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Substrate kinetics of the *Acanthamoeba castellanii* alternative oxidase and the effects of GMP

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Abstract

In *Acanthamoeba castellanii* mitochondria, the apparent affinity values of alternative oxidase for oxygen were much lower than those for cytochrome *c* oxidase. For unstimulated alternative oxidase, the K_{Mox} values were around 4–5 μM both in mitochondria oxidizing 1 mM external NADH or 10 mM succinate. For alternative oxidase fully stimulated by 1 mM GMP, the K_{Mox} values were markedly different when compared to those in the absence of GMP and they varied when different respiratory substrates were oxidized (K_{Mox} was around 1.2 μM for succinate and around 11 μM for NADH). Thus, with succinate as a reducing substrate, the activation of alternative oxidase (with GMP) resulted in the oxidation of the ubiquinone pool, and a corresponding decrease in K_{Mox}. However, when external NADH was oxidized, the ubiquinone pool was further reduced (albeit slightly) with alternative oxidase activation, and the K_{Mox} increased dramatically. Thus, the apparent affinity of alternative oxidase for oxygen decreased when the ubiquinone reduction level increased either by changing the activator or the respiratory substrate availability.

Keywords: Mitochondria; Alternative oxidase; Oxygen affinity; GMP stimulation; *Acanthamoeba castellanii*

1. Introduction

The mitochondrial respiratory chain of the amoeba *Acanthamoeba castellanii*, a non-photosynthetic amoeboid protozoon, like that of plant mitochondria, possesses both cyanide- and antimycin-resistant alternative oxidase (AOX) and conventional cytochrome *c* oxidase [1,2]. Furthermore, *A. castellanii* and higher plants share other common features at the level of the respiratory chain of the inner mitochondrial membrane, such as the presence of the non-phosphorylating rotenone-insensitive internal (matricial face) and external (cytosolic face) NADH dehydrogenases [1,3,4].

As in higher plant mitochondria, the alternative pathway of amoeba mitochondria branches from the main respiratory chain at the level of ubiquinone (Q), and electron flux through AOX is not coupled to ADP phosphorylation. While in plant mitochondria the activity of AOX is stimulated by α-keto acids and regulated by the redox state of the intermolecular disulfide bond (the reduced state is more active) [5–7], these regulations do not apply to AOX in amoeba mitochondria [8]. The amoeba cyanide-resistant AOX is strongly stimulated by purine nucleoside 5′-monophosphates AMP, GMP and IMP [1,9]. A similar effect of purine mononucleotides on the cyanide-resistant alternative pathway has also been observed in other protists and some primitive fungi [10–17]. Despite important differences between plant and amoeba AOX at the level of regulation, monoclonal antibodies developed against *Sauromatum guttatum* cross-react with the oxidase protein of *A. castellanii* mitochondria [8], as they do with proteins of a wide range of thermogenic and non-thermogenic plant species, some fungi, and trypanosomes [5,6]. This indicates

Abbreviations: AOX, alternative oxidase; BHAM, benzohydroxamate; K_{Mox}, apparent Michaelis constant of oxygen; V_{max}, apparent maximal velocity; Q or Q_{ox}, ubiquinone; Q_{red} or QH_2, ubiquinol; Q_{tot}, total endogenous pool of ubiquinone in the inner mitochondrial membrane ($Q_{ox}+Q_{red}$); Q_{red}/Q_{tot}, reduction level of the ubiquinone pool

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that the AOX protein is well conserved throughout various species.

The development of techniques for the Q redox poise measurement in isolated mitochondria [18–20] has led to a better understanding of the kinetics of electron transport between the Q pool and AOX. In plant mitochondria as well as in *A. castellanii* mitochondria, the cyanide-resistant electron flux through AOX displayed a non-linear relationship with the redox state of Q, increasing non-linearly with higher levels of Q reduction [19,21]. In *A. castellanii* mitochondria, a similar relationship was found for the kinetics of AOX contribution versus Q reduction level determined with the ADP/O method when the cytochrome pathway was active in state 3 respiration [22]. Our previous results have indicated that in isolated *A. castellanii* mitochondria, the binding of the purine nucleotide (GMP), the redox state of Q as well as the matrix pH, collaborate to set the activity of GMP-stimulated AOX [21–23]. The activation mechanism of non-plant type AOX by GMP is not yet well characterized. In our previous work, we have proposed a model explaining the pH-dependence of GMP stimulation of *A. castellanii* AOX that implicates protonation/deprotonation processes at the level of ligand (at one hydroxyl of GMP) and protein (at two conserved histidines of the N-terminal domain of GMP-dependent oxidases) with an optimum pH at 6.8 [23].

