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Effect of pH on CN-resistant respiratory activity and regulation on *Vigna uniguiculata* mitochondria

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Abstract – Mitochondria from *Vigna unguiculata* cv. Vita 5 have a cyanide insensitive alternative oxidase cross-reacting with a monoclonal antibody raised against the alternative oxidase of *Sauromatum guttatum*. In the presence of NADH as substrate and dithiothreitol, the CN-resistant respiration of *V. unguiculata* intact mitochondria was found to be significantly influenced by assay pH with an optimum value of 6.25. This effect was still observed when nigericin, known to abolish ΔpH between the matrix and the intermembrane space, was present in the reaction medium. This pH effect was shown to be reversible. Alternative oxidase activation by pyruvate, also appeared to be pH dependent with an optimum pH value of 7.25. This modulation of the alternative oxidase activity by pH has been tentatively attributed to a new protonated-deprotonated status of this protein.

alternative oxidase / AOX / cyanide resistant respiration / mitochondria / pH-dependence / pyruvate / *Vigna unguiculata*

AOX, alternative oxidase / DTT, dithiothreitol

1. INTRODUCTION

All plants are known either to possess or to be potentially able to develop a second path of electron transport, branching from the main mitochondrial respiratory chain at the ubiquinone level [4, 23]. The electron flux through the alternative pathway, mediated by alternative oxidase, depends on the level of reduction of the ubiquinone pool [12, 19]. As it bypasses complexes III and IV of the cytochrome chain, the alternative path does not produce a proton-electrochemical gradient, causing dissipation of the redox energy as heat.

Apart from its function in the process of thermogenesis in aroid species, the physiological role of the alternative oxidase (AOX) in plant metabolism still remains not well defined [29]. The alternative oxidase has been related to redirection of carbon metabolism through the control of intracellular concentration of key respiratory intermediates [30] and to the energy overflow hypothesis [16]. However, according to more recent results this hypothesis should be reevaluated [3, 17]. Furthermore, the AOX activity has been studied in connection with several treatments promoting plant stress [28, 32]. Recently, different isoforms of AOX have been shown to be encoded by a multigene family [8, 24]. The existence of a tissue-dependent expression of AOX subunits suggests a broad biochemical mechanism of metabolic control exerted by AOX during plant growth and development [2, 9, 11, 23, 26].

So far, two biochemical mechanisms, based on regulatory cysteines conserved in the plant AOX sequences, have been proposed to explain the regula-

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tion of the AOX activity. First, the activation of the AOX enzyme, existing in the inner mitochondrial membrane as a non-covalently linked dimer or as a covalently linked one, undergoes reduction of the disulfide bond that bridges regulatory cysteines of the two subunits of the enzyme homodimer [27, 31]. Second, the AOX activity is stimulated by α-keto acids, such as pyruvate [18, 30], involving the formation of a thiohemiacetal by a reaction at a sulfhydryl residue of a conserved cysteine [27, 31]. The reduced form of the enzyme is more sensitive to stimulation by pyruvate [27]. Lately, another mechanism has been proposed in which it is suggested that AOX is inactivated by its products (oxidized ubiquinone) during catalysis and this inhibition is prevented by pyruvate [12]. The AOX mechanisms will certainly be reexamined according to the new proposed structure of the AOX protein whose active site has a possible membrane binding motif [1].

Concerning the effect of pH on AOX activity, optimal values of 6.8 and 7.0 have been determined for solubilized AOX from *Sauromatum guttatum* [5] and *Arum italicum* [13], respectively. With intact hypocotyl mitochondria from *Vigna radiata*, only a slight effect of pH on CN-insensitive O_2 uptake has been observed with an optimum between 6.4 and 6.8 [6]. In contrast, in this study, using intact mitochondria from hypocotyls of a cultivar of *V. unguiculata*, Vita 5, known to be susceptible to water/salt stress treatments [7], evidence is presented of a strong pH dependence of the CN-resistant respiration (optimum pH 6.25) with an optimum for pyruvate activation at pH 7.25.

