wagner JM & Hryniewiecka L (1997). Immunological identification of the alternative oxidase of *Acanthamoeba castellanii* mitochondria. *FEBS Letters*, 411: 110-114.
15. Rhoads DM & McIntosh L (1991). Isolation and characterization of a cDNA clone encoding an alternative oxidase protein of *Sauromatum guttatum* (Schott). *Proceedings of the National Academy of Sciences, USA*, 88: 2122-2126.
16. Moore AL & Siedow JN (1991). The regulation and nature of the cyanide-resistant alternative oxidase of plant mitochondria. *Biochimica et Biophysica Acta*, 1059: 121-140.
17. Siedow JN, Whelan J, Kearns A, Wiskich JT & Day DA (1992). Topology of the alternative oxidase in soybean mitochondria. In: Lambers H & van der Plas LHW (Editors), *Molecular, Biochemical and Physiological Aspects of Plant Respiration*. SPB Academic Publishing, The Netherlands, 19-27.
18. Moore AL, Umbach AL & Siedow JN (1995). Structure-function relationships of the alternative oxidase of plant mitochondria: a model of the active site. *Journal of Bioenergetics and Biomembranes*, 27: 367-377.
19. McIntosh L (1994). Molecular biology of the alternative oxidase. *Plant Physiology*, 105: 781-786.
20. Day DA & Wiskich J (1995). Regulation of alternative oxidase activity in higher plants. *Journal of Bioenergetics and Biomembranes*, 27: 379-385.
21. Wagner AM & Krab K (1995). The alternative respiration pathway in plants: role and regulation. *Physiologia Plantarum*, 94: 318-325.
22. Krab K (1995). Kinetic and regulatory aspects of the function of the alternative oxidase in plant respiration. *Journal of Bioenergetics and Biomembranes*, 27: 387-396.
23. Vanlerberghe GC & McIntosh L (1997). Alternative oxidase: from gene to function. *Annual Review of Plant Physiology*

and Molecular Biology, 48: 703-734.
24. Schonbaum GR, Bonner WD, Storey BT & Bahr JT (1971). Specific inhibition of the cyanide-insensitive respiratory pathway in plant mitochondria by hydroxamic acids. *Plant Physiology*, 47: 124-128.
25. Parrish DJ & Leopold AC (1978). Confounding of alternate respiration by lipoxygenase activity. *Plant Physiology*, 62: 470-472.
26. Siedow JN & Grivin ME (1980). Alternative respiratory pathway. Its role in seed respiration and its inhibition by propyl gallate. *Plant Physiology*, 65: 669-674.
27. Grover SD & Laties GG (1978). Characterization of the binding properties of disulfiram, an inhibitor of cyanide-resistant respiration. In: Ducet G & Lance C (Editors), *Plant Mitochondria*. Elsevier/North Holland Biomedical Press, Amsterdam, 259-266.
28. Huq S & Palmer JM (1978). Superoxide and hydrogen peroxide production in the cyanide resistant *Arum maculatum* mitochondria. *Plant Science Letters*, 11: 351-358.
29. Siedow JN (1982). The nature of the cyanide-resistant pathway in plant mitochondria. *Recent Advancements in Phytochemistry*, 16: 47-83.
30. Minagawa N, Sakajo S, Komiyama T & Yoshimoto A (1990). A 36-kDa mitochondrial protein is responsible for cyanide-resistant respiration in *Hansenula anomala*. *FEBS Letters*, 264: 149-152.
31. Siedow JN, Umbach AL & Moore AL (1995). The active site of the cyanide-resistant oxidase from plant mitochondria contains a binuclear iron center. *FEBS Letters*, 362: 10-14.
32. Umbach AL & Siedow JN (1993). Covalent and non-covalent dimers of the cyanide-resistant alternative oxidase protein in higher plant mitochondria and their relationship to enzyme activity. *Plant Physiology*, 103: 845-854.
33. Umbach AL & Siedow JN (1996). The reaction of the soybean cotyledon mitochondrial cyanide-resistant oxidase with sulfhydryl reagents suggests that α-keto acid activation involves the formation of a thiohemiacetal. *Journal of Biological Chemistry*, 271: 25019-25026.
34. Whelan J, Millar AH & Day DA (1996). The alternative oxidase is encoded in a multigene family in soybean. *Planta*, 198: 197-201.
