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The Effect of pH on the Alternative Oxidase Activity in Isolated *Acanthamoeba castellanii* Mitochondria

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Mitochondria of *Acanthamoeba castellanii* possess a cyanide-resistant GMP-stimulated ubiquinol alternative oxidase in addition to the cytochrome pathway. In a previous work it has been observed that an interaction between the two ubiquinol-oxidizing pathways exists in intact *A. castellanii* mitochondria and that this interaction may be due to a high sensitivity of the alternative oxidase to matrix pH. In this study we have shown that the alternative oxidase activity reveals a pH-dependence with a pH optimum at 6.8 whatever the reducing substrate may be. The GMP stimulation of alternative oxidase is also strongly dependent on pH implicating probably protonation/deprotonation processes at the level of ligand and protein with an optimum pH at 6.8. The ubiquinone redox state-dependence of alternative oxidase activity is modified by pH in such a way that the highest activity for a given ubiquinone redox state is observed at pH 6.8. Thus pH, binding of GMP, and redox state of ubiquinone collaborate to set the activity of the GMP-stimulated alternative oxidase in isolated *A. castellanii* mitochondria. The high pH sensitivity of the alternative oxidase could link inactivation of the cytochrome pathway proton pumps to activation of the alternative oxidase with acceleration of redox free energy dissipation as a consequence.

KEY WORDS: Mitochondria; alternative oxidase; pH-dependence; *Acanthamoeba castellanii.*

INTRODUCTION

The mitochondrial respiratory chain of a nonphotosynthetic amoeboid protozoon *Acanthamoeba castellanii* possesses a cyanide- and antimycin-resistant ubiquinol alternative oxidase in addition to the cytochrome *c* oxidase (Edwards and Lloyds, 1978; Hryniewiecka *et al.*, 1978). Amoeba *A. castellanii* and higher plants share other common features at the level of the respiratory chain such as a presence of the nonproton pumping rotenone-insensitive internal (matrix side) and external (cytosolic side) NADH dehydrogenases (Hryniewiecka *et al.*, 1978). As in higher plant mitochondria, the alternative pathway of amoeba mitochondria branches from the main respiratory chain at the level of ubiquinone (Q), and so the electron flux through alternative oxidase is not coupled to ADP phosphorylation. While in plant mitochondria, the activity of alternative oxidase is stimulated by α-keto acids and regulated by the redox state of the enzyme (Valenberghe and McIntosh, 1997), these regulatory properties do not concern the alternative oxidase in amoeba mitochondria (Jarmuszkiewicz *et al.*, 1997). The amoeba cyanide-resistant alternative oxidase is strongly stimulated by purine nucleoside 5′-monophosphates AMP, GMP (the lowest Km of stimulation) and IMP (Edwards and Lloyds, 1978; Hryniewiecka *et al.*, 1978). A similar effect of purine mononucleotides on the cyanide-resistant alternative pathway was observed in other microorganisms: *Euglena gracilis* (Sharples and Butov, 1970), *Moniliella*

Key to abbreviations: AA, antimycin A; BHAM, benzohydroxamate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; state U, uncoupled respiration; state 3, phosphorylating respiration in the presence of added ADP; state 4, resting respiration in the absence of added ADP; Q, ubiquinone; Qred/Qtot, reduction level of ubiquinone (reduced ubiquinone versus total ubiquinone).

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tomentosa (Hanssens and Verachtert, 1976), *Neurospora crassa* (Vanderleyden *et al.*, 1980), *Paramecium tetraurelia* (Doussiere and Vignais, 1984), and *Hansemula anomala* (Sakajo *et al.*, 1997; Umbach and Siedow, 2000). Monoclonal antibodies developed against *Sauromatum guttatum* cross-react with the alternative oxidase protein of *A. castellanii* mitochondria (Jarmuszkiewicz *et al.*, 1997), as they do in a wide range of thermogenic and nonthermogenic plant species, some fungi, and trypanosomes (Valenberghe and McIntosh, 1997), indicating that this protein is well conserved throughout the species.