2. RESULTS AND DISCUSSION

2.1. Immunodetection of AOX

Immunological analysis reveals the existence in mitochondria of Vita 5 of a predominant higher molecular mass species of the oxidase (70 kDa) and a lower molecular mass band (35 kDa) detected in the absence of DTT and at the three pH values (*figure 1*), corresponding to the oxidized and reduced forms respectively. When DTT was present in the reaction medium, only one band was detected corresponding to the reduced form (*figure 1*). When mitochondria were incubated with NADH, DTT and KCN, almost all AOX proteins were also detected in their reduced form (data not shown). Apparently, the pH had no direct effect on the redox state of AOX because the protein was always under the reduced form at the three different pHs (*figure 1*). These results are comparable

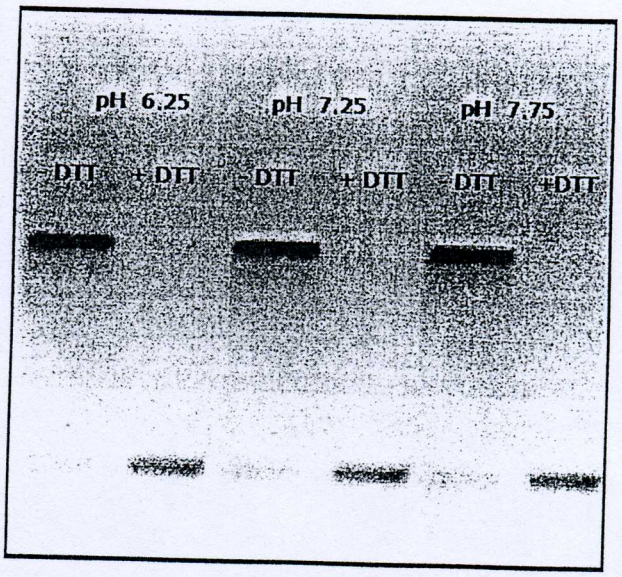

Figure 1. Immunoblot analysis of total mitochondrial proteins from *V. unguiculata* cv. Vita 5 hypocotyls' purified mitochondria at three different pHs. Samples of mitochondria (300 µg) were previously incubated in a reaction medium in the absence or in the presence of 2.5 mM DTT as described in Methods. The amount of protein loaded in each lane was 100 µg.

to those obtained with mitochondria from other plants, e.g. from soybean and *V. radiata* [29], and they showed that after mitochondrial isolation AOX is mainly a covalent oxidized dimer.

2.2. Effect of assay pH on CN-resistant respiration

The alternative oxidase mediated respiration in purified mitochondria of *V. unguiculata* has been measured with exogenous NADH as an oxidizable substrate and subsequent additions of DTT and KCN (*figure 2*). The highest CN-resistant respiration (78 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein) is observed with a lower pH (6.25) value (*figure 2A*) as compared to the rates of oxygen uptake obtained with higher pH values (i.e. 25 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein at pH 7.25 and 13 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein at pH 7.75) (*figure 2B, C*). A similar influence of assay pH on the CN-resistant respiration activity was demonstrated in the presence of antimycin A with the same values of pH (6.25, 7.25, and 7.75) in the reaction medium. As in the presence of KCN, the highest antimycin A-resistant respiration (*figure 3*) was found at lower pH value (6.25). This similarity indicates that the effect of pH on the CN-resistant respiration results from pH action and