35. Finnengan PM, Whelan J, Millar AH, Zhang Q, Smith K, Wiskich J & Day DA (1997). Differential expression of the multigene family encoding the soybean mitochondrial alternative oxidase. *Plant Physiology*, 114: 455-466.
36. Elthon TE, Nickels RL & McIntosh L (1989). Mitochondrial events during development of thermogenesis in *Sauromatum guttatum* (Schott). *Planta*, 180: 82-89.
37. Laties GG (1982). The cyanide-resistant, alternative pathway in plant mitochondria. *Annual Review of Plant Physiology*, 33: 519-555.
38. Minagawa N, Koga S, Nakano M, Sakajo S & Yoshimoto A (1992). Generation of superoxide anion detected by chemiluminescence method in the cyanide-insensitive mitochondria and the induction of the cyanide-resistant respiration in the yeast *Hansenula anomala*. In: Yagi H, Kondo M, Niki E & Yoshikawa T (Editors), *Oxygen Radicals*. Elsevier, New York, 203-206.
39. Minagawa N, Koga S, Nakano M, Sakajo S & Yoshimoto A (1992). Possible involvement of superoxide anion in the induction of cyanide-resistant respiration in *Hansenula anomala*. *FEBS Letters*, 302: 217-219.
40. Vanlerberghe GC & McIntosh L (1996). Signals regulating the expression of the nuclear gene encoding alternative oxidase of plant mitochondria. *Plant Physiology*, 111: 589-595.
41. Rhoads DM & McIntosh L (1992). Salicylic acid regulation of respiration in higher plants: alternative oxidase expression. *Plant Cell*, 4: 1131-1139.
42. Rhoads DM & McIntosh L (1993). Cytochrome and alternative pathway respiration in tobacco: Effects of salicylic acid. *Plant Physiology*, 103: 877-883.
43. Ribas-Carbo M, Lennon AM, Robinson SA, Giles L, Berry JA & Siedow JN (1997). The regulation of electron partitioning between the cytochrome and alternative pathways in soybean cotyledon and root mitochondria. *Plant Physiology*, 113: 903-911.
44. Vanlerberghe GC, Day DA, Wiskich JT, Vanlerberghe AE & McIntosh L (1995). Alternative oxidase activity in tobacco leaf mitochondria: dependence on tricarboxylic acid cycle-mediated redox regulation and pyruvate activation. *Plant Physiology*, 109: 353-361.
45. Umbach AL & Siedow AL (1997). Changes in the redox state of the alternative oxidase regulatory sulfhydryl/disulfide system during mitochondrial isolation: implications for inferences of activity *in vivo*. *Plant Science*, 123: 19-28.
46. Millar AH, Wiskich JT, Whelan J & Day DA (1993). Organic acid activation of the alternative oxidase of plant mitochondria. *FEBS Letters*, 329: 259-262.
47. Millar AH, Hoefnagel MHN, Day DA & Wiskich JT (1996). Specificity of the organic acid activation of alternative oxidase in plant mitochondria. *Plant Physiology*, 111: 613-618.
48. Day DA, Millar AH, Wiskich JT & Whelan J (1994). Regulation of alternative oxidase activity by pyruvate in soybean mitochondria. *Plant Physiology*, 106: 1421-1427.
49. Umbach AL, Wiskich JT & Siedow JN (1994). Regulation of alternative oxidase kinetics by pyruvate and intermolecular disulfide bond redox status in soybean seedling mitochondria. *FEBS Letters*, 348: 181-184.
50. Hryniewiecka L, Jenek J & Michejda J (1978). Cyanide resistance in soil amoeba *Acanthamoeba castellanii*. In: Ducet G & Lance C (Editors), *Plant Mitochondria*. Elsevier/North-Holland, Amsterdam, New York, Oxford, 307-314.
51. Hryniewiecka L & Michejda J (1988). In amoeba mitochondria phosphate mononucleotides stimulate specially the alternative respiratory pathway but not the rotenone-sensitive NADH dehydrogenase. *5th European Bioenergetic Conference Reports,* Aberystwyth, UK, 20-26 July 1998, 286 (Abstract).
52. Edwards SW & Lloyd D (1978). Properties of mitochondria isolated from cyanide-stimulated and cyanide-sensitive cultures of *Acanthamoeba castellanii*. *Biochemical Journal*, 174: 203-211.
53. Sharpless TK & Butow RA (1970). An inducible alternative terminal oxidase in *Euglena gracilis* mitochondria. *Journal of Biological Chemistry*, 245: 58-70.