In non-plant mitochondria of eukaryotic microorganisms possessing AOX, an accurate determination of its oxygen affinity has not yet been reported. In plant mitochondria, the apparent affinity of AOX for oxygen was found to be lower than that of cytochrome oxidase. Depending on the method of determination used, the apparent K_{Mox} values of plant AOX were found between 0.5–2 μM [24,25] and 10–20 μM [26]. In addition, the affinity of plant AOX for oxygen decreased as the Q pool became more reduced [26].

In this paper, we present an accurate estimation of the oxygen affinity of AOX in *A. castellanii* mitochondria, obtained using high-resolution respirometry, and compare it with the cytochrome oxidase oxygen affinity. The apparent K_{Mox} of the amoeba AOX was determined under different conditions, i.e., when the Q redox state was varied by dehydrogenase activity or GMP concentration titrations in isolated mitochondria oxidizing NADH or succinate. The effects of GMP on the dependence of AOX activity and the affinity for oxygen on the Q redox state were studied.

2. Material and methods

2.1. Cell culture and mitochondrial isolation

The soil amoeba *A. castellanii*, strain Neff, was cultured as described by Jarmuszkiewicz et al. [8]. Trophozoites of amoeba were collected 22–24 h following inoculation at the middle exponential phase (at a density of about $2–4\times10^6$ cells/ml). Mitochondria were isolated and purified on a self-generating Percoll gradient (31%) as described earlier [8]. Mitochondrial protein concentration was determined by the biuret method with bovine serum albumin (fraction V) as a standard.

2.2. Assay procedures

Oxygen uptake was measured polarographically using an oxygen electrode (Hansatech, UK) in 1.4 ml of standard incubation medium (25 °C) containing: 120 mM KCl, 20 mM Tris–HCl pH 7.4, 3 mM KH_2PO_4, 8 mM $MgCl_2$ and 0.2% (w/v) BSA, with 0.5–1 mg of mitochondrial protein. The oxidizable substrates were succinate (10 mM) or external NADH (up to 1.1 mM) in the presence of rotenone (4 μM) to block electron input from complex I. Succinate dehydrogenase was activated by 0.15 mM ATP. To titrate succinate (10 mM) oxidation, an increasing concentration of *n*-butyl malonate, a competitive inhibitor of succinate uptake, was used. The titration of NADH oxidation was performed as described previously, varying the NADH concentration (0.03–1 mM) in the presence of the enzymatic regenerating system [27]. For state 3 measurements, 1.6 mM ADP was supplied. The cytochrome pathway was inhibited with cyanide (1.5 mM). The alternative oxidase was activated with up to 1.1 mM GMP and inhibited with 1.5 mM benzohydroxamate (BHAM). Values of O_2 uptake are in nmol $O_2\times min^{-1}\times mg^{-1}$ protein. In *A. castellanii* mitochondria, no correction of respiratory rates is needed, as there is no residual rate in the presence of inhibitors of both AOX and the cytochrome *c* oxidase.

The membrane potential of mitochondria was measured simultaneously with oxygen uptake using a tetraphenylphosphonium-specific electrode according to Kamo et al. [28]. For the calculation of the membrane potential value (in mV), the matrix volume of amoeba mitochondria was assumed as 2.0 $\mu l\times mg^{-1}$ protein.

The redox state of ubiquinone (Q_{red}/Q_{tot}) in steady-state respiration was determined by an extraction technique, followed by HPLC detection according to Van den Bergen et al. [20]. As previously determined, endogenous quinone in *A. castellanii* mitochondria is Q-9 [22]. For the calibration of the peaks, commercial Q-9 (Sigma) was used. A completely oxidized extract was obtained during incubation in the absence of substrate using the evaporation/ventilation step. A completely reduced extract was obtained upon anaerobiosis and in the presence of substrates (10 mM succinate or 1 mM NADH), 1.5 mM KCN and 1.5 mM BHAM.

2.3. O_2-affinity measurements

The determination of K_{Mox} and V_{max} was performed with the high-resolution respirometry Oroboros Oxygraph and DatLab Software (Oroboros, Innsbruck, Austria) that provides a routine approach to oxygen kinetics of isolated mitochondria [29,30]. This approach secures high time resolution, background correction and internal correction for

zero signal drift that are critical for oxygen kinetics. The oxygen range can be selected for non-linear fitting and a hyperbolic function is calculated to obtain the oxygen pressure at half-maximum flux, p_{50}, and maximum flux, J_{max}, which are used to calculate K_{Mox} and V_{max}, respectively. The incubation medium was the same as for standard oxygen consumption measurements, except that the volume of the medium was 2 ml and 0.3–0.5 mg of mitochondrial protein was used.

