Interactions Between the Cytochrome Pathway and the Alternative Oxidase in Isolated *Acanthamoeba castellanii* Mitochondria

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The steady-state activity of the two quinol-oxidizing pathways of *Acanthamoeba castellanii* mitochondria, the phosphorylating cytochrome pathway (i.e. the benzohydroxamate(BHAM)-resistant respiration in state 3) and the alternative oxidase (i.e. the KCN-resistant respiration), is shown to be fixed by ubiquinone (Q) pool redox state independently of the reducing substrate (succinate or exogenous reduced nicotinamide adenine dinucleotide (NADH)), indicating that the active Q pool is homogenous. For both pathways, activity increases with the Q reduction level (up to 80%). However, the cytochrome pathway respiration partially inhibited (about 50%) by myxothiazol decreases when the Q reduction level increases above 80%. The decrease can be explained by the Q cycle mechanism of complex III. It is also shown that BHAM has an influence on the relationship between the rate of ADP phosphorylation and the Q reduction level when alternative oxidase is active, and that KCN has an influence on the relationship between the alternative oxidase activity and the Q reduction level. These unexpected effects of BHAM and KCN observed at a given Q reduction level are likely due to functional connections between the two pathways activities or to protein–protein interaction.

KEY WORDS: *Acanthamoeba castellanii*; alternative oxidase; cytochrome pathway; mitochondria; oxidative phosphorylation.

INTRODUCTION

The mitochondrial respiratory chain of the free-living nonphotosynthetic soil amoeboid protozoon amoeba

Acanthamoeba castellanii shares many common features with the respiratory chain of higher plants. These are: (i) coexistence of two constitutive ubiquinol-oxidizing pathways, the benzohydroxamate(BHAM)-resistant phosphorylating cytochrome chain, and the alternative oxidase being nonphosphorylating and insensitive to cyanide and antimycin (Edwards and Lloyd, 1978; Hryniewiecka *et al.*, 1978); (ii) the ability to oxidize external nicotinamide adenine dinucleotide (NADH) by the rotenone-insensitive external NADH dehydrogenase, located on the outer surface of the inner mitochondrial membrane (Hryniewiecka, 1980; Lloyd and Griffiths, 1968); (iii) the presence of internal nonelectrogenic rotenone-insensitive NADH dehydrogenase (Hryniewiecka, 1986).

As in higher plant and fungi mitochondria (Day *et al.*, 1995; Siedow and Umbach, 2000), the amoeba alternative pathway (BHAM-sensitive alternative oxidase, AOX) branches from the main respiratory chain at the level of ubiquinone (Q) and its activity is not coupled to

ADP phosphorylation (Hryniewiecka *et al*., 1978). The amoeba AOX protein crossed-react with monoclonal antibodies developed against *Sauromatum guttatum* protein (Jarmuszkiewicz *et al*., 1997), indicating that AOX is well-conserved throughout the species. However, considering regulation, AOX of amoeba appears to be a quite different enzyme from plant AOX. While in plant mitochondria, the activity of AOX is stimulated by α-keto acids and regulated by the redox state of the enzyme (Vanlerberghe and McIntosh, 1997), these regulations do not concern AOX in amoeba mitochondria (Jarmuszkiewicz *et al*., 1997), which, however is strongly stimulated by purine mononucleotides (like GMP) (Hryniewiecka *et al*., 1978). Regulation of AOX by purine mononucleotides occurs also in other protists and some primitive fungi (Doussiere and Vignais, 1984; Sakajo *et al*., 1997; Sharpless and Butov, 1970; Siedow and Umbach, 2000; Vanderleyden *et al*., 1980a,b).