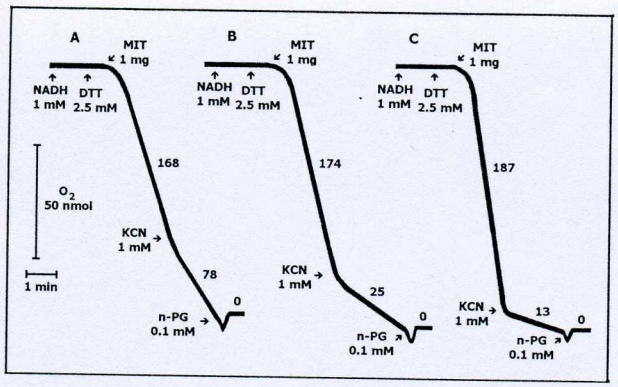

Figure 2. Effect of pH on the CN-resistant respiration. Oxygen uptake measurements were performed in the reaction medium with different pH values: 6.25 (**A**), 7.25 (**B**) and 7.75 (**C**). One mM NADH was used as a respiratory substrate in the presence of 2.5 mM DTT. Subsequent additions: 1 mM KCN and 0.1 mM n-propylgallate (n-PG). Numbers along the traces refer to rates of O_2 uptake (nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein). Representative results are shown.

could not be attributed to a decrease in the concentration of CN^-, due to the high pK value of KCN (equal to 9.3). As total O_2 uptake with NADH as substrate and DTT at the three pH values were approximately the same (*figures 2, 3*), this strongly favors the interpretation that the different pHs did not affect the Q redox state. Previously, it has been shown that NADH produces high levels of reduction of the Q-pool and a consequent AOX activation [12].

Previous work on pH measurements in soybean mitochondria had revealed that matrix pH values were kept closer to those of the reaction medium [21]. In order to have a better understanding of the pH effect on the CN-resistant respiration, this effect was investigated when the matrix pH was rendered equal to that of the intermembrane space. For this purpose, nigericin (an ionophore known to permit the entry of H^+ for K^+ exchange across the mitochondrial inner membrane, promoting ΔpH collapse) was used in the reaction medium. The CN-resistant respiration rates thus obtained were similar to those in the absence of nigericin (*table I*).

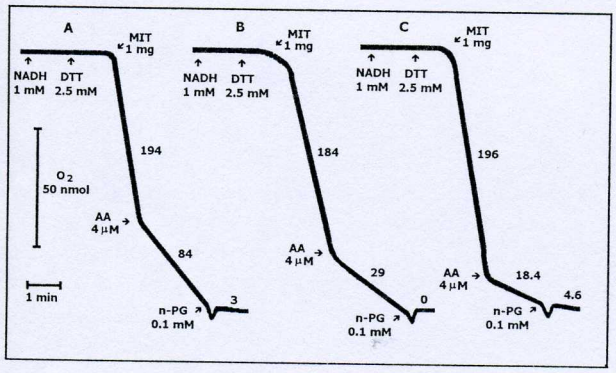

Figure 3. Effect of pH on the antimycin A (AA)-resistant alternative respiration. Experimental conditions were identical to those in *figure 2* except that KCN was replaced by 4 µM antimycin A.

Table I. Effect of pH on the CN-resistant respiration. Influence of nigericin. Activity of CN-resistant respiration was measured in the presence of 1 mM KCN with and without 100 ng nigericin·mg^{-1} mitochondrial protein. The percent values were calculated in relation to the O_2 uptake in the presence of 1 mM NADH and 2.5 mM DTT.

	pH 6.25		pH 7.25		pH 7.75	
Nigericin	+	–	+	–	+	–
% CN-resistant respiration	53	46	13	14	7	7

The effect of assay pH on the CN-resistant respiration was investigated in a pH range of 5.75 to 8.25 (*figure 4*). The highest rate of the CN-resistant respiration (68 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein) was obtained at pH 6.25. Increase in pH over values of 6.25 and decrease in pH below 6.25 resulted in a progressive decrease in the oxygen uptake.

2.3. Reversibility of assay pH effect on CN-resistant respiration

Oxygen uptake of the CN-resistant respiration was measured in the presence of NADH, DTT and KCN