3. Results and discussion

3.1. Oxygen affinity of the cytochrome and alternative oxidases in A. castellanii mitochondria

Accurate measurements of respiration at low oxygen concentrations and determination of apparent affinity for oxygen and apparent maximal respiratory velocity were made with high-resolution respirometry [29,30]. The apparent maximal respiratory velocity (V_{max}) values calculated from non-linear fitting and the hyperbolic function of oxygen pressure corresponded very closely to the maximum steady-state rates measured simultaneously by the oxygraph.

For measurements of cytochrome pathway respiration, 1.5 mM BHAM was added to inhibit AOX. Measurements of the apparent affinity of cytochrome *c* oxidase for oxygen in isolated *A. castellanii* mitochondria gave values (Table 1) that were not significantly different from the values cited previously for these mitochondria [31]. Apparent K_{Mox} values did not differ considerably when different respiratory substrates were oxidized. These values ranged from 0.39 to 0.56 μM oxygen both for mitochondria respiring in the presence (state 3) or absence (state 4) of ADP. As shown in Table 1, the apparent K_{Mox} values of cytochrome *c* oxidase were found to be higher in phosphorylating mitochondria (or uncoupled mitochondria, data not shown) compared to those of mitochondria respiring in the absence of ADP (state 4). However, it can be concluded that in *A. castellanii* mitochondria the cytochrome *c* oxidase has a low K_M for oxygen, although higher when compared with animal and plant mitochondria (0.1–0.3 μM) [25,32,33].

Table 1
The apparent maximal velocity (V_{max}) and the oxygen Michaelis constant (K_{Mox}) of respiration via the cytochrome *c* and alternative oxidases in *A. castellanii* mitochondria

Conditions	V_{max} (nmol O_2/min/mg pr.)	K_{Mox} (μM)
COX		
Succinate		
No ADP	43.3±3.5	0.39±0.08
+1 mM ADP	160±8	0.52±0.11
NADH		
No ADP	47.2±3.6	0.46±0.06
+1 mM ADP	182±10	0.56±0.06
AOX		
Succinate		
Unstimulated	13.2±2.5	5.11±0.31
1 mM GMP-stimulated	66.6±4.2	1.21±0.18
NADH		
Unstimulated	15.4±2.6	4.91±0.41
1 mM GMP-stimulated	198±11	11.3±0.9
Succinate+NADH		
Unstimulated	15.8±4	5.06±0.22
1 mM GMP-stimulated	218±13	12.5±1.0

Respiratory substrates were 10 mM succinate or/and 1 mM NADH. For cytochrome *c* oxidase measurements, 1.5 mM BHAM was added. Where indicated, 1 mM ADP was added to ensure phosphorylating (state 3) respiration. For AOX measurements, 1.5 mM cyanide was added. Where indicated, 1 mM GMP was supplied to ensure maximum activity. Mean values±S.D. are given for 12 determinations with different mitochondrial preparations.

In order to measure AOX mediated respiration, the cytochrome pathway was inhibited with 1.5 mM KCN. In *A. castellanii* mitochondria, the K_{Mox} values for AOX were much higher than those for the cytochrome *c* oxidase (Table 1). For unstimulated AOX, K_{Mox} values ranged from 4.91 to 5.11 μM both in mitochondria oxidizing 1 mM external NADH or 10 mM succinate (or both together, not shown). However, in the case of AOX fully stimulated by 1 mM GMP, K_{Mox} values differed considerably in comparison with the absence of GMP and varied when different respiratory substrates were oxidized. Analysis of different sets of data for cyanide-insensitive GMP-stimulated respiration gave the apparent K_{Mox} of 1.21±0.18 μM and 11.3±0.9 μM, with succinate and NADH as substrates, respectively. In *A. castellanii* mitochondria simultaneously oxidizing succinate and NADH, the observed K_{Mox} values (as well as V_{max} values) of unstimulated AOX were the same as for each respiratory substrate alone (Table 1). Similarly, for the GMP-stimulated AOX, the K_{Mox} and V_{max} values did not significantly differ either when with the two substrates were oxidized simultaneously or with NADH alone. This indicates that when mitochondria oxidized NADH alone or the two substrates simultaneously, the AOX activity is saturated by ubiquinol (QH_2). With isolated plant mitochondria, the K_{Mox} of AOX has been reported to be quite different for different approaches, i.e., 0.5–2 μM [24,25] and 10–20 μM [26]. This difference in the K_{Mox} values for plant alternative oxidases does not seem to result from the plant material, the presence of pyruvate (an activator of the plant AOX) or the used respiratory substrate. However, it was observed that the K_{Mox} varied with the redox poise of the Q pool [26].