Using the ADP/O method, we have previously determined how the calculated contributions of both the cytochrome and alternative pathways in isolated amoeba mitochondria respiring with succinate change when the steady-state rate of the Q-reducing pathway is varied (Jarmuszkiewicz *et al*., 1998). Our results showed that with increasing level of Q reduction the cytochrome pathway contribution passed through an unexpected maximum. The decrease in the cytochrome pathway contribution with increasing Q reduction level may either be inherent to the cytochrome pathway kinetics (in particular it might be due to the Q cycle mechanism of complex III) or be due to an unknown factor of regulation. It was proposed (Jarmuszkiewicz *et al*., 1998) that the concentration of this putative factor was varied during titration of the succinate sustained-respiration with *n*-butyl malonate (a competitive inhibitor of succinate uptake) used to decrease the reduction level of the Q pool.

In this study, we have investigated the state 3 respiration using another reducing substrate (external NADH) at various concentrations in order to modulate the Q redox state. We have compared kinetics of the two ubiquinol-oxidizing pathways, the cytochrome pathway and the alternative oxidase, for external NADH and succinate. These kinetics (activities versus Q redox state) are independent of the reducing substrate that supplies the Q pool with electrons. However, the observation that the activities of the cytochrome and alternative pathways at a given Q redox state are dependent on each other led us to propose new types of connections between the two oxidizing pathways different from the common-substrate sharing. Therefore, the optimum observed in the calculated contribution of the cytochrome pathway might be linked to the AOX activity. On the other hand, in order to evidence that the complex III

kinetics can also be responsible for a decrease in the cytochrome pathway activity at high Q reduction level, the behaviour of BHAM-resistant respiration in state 3 has been studied when complex III was inhibited partially by myxothiazol.

MATERIALS AND METHODS

Cell Culture and Mitochondrial Isolation

Soil amoeba *Acanthamoeba castellanii*, strain Neff, was cultured as described previously (Jarmuszkiewicz *et al*., 1997). Trophozoites of amoeba were collected 22–24 h following inoculation at the middle exponential phase (at a density of about 2–4×10^6 cells/mL). Mitochondria were isolated and purified on a self-generating Percoll gradient (31%) as described before (Jarmuszkiewicz *et al*., 1997). Mitochondrial protein concentration was determined by the biuret method with bovine serum albumin (fraction V) as a standard.

Assay Procedures

Oxygen uptake was measured polarographically using an oxygen electrode (Hansatech, UK) in 2 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% (wt/vol) 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 (20 μM) to inhibit electron flux through complex I. Succinate dehydrogenase was activated by the 0.15 mM ATP. To titrate succinate oxidation increasing concentration of *n*-butyl malonate (nBM), a competitive inhibitor of succinate uptake, was used. Titration of NADH oxidation was performed as described previously varying NADH concentration in the presence of enzymatic regenerating system (Hoefnagel and Wiskich, 1996). The cytochrome pathway was inhibited with myxothiazol (up to 80 μM) or with cyanide (1.5 mM). The alternative oxidase (AOX) was activated with 0.6 mM GMP and inhibited with 1.5 mM benzohydroxamate (BHAM). For state 3 measurements, 0.21 mM (pulse) or 1.6 mM (saturating) ADP was supplied. The total amount of oxygen consumed during state 3 respiration was used for calculation of the ADP/O ratio. Values of O_2 uptake are in nanomoles of O per minute per milligram of protein.

Redox state of ubiquinone in steady-state respiration was determined by an extraction technique followed by HPLC detection according to Van den Bergen *et al*.

(1994). As previously found, endogenous ubiquinone in *A. castellanii* mitochondria is Q-9 (Jarmuszkiewicz *et al*., 1998). For calibration of the peaks commercial Q-9 (Sigma) was used. A completely oxidized extract was obtained during incubation in the absence of substrate using 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. An inactive Q pool contains ubiquinol that can never be oxidized and ubiquinone that can never be reduced.

RESULTS

Behavior of the BHAM-Resistant Respiration in State 3 When the Ubiquinone Reduction Rate is Modulated: Effect of Myxothiazol

In isolated mitochondria of *A. castellanii*, the phosphorylating BHAM-resistant respiration (the cytochrome pathway activity in state 3) in the presence of 10 mM succinate (+ rotenone) decreases when succinate uptake is inhibited by increasing concentration of nBM (Fig. 1(A), left part, $\triangle$) and is enhanced by NADH additions up to

0.4 mM (Fig. 1(A), right part, ■). At higher NADH concentrations, a slight decrease is observed. With external NADH as a single substrate, the respiratory rate increases with the NADH concentration and reaches a saturation value (Fig. 1(A), right part, ○). At any NADH concentration, the respiratory rates in the presence of 10 mM succinate are higher than with NADH alone.