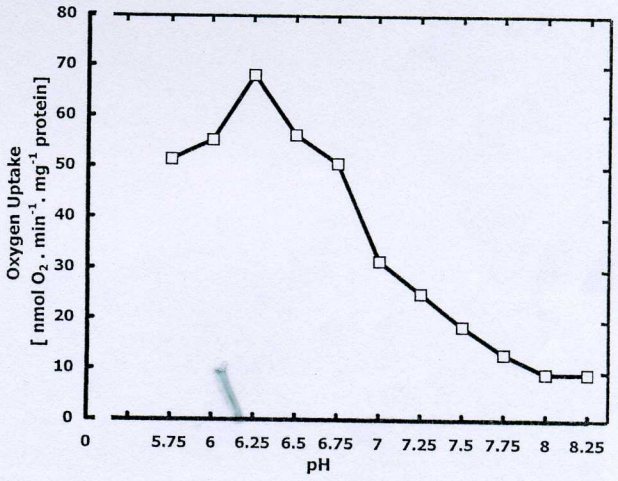

Figure 4. Determination of the pH-dependence of the KCN-resistant respiration over a pH range of 5.75 to 8.25. Oxygen uptake (nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein) was measured in the reaction medium with different pH values, with 1 mg mitochondrial protein in the presence of 1 mM NADH, 2.5 mM DTT and 1 mM KCN.

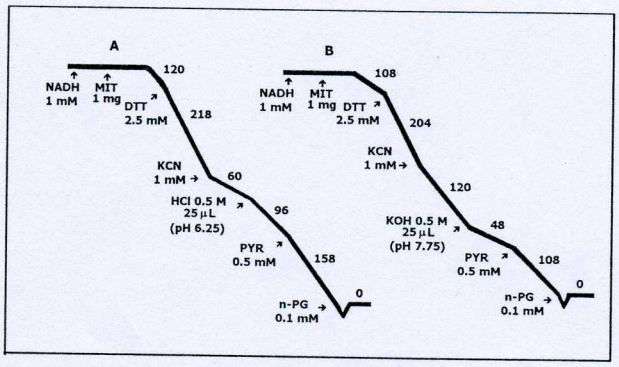

Figure 5. Effect of pH reversibility on CN-resistant respiration in the presence or in the absence of pyruvate. **A**, Initial pH value of 7.75 in the reaction medium reversed with HCl to pH 6.25; **B**, initial pH value of 6.25 in the reaction medium reversed with KOH to pH 7.75. Numbers along the traces refer to rates of O_2 uptake (nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein).

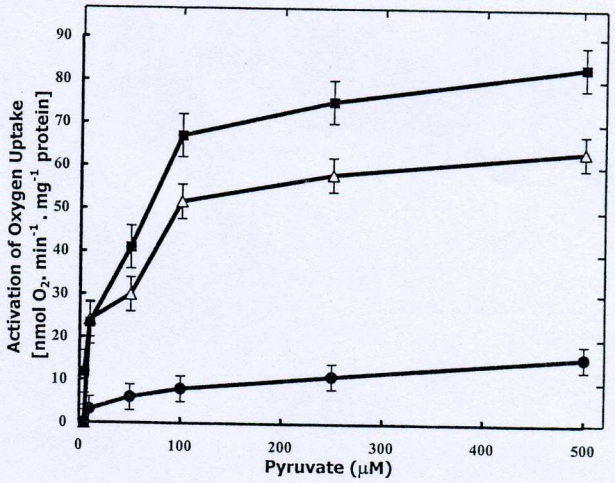

Figure 6. Effect of pH on pyruvate activation of the CN-resistant respiration. Oxygen uptake (nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$protein) was measured in the reaction medium with different pH values: (△), 6.25; (■), 7.25; (●), 7.75, with 1 mg mitochondrial protein in the presence of 1 mM NADH, 2.5 mM DTT and 1 mM KCN.

(*figure 5*). The lowest CN-resistant respiratory rates obtained at pH 7.75, increased when a decrease of pH (6.25) was induced in the reaction medium (*figure 5A*). In contrast, when the initial pH was at 6.25, the highest CN-resistant respiratory rates decreased when the value of pH was increased to 7.75 in the reaction medium (*figure 5B*). This prompt reversibility of the pH effect on CN-resistant respiration strongly suggests a physiological role for such modulation.