Besides the important difference at the level of regulation, the alternative oxidases of plants and microorganisms (several fungi and protozoa) differ at the level of their molecular organization (Joseph-Horne, 2001; Siedow and Umbach, 2000). The primary sequences of the plant and fungal alternative oxidases differ mainly in their N terminus. On the basis of protein sequence analysis and cross-linking studies, it is proposed that the plant oxidase is dimeric and that the fungitype oxidase is monomeric (Siedow and Umbach, 2000). There are two structural models that can be applied to the plant and fungitype oxidases. In the Siedow–Umbach–Moore model, the alternative oxidase is an integral membrane protein with two transmembrane helices and part exposed into the intermembrane space (Siedow *et al.*, 1995). In a more recent, now widely accepted model (the Anderson–Nordlund model), the alternative oxidase is an interfacial membrane protein (matrix phase) without any external part (Albury *et al.*, 2002; Andersson and Norlund, 1999; Berthold *et al.*, 2000).

Recently, it has been observed that kinetic behavior of the alternative oxidase is influenced by the cytochrome pathway activity and vice versa in *A. castellanii* mitochondria (Jarmuszkiewicz *et al.*, 2002). This newly observed type of interplay between the two ubiquinol-oxidizing pathways could be explained either by a direct interaction or by an indirect connection (not mutually exclusive) between the two pathways. Considering the indirect connection, activation of the alternative oxidase in the cyanide-inhibited mitochondria at fixed QH_2 poise has been proposed to be due to a high sensitivity of the alternative oxidase to the matrix pH which is lower when the cytochrome pathway proton pumps are inactive. This link between the two ubiquinol-oxidizing pathways may be important for the cell physiology. Thus, it is particularly relevant to study the pH-sensitivity of *A. castellanii* alternative oxidase and the mechanisms responsible for this sensitivity.

The aim of this work is to determine the pH-sensitivity of the cyanide-resistant (or antimycin-resistant) respiration, sustained by the alternative oxidase activity, in intact isolated *A. castellanii* mitochondria using different oxidizable substrates. This is the first attempt to determine pH-dependence of the nonplant GMP-stimulated alternative oxidase. We show that the pH optimum of the alternative oxidase-mediated respiration is lower than that of the cytochrome pathway and also that pH-dependence of the alternative oxidase activity is directly related to the binding of GMP on the alternative oxidase and to the redox state of ubiquinone.

MATERIAL AND METHODS

Cell Culture and Mitochondrial Isolation

Soil amoeba *A. castellanii*, strain Neff, was cultured as described by Jarmuszkiewicz *et al.* (1997). Trophozoites of amoeba were collected 48 h following inoculation at the late exponential phase (at density 5–6 $\times$ 10^6 cells/mL). Mitochondria were isolated and purified on a self-generating Percoll gradient (31%) as described previously (Jarmuszkiewicz *et al.*, 1997). Protein concentration was estimated by the biuret method (Gornall *et al.*, 1949).

Oxygen Uptake

Oxygen uptake was measured polarographically with a Rank Brothers (Cambridge, United Kingdom) oxygen electrode in 3 mL of the medium (25°C) containing 120 mM KCl, 10 mM Tris/Cl, 5 mM MOPS, 5 mM MES, 3 mM KH_2PO_4, 8 mM $MgCl_2$, and 0.2% BSA, with 1–2 mg of mitochondrial protein. The pH values of the reaction medium were adjusted according to experimental need. Exogenous NADH (1 mM), succinate (10 mM) plus rotenone (15 μM) plus 0.17 mM ATP, and malate (10 mM) were used as respiratory substrates. KCN (1.5 mM) or antimycin A (AA, 1.5 μg/mg of mitochondrial protein) and benzohydroxymate (BHAM, 1.5 mM) were used as inhibitors of the cytochrome pathway and of alternative oxidase, respectively. To fully activate the alternative oxidase, 0.8 mM GMP was added to the incubation medium. Measurements were performed in the absence (state 4) or presence (state 3) of 1.5 mM ADP or in the presence of 1.5 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, uncoupled state, state U). Values of O_2 uptake are presented in nmol $O \times min^{-1}$ mg^{-1} protein.

Redox State of Ubiquinone

The redox state of ubiquinone (Q) in steady-state respiration was determined by an extraction technique as

described previously (Van den Bergen *et al.*, 1994). To decrease the ubiquinone redox state (Qred/Qtot), increasing concentration (10–30 mM) of *n*-butyl malonate (an inhibitor of succinate uptake) or decreasing concentration of NADH in the presence of enzymatic regenerating system (Hoefnagel and Wiskich, 1996) was used, when succinate or external NADH was reducing substrates, respectively.