As with the K_{Mox} values, the apparent V_{max} values were almost the same for unstimulated cyanide-resistant respiration with both substrates (13.2–15.4 nmol $O_2 \times min^{-1} \times mg^{-1}$ pr.), and significantly different for GMP-stimulated cyanide-resistant respiration when succinate (around 66 nmol $O_2 \times min^{-1} \times mg^{-1}$ pr.) and external NADH (around 200 nmol $O_2 \times min^{-1} \times mg^{-1}$ pr.) were oxidized by *A. castellanii* mitochondria (Table 1). The difference in the maximal activity of *A. castellanii* AOX (at a high GMP concentration) with different substrates has been already reported in our previous studies [21]. However, in *A. castellanii* mitochondria, AOX activity is independent of the reducing substrate

that supplies the Q pool with electrons when presented versus the Q redox state varied by substrate availability titration [21].

3.2. Effect of GMP on alternative oxidase activity and affinity for oxygen

In order to elucidate the effect of GMP on *A. castellanii* AOX activity and on the affinity for oxygen, we determined the apparent affinity of cyanide-resistant respiration for stimulation by GMP using an increasing concentration of the nucleotide (Fig. 1). Fig. 1A shows the calculated apparent maximal respiratory velocity as a function of GMP concentration and Fig. 1B shows the calculated apparent affinity for oxygen as a function of GMP concentration, both for the cyanide-resistant AOX-mediated respiration with a saturating concentration of succinate or external NADH. Concentrations of GMP causing a half-maximum effect on respiration and apparent affinity for oxygen were similar for the given respiratory substrate,

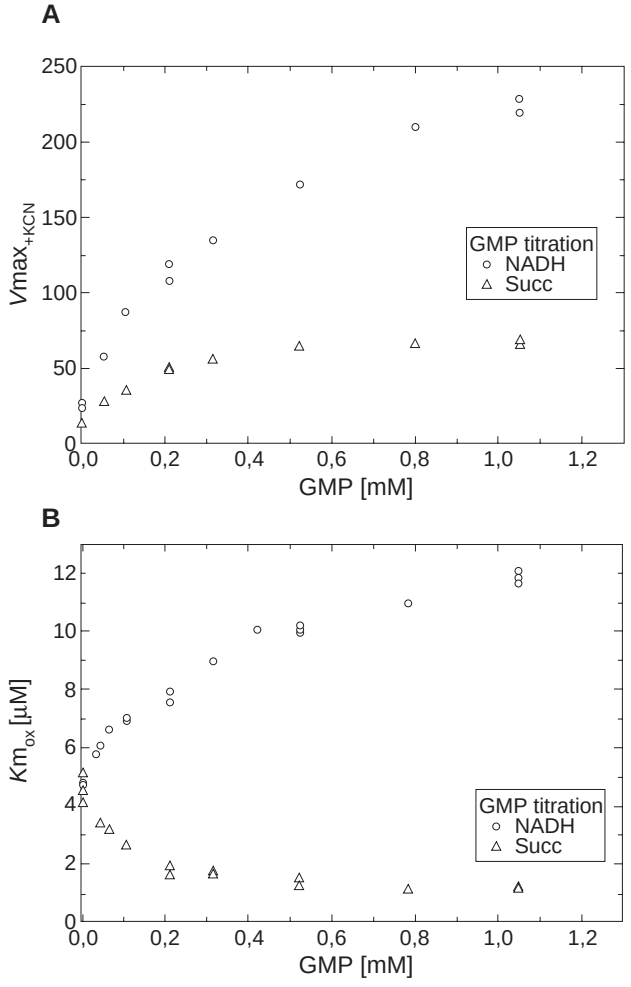

Fig. 1. The effect of GMP concentration on the cyanide-resistant AOX-mediated respiration (the apparent maximal velocity, V_{max}) (A) and on the apparent Michaelis constant of oxygen (K_{Mox}) (B), in mitochondria oxidizing 1 mM NADH or 10 mM succinate in the presence of 1.5 mM cyanide.

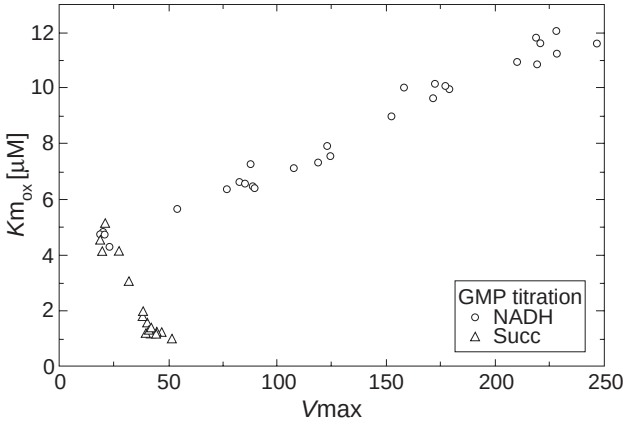

Fig. 2. The apparent Michaelis constant of oxygen (K_{Mox}) versus the apparent maximal velocity (V_{max}) in mitochondria oxidizing 1 mM NADH or 10 mM succinate in the presence of 1.5 mM cyanide. Assay conditions as in Fig. 1.