54. Hanssens L & Verachtert H (1976). Adenosine 5'-monophosphate stimulates cyanide-insensitive respiration in mitochondria of *Moniliella tomentosa*. *Journal of Bacteriology*, 125: 829-836.
55. Vanderleyden J, Van den Eynde E & Verachtert H (1980). Nature of the effect of adenosine 5'monophosphate on the cyanide-insensitive respiration in mitochondria of *Moniliella tomentosa*. *Biochemical Journal*, 186: 309-316.
56. Doussiere J & Vignais PV (1984). AMP-dependence of the cyanide-insensitive pathway in the respiratory chain of *Paramecium tetraurelia*. *Biochemical Journal*, 220: 787-794.
57. Vanderleyden J, Peeters C, Verachtert H & Bertrandt H (1980). Stimulation of the alternative oxidase of *Neurospora crassa* by nucleoside phosphates. *Biochemical Journal*, 188: 141-144.

58. Sakajo S, Minagawa N & Yoshimoto Y (1997). Effects of nucleotides on cyanide-resistant respiratory activity in mitochondria isolated from antimycin A-treated yeast *Hansenula anomala*. *Bioscience of Biotechnology and Biochemistry,* 61: 397-399.
59. Vanderleyden J, Kurth J & Verachtert H (1979). Characterization of cyanide-insensitive respiration in mitochondria and submitochondrial particles of *Moniliella tamentosa*. *Biochemical Journal*, 182: 437-443.
60. Jarmuszkiewicz W, Wagner AM & Hryniewiecka L (1996). Regulation of the activity of the alternative oxidase in amoeba *A. castellanii* mitochondria. *Biochimica et Biophysica Acta*, *9th European Bioenergetic Conference Reports,* Louvain la Neuve, Belgium, 17-22 August 1996, 36 (Abstract C. V-5).
61. Li Q, Ritzel RG, McLean LLT, McIntosh L, Ko T, Bertrand H & Nargang FE (1996). Cloning and analysis of the alternative oxidase gene of *Neurospora crassa*. *Genetics*, 142: 129-140.
62. Ribas-Carbo M, Wiskich JT, Berry JA & Siedow JN (1995). Ubiquinone redox behaviour in plant mitochondria during electron transport. *Archives of Biochemistry and Biophysis*, 317: 156-160.
63. Bahr JT & Bonner WD (1973). Cyanide-insensitive respiration. I. The steady states of skunk cabbage spadix and bean hypocotyl mitochondria. *Journal of Biological Chemistry*, 248: 3441-3445.
64. Bahr JT & Bonner WD (1973). Cyanide-insensitive respiration. II. Control of the alternative pathway. *Journal of Biological Chemistry,* 248: 3446-3450.
65. Lambers H (1980). The physiological significance of cyanide-resistant respiration in higher plants. *Plant Cell and Environment*, 3: 293-302.
66. Lambers H (1982). Cyanide-resistant respiration: a non-phosphorylating electron transport pathway acting as an energy overflow. *Physiologia Plantarum*, 55: 478-485.
67. Moore AL, Dry IB & Wiskich JT (1988). Measurement of the redox state of the ubiquinone pool in plant mitochondria. *FEBS Letters*, 235: 76-80.
68. Dry JB, Moore AL, Day DA & Wiskich JT (1989). Regulation of alternative pathway activity in plant mitochondria: nonlinear relationship between electron flux and the redox poise of the quinone pool. *Archives of Biochemistry and Biophysics*, 273: 148-157.
69. Van den Bergen CWM, Wagner AM, Krab K & Moore AL (1994). The relationship between electron flux and the redox poise of the quinone pool in plant mitochondria. *European Journal of Biochemistry*, 226: 1071-1078.
70. Hoefnagel MHN & Wiskich JT (1996). Alternative oxidase activity and the ubiquinone redox level in soybean cotyledon and *Arum* spadix mitochondria during NADH and succinate oxidation. *Plant Physiology*, 110: 1329-1335.
71. Ikuma H, Schindler FJ & Bonner WD (1964). Kinetic analysis of oxidases in tightly coupled plant mitochondria. *Plant Physiology*, 39: S-1x.
72. Bendall DS & Bonner WD (1971). Cyanide-insensitive respiration in plant mitochondria. *Plant Physiology*, 47: 236-245.