RESULTS

The influence of assay pH on amoeba *A. castellanii* respiration was studied in a pH range from 6.2 to 8.2 with respiratory substrates that differ in topology of electron input into ubiquinone (i.e., from complex I or internal rotenone-insensitive NADH dehydrogenase with malate, from complex II with succinate (plus rotenone), and from external NADH dehydrogenase with exogenous NADH). The pH-dependence of benzohydroxamate (BHAM)-resistant, cyanide-sensitive respiration, representing the cytochrome pathway-mediated respiration was determined with mitochondria respiring in resting state (state 4, in the absence of added ADP), in phosphorylating state (state 3, in the presence of ADP), and in uncoupled state when the mitochondrial, electrochemical gradient was collapsed by a protonophore, FCCP. For each type of substrate, as shown in Fig. 1 with succinate (plus rotenone) as an example, almost the same pH-dependent respiratory rate profile was observed in the phosphorylating and uncoupled states, indicating that the electron flux is essentially controlled by the respiratory chain capacity at each tested pH. Profiles of states 4 and 3 respiration versus pH are such that respiratory control ratio remains constant (2.56 ± 0.1, SD, $n = 7$) within the pH range. These observations mean that the pH-dependence observed for the BHAM-resistant cyanide-sensitive respiration characterizes mainly the cytochrome pathway as it is independent of the electron source (i.e., reducing paths through different dehydrogenases) and the energized state of mitochondria. The highest cytochrome pathway-mediated respiration was reached with external NADH, while the lowest was observed with malate (data not shown) confirming our previous results obtained at pH value 7.4 (Jarmuszkiewicz and Hryniewiecka, 1994). In this study, a single pH optimum (at pH 7.4) was observed for all substrates and all respiratory states, indicating that the pH optimum characterizes the cytochrome pathway activity (UQH_2-O_2 span) only.

The effect of assay pH on the alternative oxidase-mediated respiration was measured in the presence of cyanide both in the absence or in the presence of GMP, an activator of the amoeba alternative oxidase. In the presence of cyanide, the internal (matrix) pH is expected to be very

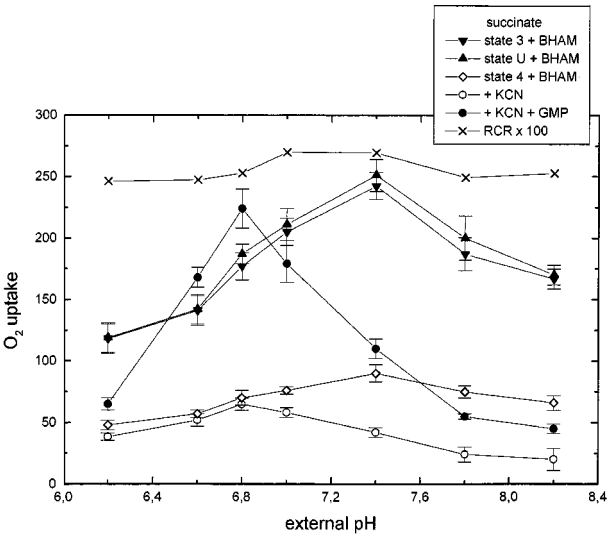


Fig. 1. pH-dependence profile of cyanide-resistant respiration and BHAM-resistant respiration in amoeba mitochondria. Respiration with 10 mM succinate (plus rotenone) in state 4 (no ADP), in state 3 (in the presence of 1.5 mM ADP), or in uncoupled state (state U, in the presence of 1.5 μM FCCP) was inhibited by 1.5 mM BHAM or 1.5 mM KCN. To activate the alternative oxidase, 0.8 mM GMP was added where indicated. Respiratory control ratio (RCR, state 3/state 4 respiration) is given as $\times 100$. Values of O_2 uptake are presented in nmol O $\times$ min^{-1} mg^{-1} protein. Each set of data represents the mean of 3–5 experiments.

close to the external (assay) pH as the H^+ pumps of the cytochrome pathway are inactive. Thus, the assay-pH dependence of the cyanide-resistant respiration (the alternative oxidase activity) could actually represent the sensitivity of alternative oxidase to matrix pH. As shown for succinate (plus rotenone) as an example (Fig. 1), for all three substrates (data not shown), the pH optimum of the unstimulated and GMP-stimulated alternative oxidase-mediated respiration was at 6.8. This identical pH optimum with the three substrates means that it is a property of the ubiquinol oxidizing step, i.e., alternative oxidase only. To exclude the possibility that pH effect on the cyanide-resistant alternative oxidase-mediated respiration could be partly related to a decrease in the CN^- concentration, because of a high pKa value of KCN (equal to 9.3), another inhibitor of the cytochrome pathway (i.e. antimycin A) was used. As in cyanide-resistant respiration, both unstimulated and GMP-stimulated AA-resistant respiration with succinate (plus rotenone) as an example (Table I) revealed the highest activity at pH 6.8. Thus, the pH optimum of the alternative oxidase (6.8) is actually lower than the pH optimum of the cytochrome pathway (7.4).