although almost 3 times higher for NADH compared to succinate. The $S_{0.5}$ values for GMP, ranging from around 0.260 to 0.300 mM (for NADH) and from around 0.10 to 0.130 mM (for succinate), correspond to the GMP concentration that induces 50% stimulation of AOX activity (Fig. 1A) and 50% modification of K_{Mox} (Fig. 1B). Thus, there was a surprising difference between the AOX activity when supplied with electrons from succinate dehydrogenase and NADH dehydrogenase. Besides the difference in $S_{0.5}$ values for GMP, the different behavior of the apparent affinity for oxygen was observed with the increasing concentration of GMP. With succinate, the apparent K_M of AOX for oxygen decreased, while with NADH it increased, with the increasing concentration of GMP and thereby with an increasing maximal respiratory rate (Fig. 2). As it was observed that in plant mitochondria the K_{Mox} of AOX varied with the redox poise of the Q pool [26], it was important to check how a reduction level of ubiquinone influences the affinity of the oxidase for the second substrate, i.e. oxygen in *A. castellanii* mitochondria.

3.3. Effect of GMP on the dependence of alternative oxidase activity and affinity for oxygen on the ubiquinone redox state

It is well established that AOX activity depends on the Q redox state. Fig. 3A shows the relationship between AOX activity (the cyanide-resistant respiration) and the Q redox state in *A. castellanii* mitochondria oxidizing succinate or NADH (at saturating concentrations) during GMP titration. With increasing concentration of GMP (from 0.05 to 1.05 mM), an increase in the AOX activity was accompanied by a decrease or no significant change in the Q reduction level with succinate and external NADH, respectively. This difference between the two substrates disappeared when the apparent K_{Mox} values were plotted versus the Q redox state that gave a single relationship (Fig. 3B). Namely, in *A. castellanii* mitochondria at saturating concentrations of the