2.4. Pyruvate activation on CN-resistant respiration at different pHs

The activation of CN-resistant respiration by pyruvate at different pHs was studied in the presence of DTT. *Figure 6* shows the stimulatory effect of increasing concentration of pyruvate (0–500 µM) on the CN-resistant respiration, expressed as the difference in O_2 uptake values in the presence or absence of pyruvate at pH 6.25, 7.25, and 7.75. The curves presented a hyperbolic shape and attained a plateau between 300 to 500 µM pyruvate. At these values the highest stimulation was observed at pH 7.25 (300 %) followed by 100 and 80 %, respectively at 7.75 and 6.25 pH values.

The activation of CN-resistant respiration by pyruvate exhibited a pH optimum around neutrality and has to be considered with caution because pyruvate stimulates AOX from the matrix side of mitochondria [16] and because pyruvate transport is a pyruvate + H^+ symport [26]. Thus, at the equilibrium of pyruvate distribution between both mitochondrial compartments, the ratio of the internal-pyruvate concentration over the external pyruvate concentration is equal to the ratio between the external-H^+ concentration over the internal-H^+ concentration. Data (reported in *figure 6*) as a function of the external-pyruvate concentration give a relevant presentation if $[H^+]_{out}/[H^+]_{in}$ is not pH dependent (i.e. if ΔpH is constant whatever the external pH) so $[pyr]_{in}/[pyr]_{out}$ can also be assumed to be pH independent. On the other hand, if the internal pH is considered as constant whatever the external pH, data should be better analyzed when plotted as a function of $([pyr]_{out} \times [H^+]_{out})$. Such a plot (not shown) leads to the same conclusion, i.e. the stimulation by pyruvate is clearly higher at pH 7.25 compared to pH 7.75 and 6.25. Thus, it can be proposed safely that the reduced AOX dimer (E_{red}) has two protonated forms (H^+E_{red} and $H^+H^+E_{red}$) and that H^+E_{red} is more readily activatable by pyruvate compared with E_{red} and $H^+H^+E_{red}$. This more efficient activation of H^+E_{red} suggests that its affinity for pyruvate is higher and/or that this single-protonated form has the highest activity ratio: pyr-loaded AOX/pyr-free AOX.

In these conditions, it appears that the AOX activity is probably not only under the influence of the redox status of the dimer, but also of its protonated status according to *figure 7* in which the most active form is H^+E_{red}-pyr followed by H^+E_{red}.

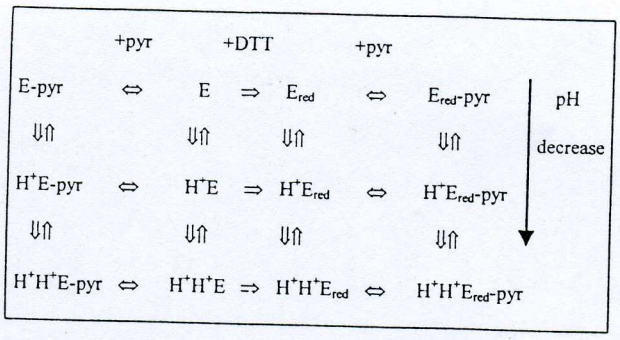

Figure 7. Schematic model proposed for AOX activity. E, enzyme (AOX); E_{red}, AOX reduced dimer; E_{red}-pyr, AOX reduced dimer pyruvate-loaded; H^+E_{red} and $H^+H^+E_{red}$, two protonated forms of reduced AOX dimer; H^+E_{red}-pyr and $H^+H^+E_{red}$-pyr, two protonated forms of reduced AOX dimer pyruvate-loaded.

3. CONCLUSION

The level of alternative oxidase in plant mitochondria is known to increase in response to several developmental, environmental and chemical signals [9, 22]. In the present study, CN-resistant respiration of mitochondria from *V. unguiculata* has been shown to vary in response to a pH signal, when maximal rates were detected at a pH optimal value of 6.25 which shifted to 7.25 during its activation by pyruvate. Such pH effect on alternative oxidase activity has been tentatively attributed to a new protonated-deprotonated status of this protein. The pH signal on alternative oxidase activity might occur in response to exposure of plant tissues to different stress conditions. Identification of these signals could contribute to a better understanding of the physiological role of the alternative mitochondrial electron transport pathway.