As shown in Fig. 2, stimulation of the cyanide-resistant alternative oxidase-mediated respiration by GMP is strongly influenced by the assay pH whatever the

Table I. Effect of Assay pH on Antimycin-Resistant Respiration with Succinate (Plus Rotenone) as an Oxidizable Substrate. Succinate (10 mM) Oxidation was Inhibited by 1.5 μg/mg of Mitochondrial Protein Antimycin A in the Absence or Presence of 0.8 mM GMP. Values of O_2 Uptake are Presented in nmol O $\times$ min^{-1} mg^{-1} protein. Data are Mean $\pm$ SD of Three Experiments

	Assay pH		
Antimycin-resistant respiration	6.2	6.8	7.4
−GMP	35 ± 4	63 ± 2	38 ± 6
+GMP	70 ± 5	302 ± 12	91 ± 8

reducing substrate may be. For every substrate, the highest effect of GMP was observed at the same pH (6.8) as the pH optimum observed with succinate for cyanide-resistant respiration (±GMP) (Fig. 1). This important observation indicates that the binding of GMP, that is responsible for allosteric stimulation, is not independent of pH. An increase in the binding for pH higher than 6.2 could be due to the deprotonation of the second hydroxyl of the phosphomonoester and a decrease in binding for pH higher than 6.8 could be due to changes in protonation of the GMP-binding site of the protein.

Activity of the amoeba alternative oxidase depends upon the Q reduction level and increases sharply for Qred/Qtot ratio higher than 50% (Jarmuszkiewicz *et al.*, 1998). Figure 3 shows the influence of Q redox state on the cyanide-resistant GMP-stimulated steady-state rate respiration at three pH values (6.2, 6.8, and 7.4) when Q redox state was decreased by increasing the concentration

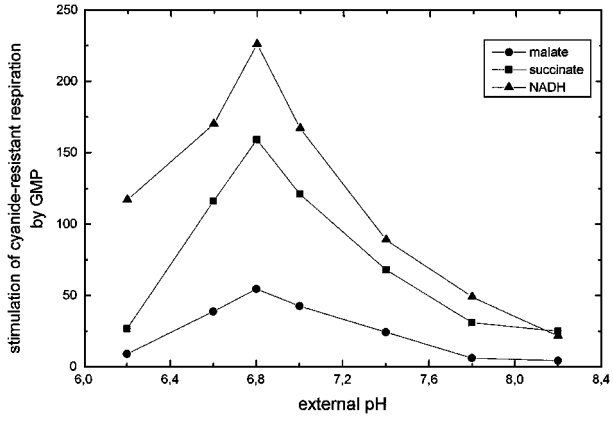


Fig. 2. Effect of assay pH on stimulation of cyanide-resistant respiration by GMP with different substrates. Substrates: 10 mM malate, 10 mM succinate (plus rotenone), 1 mM NADH. Respiration was inhibited by 1.5 mM cyanide in the absence or presence of 0.8 mM GMP. Effect of GMP on the alternative oxidase activity at different assay pH value presented as the difference between the cyanide-resistant respiration in the presence of GMP minus the cyanide-resistant respiration in the absence of activator.

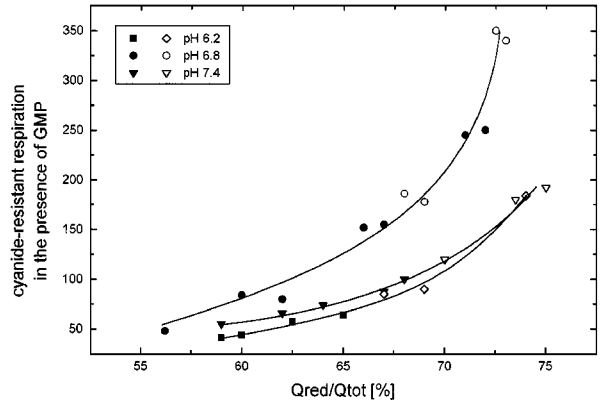


Fig. 3. Influence of assay pH on Q redox state-dependence of the alternative oxidase activity. GMP-stimulated cyanide-resistant respiration with succinate (solid symbols) and external NADH (open symbols) versus Q reduction level at three pH values, i.e., 6.2, 6.8, and 7.4, is shown. Level of Q reduction was varied by titrations of succinate and NADH oxidation as described under Material and Methods.