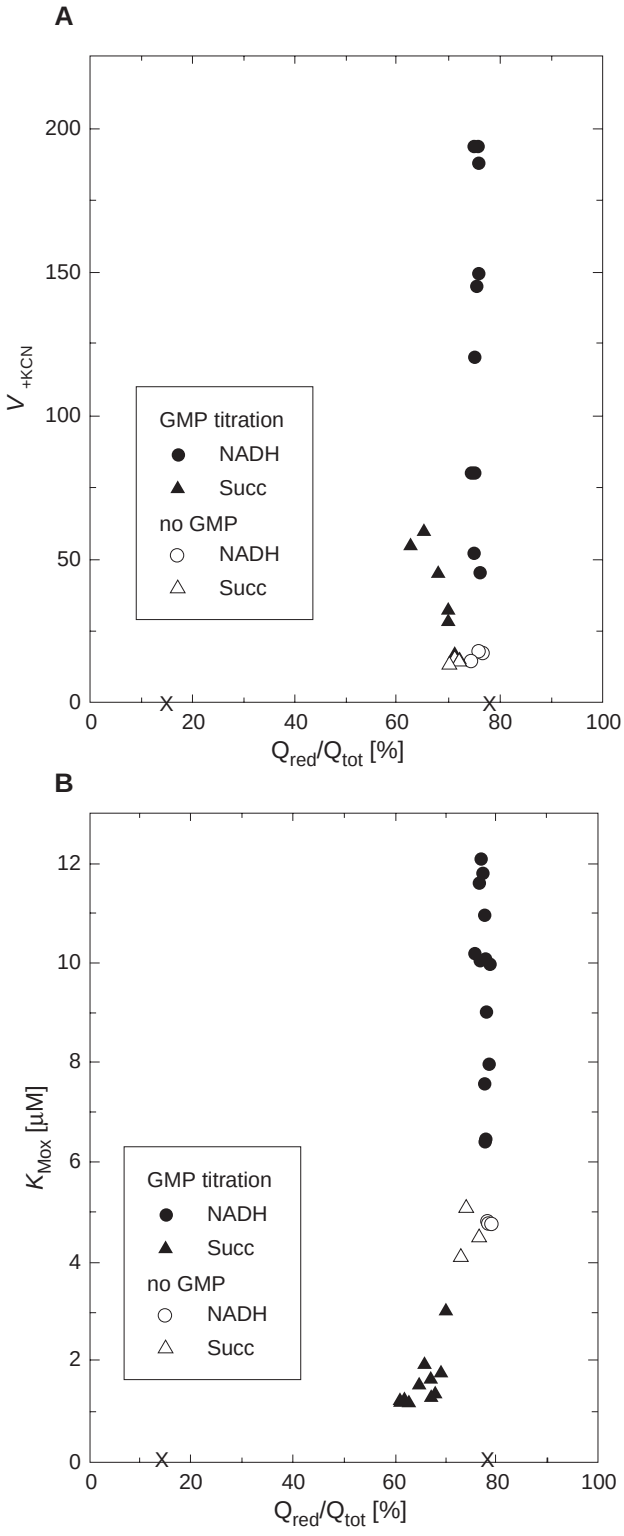

Fig. 3. The effect of GMP on the dependence of AOX activity (A) and affinity for oxygen (B) on the Q redox state in mitochondria oxidizing 1 mM NADH or 10 mM succinate in the presence of 1.5 mM cyanide. GMP concentration was as in Fig. 1 (0–1.05 mM). The asterisks correspond to values of completely oxidized and reduced Q_{red}/Q_{tot}.

two substrates, we observed that when changing the availability of the AOX activator, the value of the K_{Mox} varied with the Q redox poise. For more reduced Q (during GMP titration with succinate as substrate), a larger K_{Mox} was observed until around 76% of the Q reduction level that is the maximal value of the Q pool reduction in the studied *A. castellanii* mitochondria. This value was characteristic of unstimulated AOX activity for both substrates. For a higher activity of AOX that can be reached with NADH in the presence of GMP, a further increase of the K_{Mox} value with increasing concentration of GMP occurred with no detectable change in the Q reduction level, indicating a very steep dependence of K_{Mox} on the Q redox poise under these conditions. The increase in the K_{Mox} value with a higher Q reduction level has been reported previously for plant AOX [26]. However, the interpretation of data presenting the calculated apparent K_{Mox} value versus the Q reduction level depends on the constancy of the redox state of the ubiquinone pool over the course of the reaction. The redox poise of the Q pool measured using the voltametric method [18] was found to remain constant until just before the reaction reached anaerobiosis during the measurement of the oxygen K_M [26]. Following this observation, we assume no appreciable change of the Q reduction level during the course of the reaction when the concentration of oxygen is decreasing, in our experimental conditions. Moreover, in the applied method of determination of the *A. castellanii* AOX K_{Mox} value, based on non-linear fitting, all rate measurements were taken at oxygen concentration (above 3 μM) when the Q pool reduction should remain unchanged [26].

3.4. Effect of ubiquinone-reducing pathway activity on alternative oxidase activity and affinity for oxygen and their dependence on the ubiquinone redox state

As shown in Figs. 4 and 5, in the absence or presence of 1.05 mM GMP, the behavior of AOX is not significantly different when the two titrations of Q-reducing pathway

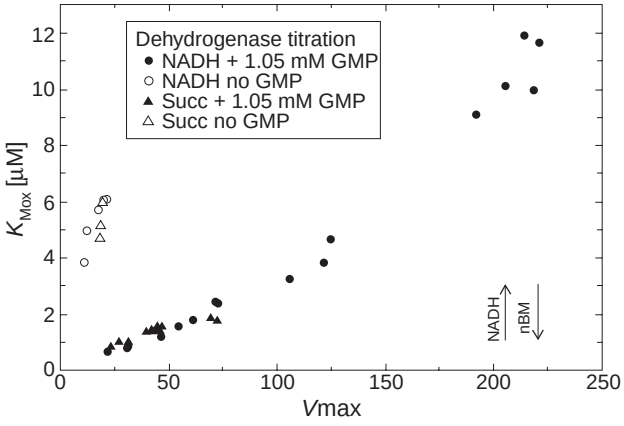

Fig. 4. The apparent Michaelis constant of oxygen (K_{Mox}) versus the apparent maximal velocity (V_{max}) during substrate dehydrogenase titration in mitochondria oxidizing NADH or succinate in the presence of 1.5 mM cyanide and in the absence or presence of 1.05 mM GMP. The titrations were performed as described under Material and methods. Arrows indicate the direction of increasing concentrations of the titrated compounds, i.e., NADH or *n*-butyl malonate (*n*-BM) (for succinate oxidation).