4. METHODS

4.1. Plant material

Seeds of Vita 5, a cultivar of *Vigna unguiculata* (L.) Walp, known to be a susceptible to salt stress [7], were obtained from the seed bank of the Departamento de Fitotecnia, Universidade Federal do Ceará, Fortaleza, Ceará, Brasil. Seeds were surface sterilized for 5 min in 0.5 % NaOCl, rinsed with water, and germinated in the dark, at 28 °C, in plastic pots containing vermiculite soaked with distilled water.

4.2. Isolation and purification of mitochondria

Mitochondria were isolated from hypocotyls of fresh plants. Hypocotyls (800 g) were cut into 1.5 L cold extraction medium containing 0.3 M mannitol 0.1 % BSA (w/v), 4 mM cysteine, 0.1 M EDTA and 70 mM K_2HPO_4, the pH being adjusted to 8.0. After homogenization in a mixer (Walita), the homogenate was filtered through a 150-μm nylon net. The hypocotyl fragments remaining in the net were again blended in the mixer with 1.5 L extraction medium. Afterwards, the homogenization and filtration steps were repeated as described above. The two successive filtrates were pooled and submitted to differential centrifugation according to Silva Lima and Pinheiro [25]. The washing medium contained 0.3 M mannitol, 10 mM KH_2PO_4 (pH 7.2) and 0.1 % BSA (w/v). The purification of the mitochondria was performed as previously described by Jolivet et al. [14] and only mitochondria > 95 % intact, as judged by their impermeability to cytochrome c, were used [20].

4.3. Eletrophoresis and western blotting analysis

SDS-PAGE was carried out in a Bio-Rad Mini-PROTEAN II cell. Samples of purified mitochondria (300 μg) were previously incubated for 15 min in 0.9 mL reaction medium (pH 7.25, without BSA) in the absence or in the presence of 2.5 mM DTT at room temperature with gentle shaking. Then, mitochondria were pelleted in a microcentrifuge for 10 min at 4 °C. The mitochondrial pellets were resuspended in a sample buffer (2 % SDS (w/v), 2 % glycerol), 500 mM Tris-HCl (pH 6.8), and 0.05 % (w/v) bromophenol blue tracking dye) and boiled for 3 min. Electrophoresis of these samples (100 μg protein loaded for each lane) was carried out with the buffer system of Laemmli [15] using a 4 % (w/v) stacking gel and a 12 % (w/v) polyacrylamide resolving gel. After 4 h protein transfer (200 mA), the nitrocellulose membrane was probed with a 1:100 dilution of a monoclonal antibody raised against the alternative oxidase proteins of *Sauromatum guttatum* (Alternative Oxidase monoclonal antibodies from GT Monoclonal Antibodies GTMA). Visualization was revealed with diaminobenzidine tetrahydrochloride/$NiCl_2$/H_2O_2 solution.

4.4. Assay procedures

Oxygen uptake was determined with a Clark electrode at 26 °C and performed in 1.9 mL reaction medium containing 0.3 M mannitol, 6 mM $MgCl_2$, 10 mM KCl, 10 mM MOPS, 10 mM KH_2PO_4 and 0.1 % BSA. pH values of the reaction medium, adjusted according to experimental need, are indicated in the legends of the corresponding figures. Oxygen uptake

was measured with 1 mg mitochondrial protein. Exogenous NADH (1 mM) was used as a respiratory substrate in the presence of 2.5 mM DTT. KCN (1 mM) or antimycin A (4 μM) and n-propylgallate (100 μM) were used as inhibitors of the cytochrome pathway and of the alternative pathway, respectively. Nigericin (13 nM), an antibiotic, was used to abolish ΔpH. For pH reversibility studies, 0.5 N KOH or 0.5 N HCl were reciprocally added in the assay reaction medium and are indicated in the legends of the corresponding figures. Protein concentration was determined by the procedure of Gornall et al. [10].

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