When the succinate-sustained BHAM-resistant respiration partially inhibited (around 50%) by myxothiazol, an inhibitor of complex III, is titrated with nBM, an unexpected stimulatory effect of nBM at low concentrations is observed (Fig. 1(B), left part, $\triangle$). During succinate-sustained respiration in the presence of myxothiazol, increasing concentration of NADH surprisingly decreases the respiratory rate (Fig. 1(B), right part, ■). In the absence of succinate, NADH-sustained respiration in the presence of myxothiazol first increases with the increasing NADH concentration, passes through a maximum and then decreases (Fig. 1(B), right part, ○). Except at a very low NADH concentration, respiration with NADH alone is higher than with both substrates together (Fig. 1(B), right part). In the presence of myxothiazol, the maximal respiratory rate observed with NADH (Fig. 1(B), right part, ○) is almost the same as that with succinate (Fig. 1(B),
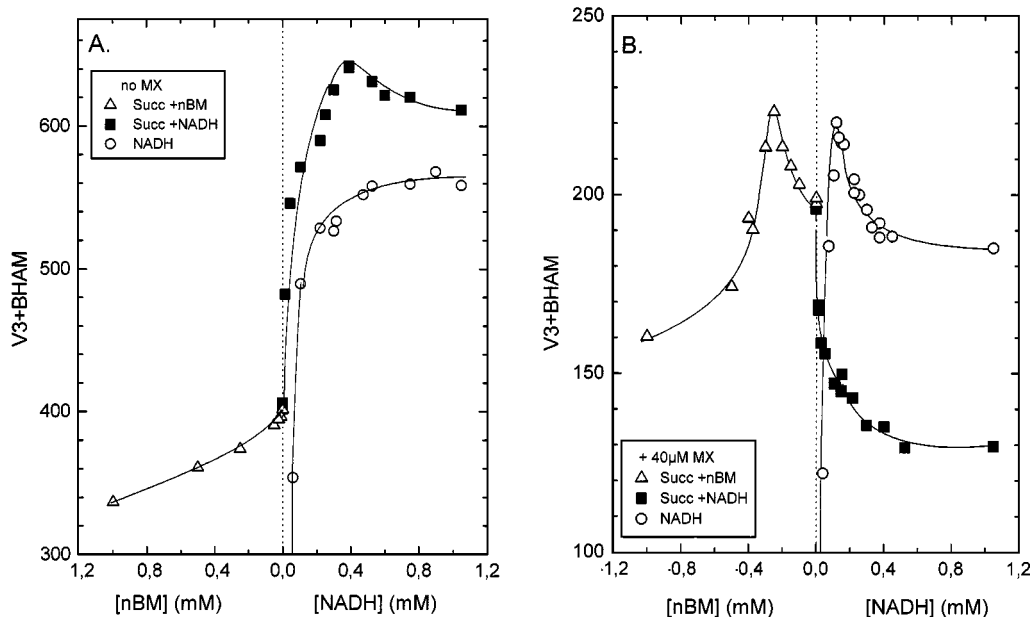