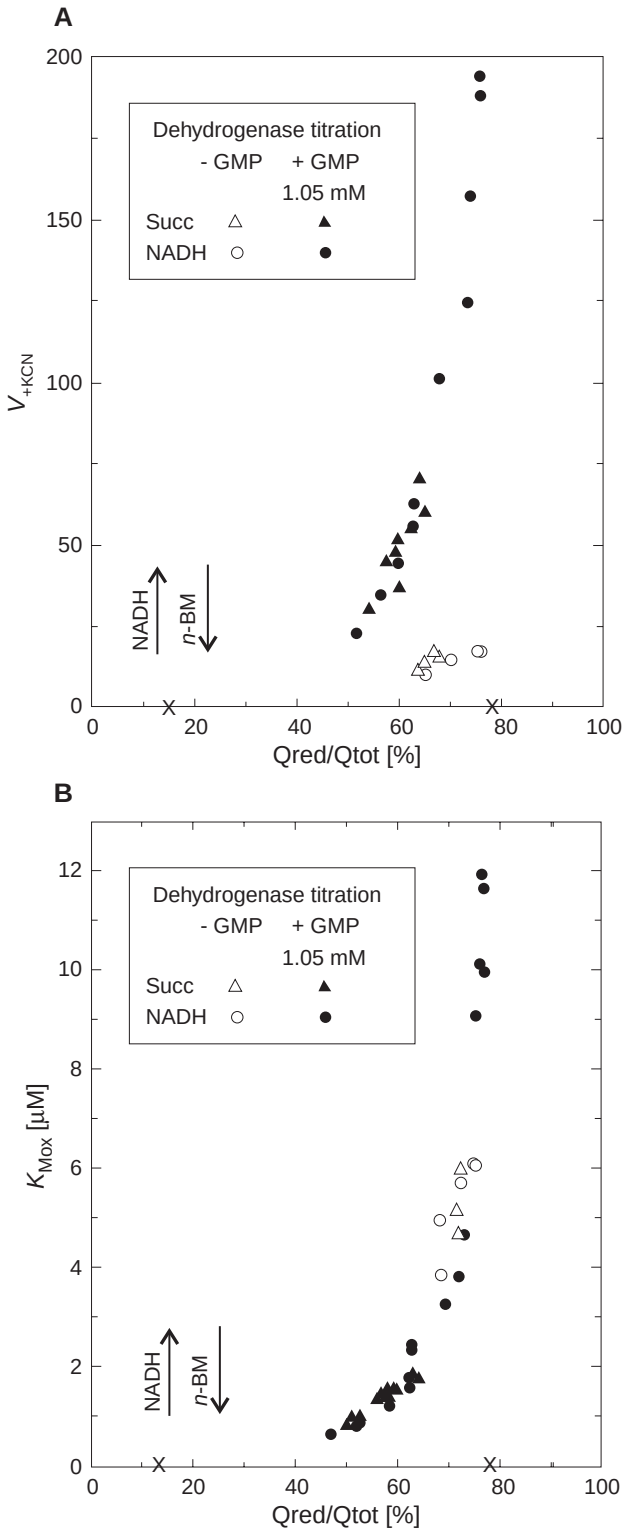

Fig. 5. Dependence of the AOX activity (A) and the Michaelis constant of oxygen (B) on Q redox state during substrate dehydrogenase titration in mitochondria oxidizing NADH or succinate in the presence of 1.5 mM cyanide and in the absence or presence of 1.05 mM GMP. The titrations were performed as described under Material and methods. The asterisks correspond to values of completely oxidized and reduced Q_{red}/Q_{tot}. Arrows indicate the direction of increasing concentrations of the titrated compounds, i.e., NADH or *n*-butyl malonate (*n*-BM) (for succinate oxidation).

activity (with succinate or external NADH) are compared. The substrate dehydrogenase activity was varied by titration of cyanide-resistant succinate-sustained respiration with *n*-butyl malonate or by titration of the cyanide-resistant NADH-sustained respiration with different NADH concentrations. Data presenting the effect of Q-reducing pathway activity on AOX activity and affinity for oxygen (Fig. 4) and their dependence on the Q redox state (Fig. 5A and B) gave single relationships with respect to succinate and NADH (for the same conditions minus or plus GMP), indicating that the two concerned dehydrogenases have no direct interaction with AOX and that the link between the Q-reducing pathway and QH_2-oxidizing pathway (AOX) activities occurs through a single homogenous active Q pool. This is in agreement with our previous results obtained for *A. castellanii* AOX activity in state 3 respiration, when the cytochrome pathway was active [21].

Fig. 5A shows that the *A. castellanii* AOX activity (both unstimulated and GMP-stimulated) increased non-linearly with higher levels of Q reduction altered with variation in respiratory substrate availability as observed for plant AOX [19]. In the absence or presence of a high GMP concentration, the K_M of AOX for oxygen decreased with the lowering of dehydrogenase activity, thereby with lowering of the AOX activity and the redox poise of the Q pool alike (Figs. 4, 5B). As in Fig. 3B, the highest K_{Mox} values were obtained for NADH oxidation at high respiratory substrate concentration and in the presence of a high concentration of GMP, i.e., under conditions that gave the highest activity of AOX, however, at the maximal, unchangeable range of the Q reduction level (Fig. 5B).

3.5. Regulation of A. castellanii alternative oxidase: interplay between substrates and activator

The activation mechanism of non-plant type AOX (including *A. castellanii* AOX) by GMP is not yet well characterized. At present, direct evidence that GMP binds to AOX protein to stimulate its activity has not yet been obtained. However, a clear indication can be found in Fig. 5A, which demonstrates that in *A. castellanii* mitochondria, for a given Q reduction level, GMP increases the apparent maximal rate of AOX.