of *n*-butyl malonate or decreasing the concentration of NADH, with succinate or external NADH as reducing substrates, respectively. At a given pH, the same rate (the cyanide-resistant GMP-stimulated respiratory rate) versus Qred/Qtot relationship is observed with both substrates. When NADH was reducing substrate, respiratory rate and Q redox state were higher than those with succinate as observed previously for pH value 7.4 (Jarmuszkiewicz *et al.*, 2002). At a given Q reduction level, the cyanide-resistant respiration (+GMP) with succinate or NADH depends strongly on the value of assay pH. The highest cyanide-resistant respiration was observed at pH 6.8 for each given Q redox state whatever the reducing substrate (internal succinate or external NADH). This strongly supports the idea that pH effect on the cyanide-resistant respiration at a given Q redox state is solely characteristic of AOX.

DISCUSSION

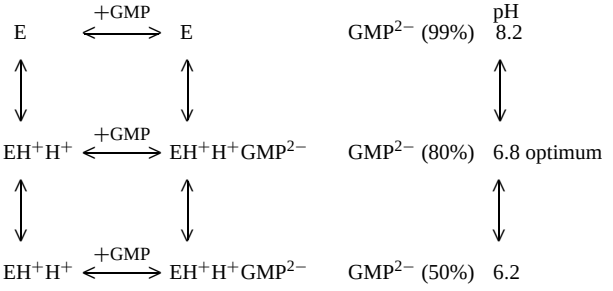
In plant mitochondria, the effect of assay pH on the alternative oxidase-mediated respiration depends on plant species. With intact mitochondria, pH had little or no effect on cyanide-resistant respiration as observed in corn (*Zea mays* L.) shoot mitochondria and in mung bean (*Vigna radiata* L.) hypocotyl mitochondria (Elthon *et al.*, 1986). However, in voodoo lily (*Sauromatum guttatum*) appendix and vita bean (*Vigna uniculata* L.) hypocotyl mitochondria, a pH dependence of the cyanide-resistant respiration with external NADH revealed an optimum between 6.8 and 7.2 or at 6.25, respectively (Elthon *et al.*, 1986; Lima *et al.*, 2000). In isolated *V. uniculata* mitochondria, an activation of the cyanide-resistant respiration

by pyruvate (an activator of the plant alternative oxidase) was also pH-dependent, although pH optimum (near neutrality) was significantly different from that in the absence of pyruvate (Lima *et al.*, 2000). For activity of solubilized, partially purified AOX from *S. guttatum* (Elthon and McIntosh, 1986) and *A. italicum* (Hoefnagel *et al.*, 1997), optimal values of 6.8 and 7.0 were determined, respectively. In the case of *A. italicum* solubilized alternative oxidase, the observed pH optimum was the same in the presence or absence of pyruvate (Hoefnagel *et al.*, 1997). So far, to our knowledge, nothing was known about pH regulation of the GMP-stimulated nonplanttype alternative oxidase.

The results shown in Fig. 1 demonstrate that the cytochrome pathway and the alternative oxidase have different assay pH optima (7.4 and 6.8, respectively) when their activities are measured with intact *A. castellanii* mitochondria. These two pH optima are not influenced by the various ways ubiquinone can be reduced or by the energy state of the mitochondrial membrane. This indicates that the two pH optima are independent of the origin of ubiquinone-reducing electrons and are solely related to the ubiquinol-oxidizing pathways, i.e., the cytochrome pathway or the alternative oxidase. Moreover, the assay pH-dependence of the alternative oxidase activity measured in the presence of cyanide could actually represent the sensitivity of alternative oxidase to matrix pH.

The activation mechanism of alternative oxidase by purine nucleoside 5′-monophosphates (like GMP) in microorganisms is not yet well characterized. Purine nucleotides are supposed to act from outside the inner mitochondrial membrane (Doussiere and Vignais, 1984; Vanderleyden *et al.*, 1979). In mitochondria of *Paramecium tetraurelia*, stimulation of the cyanide-resistant respiration by the non-penetrating inner membrance complex AMP-agarose was observed (Doussiere and Vignais, 1984). However, it is difficult to reconcile these observations with the new topological model of alternative oxidase as an interfacial membrane protein on the matrix side of the inner mitochondrial membrane (Andersson and Norlund, 1999; Berthold *et al.*, 2000).