Fig. 1. BHAM-resistant state 3 respiration in isolated *A. castellanii* mitochondria oxidizing succinate and/or external NADH. The assays were carried out in state 3 (with 1.6 mM ADP) in the presence of 1.5 mM BHAM, in the absence (A) or presence (B) of 40 μM myxothiazol (MX). Titrations of succinate (Succ) and NADH oxidation were performed as described under "Materials and Methods." Titrations with increasing NADH concentration (right part of (A) and (B)) in the absence (○) and presence (■) of 10 mM succinate. Mitochondria oxidizing succinate titrated with nBM ($\triangle$) (left part of (A) and (B)). State 3 respiratory rates (V_{3+BHAM}) are in nanomoles of O per minute per milligram of protein. For each panel, shown data come from a single representative mitochondrial respiration.
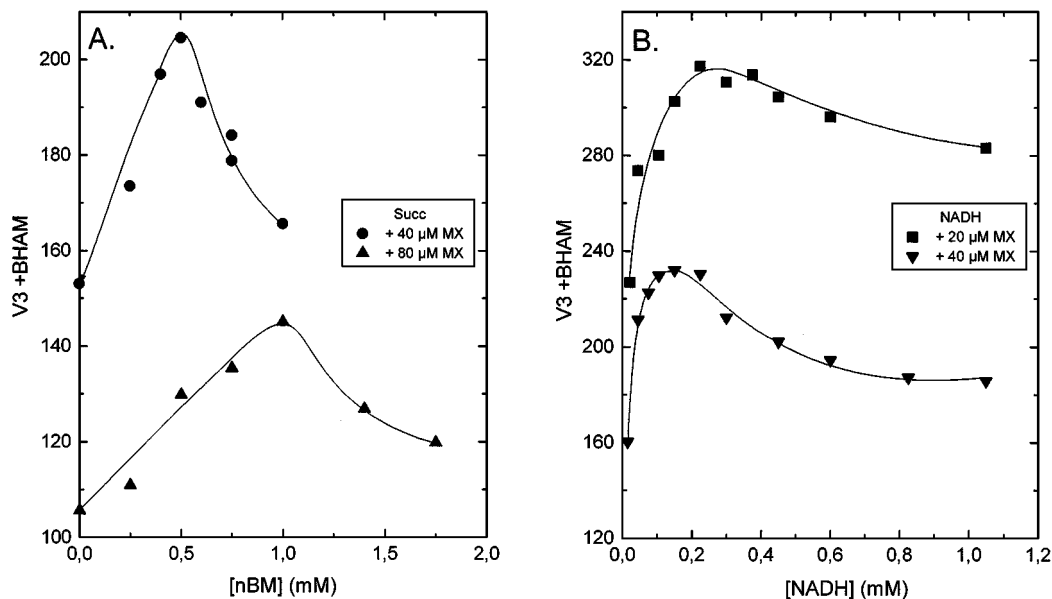
Fig. 2. Effect of myxothiazol concentration on BHAM-resistant state 3 respiration with succinate or external NADH. Mitochondria were respiring in state 3 (with 1.6 mM ADP) in the presence of 1.5 mM BHAM and 20 or 40 or 80 μM myxothiazol (MX). Titrations of succinate (Succ) and NADH oxidation were performed as described under "Materials and Methods." (A) Succinate oxidation titrated with an increasing concentration of nBM. (B) Titrations with increasing NADH concentration. For each comparison, shown data come from a single representative mitochondrial preparation. State 3 respiratory rates (V_{3+BHAM}) are in nanomoles of O per minute per milligram of protein.

left part, $\triangle$) suggesting that both maxima could have a common origin independent of the Q-reducing dehydrogenase and thereby be linked to the kinetics of the cytochrome pathway.

The behavior of the phosphorylating cytochrome pathway steady-state activity in response to variations of the Q pool redox state in the presence of myxothiazol, is presented in Fig. 3(C). As expected from the results of Fig. 1(B), the respiratory rate passes through a maximum for both respiratory substrates when the Q redox state is modulated by nBM for succinate or by variation of the concentration for NADH.

Surprising maxima in respiratory rates may be either due to the kinetic properties of complex III, including a decrease in activity when the ubiquinone pool becomes reduced enough (at low nBM concentration or at high NADH concentration), or due to a substrate inhibition kinetics of complex IV by the reduced cytochrome c (in that case reduction level of cytochrome c increases when the nBM concentration decreases and when the NADH concentration increases). In the case of substrate inhibition of complex IV, the same behavior would have been expected in the absence of complex III (QH$_2$-cytochrome c oxidoreductase) inhibition by myxothiazol i.e. when higher cytochrome c reduction levels are reached. However, as shown in Fig. 1, an optimum in respiration is clearly ob-

served only in the presence of myxothiazol, indicating that the cytochrome c reduction state is not responsible for the maximum.