73. Millar AH, Bergersen FJ & Day DA (1994). Oxygen affinity of terminal oxidases in soybean mitochondria. *Plant Physiology and Biochemistry*, 32: 847-852.
74. Barzu O & Satre M (1970). Determination of oxygen affinity of respiratory systems using oxyhemoglobin as oxygen donor. *Analytical Biochemistry*, 36: 428-433.
75. Rawsthorne S & LaRue TA (1986). Metabolism under microaerobic conditions of mitochondria from cowpea nodules. *Plant Physiology*, 81: 1097-1102.
76. Ribas-Carbo M, Berry JA, Azcon-Bieto J & Siedow JN (1994). The reaction of the plant mitochondrial cyanide-resistant alternative oxidase with oxygen. *Biochimica et Biophysica Acta*, 1188: 205-212.
77. Day DA, Krab K, Lambers H, Moore AL, Siedow JN, Wagner AM & Wiskich JT (1996). The cyanide-resistant oxidase: to inhibit or not to inhibit, that is the question. *Plant Physiology*, 110: 1-2.
78. Millar AH, Atkin OK, Lambers H, Wiskich JT & Day DA (1995). A critique of the use of inhibitors to estimate partitioning of electrons between mitochondrial respiratory pathways in plants. *Physiologia Plantarum*, 95: 523-532.
79. Guy RG, Berry JA, Fogel ML & Hoerinng TC (1989). Differential fractionation of oxygen isotopes by cyanide-resistant and cyanide-insensitive respiration in plants. *Planta*, 177: 483-491.
80. Guy RG, Berry JA, Fogel ML, Turpin DH & Weger HG (1992). Fractionation of the stable isotopes of oxygen during respiration by plants - the basis for a new technique. In: Lambers H & van der Plas LHW (Editors), *Molecular, Biochemical and Physiological Aspects of Plant Respiration*. SPB Academic Publishing, Hague, 442-453.
81. Robinson SA, Yakir D, Ribas-Carbo M, Giles L, Osmond CB, Siedow JT & Berry JA (1992). Measurements of the engagement of cyanide-resistant respiration in crassulacean acid metabolism plant *Kalanchoe daigremontiana* with the use of on-line oxygen isotope discrimination. *Planta*, 164: 415-422.
82. Robinson SA, Ribas-Carbo M, Yakir D, Giles L, Reuveni Y & Berry JA (1995). Beyond SHAM and cyanide: opportunities for studing the alternative oxidase in plant respiration using oxygen isotope discrimination. *Australian Journal of Plant Physiology*, 22: 487-496.
83. Ribas-Carbo M, Berry JA, Yakir D, Giles L, Robinson SA, Lennon AM & Siedow JN (1995). Electron partitioning between the cytochrome and alternative pathways in plant mitochondria. *Plant Physiology*, 109: 829-837.
84. Jarmuszkiewicz W, Sluse-Goffart CM, Hryniewiecka L, Michejda J & Sluse FE (1996). Determination of the respective contributions of the cytochrome and alternative oxidase pathways in *Acanthamoeba castellanii* mitochondria. In: Westerhoff HV, Snoep JL, Wijker J, Sluse FE & Kholodenko BN (Editors), *BioThermoKinetics of the Living Cell*. BTK Publishing, Amsterdam, The Netherlands, 31-34.
85. Jarmuszkiewicz W, Sluse-Goffart CM, Hryniewiecka L, Michejda J & Sluse FE (1998). Electron partitioning between the two branching quinol-oxidizing pathways in *Acanthamoeba castellanii* mitochondria during steady-state 3 respiration. *Journal of Biological Chemistry* (in press).
86. Lambowitz AM, Smith EW & Slayman CW (1972). Oxidative phosphorylation in *Neurospora* mitochondria. Studies on wild-type, *poky* and chloramphenicol induced wild type. *Journal of Biological Chemistry,* 247: 4859-4865.
87. Meese BJD (1975). Thermogenic respiration in aroids. *Annual Review of Plant Physiology*, 26: 117-126.
88. Purvis AC & Shewfelt RL (1993). Does the alternative pathway ameliorate chilling injury in sensitive plant tissues? *Physiologia Plantarum*, 88: 712-718.
89. Purvis AC (1997). Role of the alternative oxidase in limiting superoxide production by plant mitochondria. *Physiologia Plantarum*, 100: 165-170.
90. Kromer S (1995). Respiration during photosynthesis. *Annual Review of Plant Physiology and Molecular Biology*, 46: 45-70.