In amoeba *A. castellanii* mitochondria, stimulation of the alternative oxidase activity by GMP exhibits a clear pH optimum, indicating that the GMP binding on the enzyme is pH-dependent. When pK_a values of the various protonation sites of GMP are analyzed, only one hydroxyl of the phosphomonoester (p$K_a = 6.2$) could take place in protonation/deprotonation events in the studied pH range (6.2–8.2). Thus, GMP occurs in 99, 80, or 50% as GMP^{2-} at pH 8.2, 6.8, and 6.2, respectively. At the level of the alternative oxidase protein, two regions can be defined: the most conserved sequence which comprises the diiron-binding helices and the large hydrophilic N-terminal domain (Andersson and Norlund, 1999; Berthold *et al.*, 2000. The structure of the N-terminal region is not known. In the plant alternative oxidase, it contains the cysteine responsible for the redox state-pyruvate-dependent regulation. In the GMP-dependent alternative oxidase (protozoan- and fungitype alternative oxidase), the N-terminal region contains no cysteine but two histidines, one conserved in all types of alternative oxidases and second conserved specifically in the GMP-dependent oxidase. This pair of histidines could play a role in GMP^{2-} binding, considering that the pK_a of their imidazol rings in the protein can fit for the protonation/deprotonation events in the tested pH range. Therefore, the two imidazol rings could be deprotonated (neutral) at pH 8.2 and protonated (1+) at pH 6.8 and 6.2. A simple scheme explaining the pH-dependence (with optimum at 6.8) of GMP stimulation can be proposed:



This scheme accounts for (i) a very weak activity of the alternative oxidase and its weak stimulation by GMP at pH 8.2, (ii) a peak activity of the alternative oxidase and its peak stimulation by GMP at pH 6.8, and (iii) a decrease in activity of the alternative oxidase and in its activation by GMP at pH below 6.8. We have proposed the two histidines as candidates for protonation/deprotonation events because of their position in the hydrophilic N-terminal domain of the protein which contains not only the most variable sequence regions between the plant and nonplant GMP-stimulated alternative oxidases but also the regulatory cysteine in the plant oxidase.

When the pH effect on the GMP-stimulated cyanide-resistant respiration was measured for various Q redox state (Fig. 3), with succinate (plus rotenone) or external NADH as oxidizable substrates, the highest activity was observed at pH 6.8. This observation confirms that in these conditions the alternative oxidase could be the sole target of pH and leads to proposition that QH_2-binding could be also dependent on pH as GMP-binding.

From this analysis, it can be concluded that pH modifies the activity of alternative oxidase through

protonation/deprotonation events that in turn modulate the binding of the alternative oxidase allosteric regulator (GMP). The Q redox state-dependence of the alternative oxidase activity is also modified by pH perhaps through the binding of QH_2. Thus, these mechanistic steps could be responsible for the pH-dependence of the alternative oxidase activity and lead to a high pH-sensitivity within the physiological range of pH.

Large changes in pH are necessary to alter significantly the cyanide-resistant respiration as shown in Fig. 1. A decrease in pH from 8.2 to 6.8 in the aqueous environment of the alternative oxidase (matrix) could lead to the almost five times increased activity of the alternative oxidase when GMP is present. As previously observed, at a fixed QH_2 poise, the cyanide-inhibited respiration was maximally 2.5 time higher than the alternative oxidase contribution (measured in the absence of cyanide) (Jarmuszkiewicz *et al.*, 1998). If this activation by cyanide were solely linked to a pH change, it would imply a pH decrease from 8 to 7.4. This can reasonably occur in matrix space when the cytochrome pathway proton pumps become inactive and stop to extract protons from matrix. Such a pH connection would link inactivation of the respiratory chain proton pumps with activation of the alternative oxidase in mitochondria containing GMP-activated alternative oxidase (nonplanttype). A consequence would be an increase in the ability of the alternative oxidase to oxidize the overreduced Q pool, which is the result of the cytochrome pathway slow down, in this way allowing redoxfree energy dissipation.

This link between the two pathways could be important for physiology and energy-metabolic balance of the cell (Jarmuszkiewicz *et al.*, 2001; Sluse and Jarmuszkiewicz, 2002).

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