In *A. castellanii* mitochondria, the AOX activity and affinity for oxygen are independent of the reducing substrate that supplies the Q pool with electrons both in the presence or absence of GMP when compared to a given Q redox state (Figs. 3 and 5). When the Q reduction level decreases either by changing the activator (Fig. 3B) or respiratory substrate (Fig. 5B) availability, the K_{Mox} decreases. Moreover, the K_{Mox} versus Q_{red}/Q_{tot} relationships (Figs. 3B and 5B) obtained in both cases are very close together. Thus, titration results suggest that GMP and oxygen binding to AOX are independent. In other words, the K_{Mox} of AOX might not be affected directly by the presence or absence of GMP, but rather GMP affects the Q reduction level, which in turn

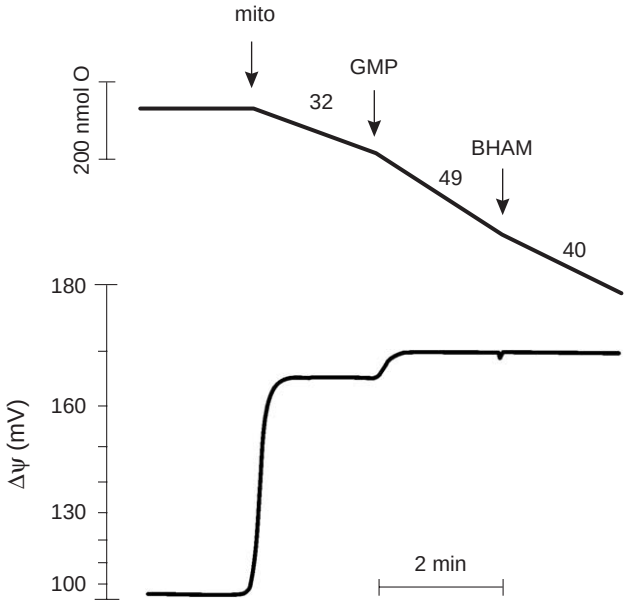

Fig. 6. The effect of GMP on oxygen consumption and membrane potential ($\Delta\Psi$) in mitochondria oxidizing NADH (at not saturating concentration) in the absence of cyanide. The concentration of NADH (0.03 mM) was maintained by the enzymatic regenerating system. The measurement was performed in the presence of 4 μM rotenone in the incubation medium. Additions (where indicated): 1 mM GMP, 1.5 mM BHAM. Numbers on the trace refer to oxygen consumption rates in nmol $O_2 \times min^{-1} \times mg^{-1}$ protein.

affects the K_{Mox}. As a consequence, the K_{Mox} increase with increasing concentration of GMP during NADH oxidation (Fig. 1B) should be due to a slight (undetectable, Fig. 3B) increase in the Q reduction that could occur only if GMP activates the external NADH dehydrogenase. For an apparently maximal Q reduction level (around 76%), unstimulated and GMP-stimulated AOX activities yield two different K_{Mox} values (5 and 12, respectively) (Fig. 3B), suggesting that GMP has a direct effect on the AOX affinity for oxygen. However, the possible activation of external NADH dehydrogenase by GMP (see section 3.6) might lead to a higher Q reduction level (although not detectable in the range close to maximum), and consequently to a higher K_{Mox}, following a very steep dependence of K_{Mox} on the Q redox poise under these conditions.

3.6. Possible activation of A. castellanii external NADH dehydrogenase by GMP

The higher GMP concentration inducing a 50% stimulation of AOX activity, observed with NADH compared to succinate (Fig. 1A), supports the possibility that GMP activates not only AOX but also external NADH dehydrogenase in *A. castellanii* mitochondria. Fig. 6 reports an experiment also consistent with the proposed hypothesis. Namely, when mitochondria oxidized external NADH at a low concentration (leading to low activity of external NADH dehydrogenase), in the absence of inhibitors of AOX and the cytochrome pathway, the addition of 1 mM GMP resulted in a stimulation of oxygen uptake that was accompanied by an increase in the membrane potential. The increase in the membrane potential and subsequently observed only partial inhibition of GMP-stimulated respiration by benzohydroxamate indicates that the nucleotide stimulates not only AOX but also the external NADH dehydrogenase. Indeed, in the described conditions, the GMP-activated NADH dehydrogenase would supply more electrons to the cytochrome pathway and as a consequence would lead to an increased membrane potential and increased respiration, not sensitive to the AOX inhibitor. However, these preliminary results are just a starting point for the further studies necessary to elucidate the possible activation of external NADH dehydrogenase in *A. castellanii* mitochondria.

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