As in the presence of myxothiazol the Q pool could reach a higher reduction level, application of myxothiazol could make possible the observation of an optimum in respiration followed by a decrease when the Q pool reduction level increases above an optimal value imposed by the kinetics of complex III. Consistently, as shown in Fig. 2, the nBM (that decreases the Q reduction rate) concentration necessary to reach the optimum succinate-sustained respiration increases with myxothiazol (that decreases the QH$_2$ oxidation rate) concentration (Fig. 2(A)) and the NADH (that increases the Q reduction rate) concentration necessary to reach the optimum NADH-sustained respiration increases when myxothiazol concentration is decreased (Fig. 2(B)). Thus, these maxima in respiration appear to be linked to the kinetic properties of complex III.

Steady State Kinetics of QH$_2$-Oxidase Activity in the Presence of BHAM or KCN: Comparison Between Succinate- and External NADH-Sustained Respiration

If the redox state of the membranous Q pool is the only link between the substrate-Q oxidoreductase part

(nonproton pumping) and the QH_2-oxidase part of the respiratory chain, the relationship between the QH_2-oxidase activity and the steady state Q_{red}/Q_{tot} ratio should be independent of the kinetic parameters of the upstream processes. Therefore, a unique relationship (rate versus Q_{red}/Q_{tot}) should be obtained using succinate or external NADH as respiratory substrate providing that diffusion of Q and QH_2 be fast enough to insure homogeneity in the membranous Q pool. Considering possible kinetic limitation by transmembrane diffusion of Q and QH_2 and the known membranous topology of the enzyme catalytic sites, differences between two substrates (at fixed overall Q_{red}/Q_{tot} ratio) can be predicted. The complex III activity (QH_2 oxidation on the cytosolic side of the inner mitochondrial membrane) would be favored (i.e. its QH_2 oxidation site would see a higher localized Q_{red}/Q_{tot} ratio) by external NADH (reducing Q on the cytosolic side) compared with succinate (reducing Q on the matricial side). In contrast, succinate would be a better substrate than external NADH for the alternative oxidase (QH_2 oxidation on the matricial side of the membrane).

As shown in Figs. 3(A) and (C), in the absence or presence of myxothiazol, the behavior of the cytochrome pathway (the BHAM-resistant respiration) in state 3 versus the Q reduction level is not significantly different when the succinate-sustained respiration is titrated with nBM or when the NADH-sustained respiration is varied by different NADH concentrations. In the absence of myxothiazol (Fig. 3(A)), comparison between substrates is possible only for Q_{red}/Q_{tot} values below 40%. For higher Q reduction values, no influence of 10 mM succinate on the relationship between respiratory rate and Q redox state with increasing concentrations of NADH is observed (Fig. 3(B)). Moreover, as shown in Fig. 3(D), the kinetics of the GMP-activated alternative oxidase (the KCN resistant respiration) is also independent of the way the Q redox state is modulated. Thus, the results presented in Fig. 3 strongly suggest that the two concerned dehydrogenases have no direct interaction with either the complexes of the phosphorylating cytochrome pathway or with the alternative oxidase and that the link between Q-reducing pathway(s) and QH_2-oxidizing pathway(s) activities occurs through a single homogenous active Q pool.

The relationships between respiratory rate and the Q reduction level presented in Fig. 3 exhibit quite classical profiles for both QH_2-oxidizing pathways, the cytochrome and alternative oxidase pathways, except that of the cytochrome pathway partially inhibited with myxothiazol (Fig. 3(C)). When comparing Figs. 3(A) and (B) with Fig. 3(C), it is evident that partial inhibition of the BHAM-resistant respiration by myxothiazol allows

higher Q reduction levels to be reached and that the decrease in respiration occurs in this high reduction range (above 80%).

A maximum in the contribution of the cytochrome pathway in state 3 respiration plotted versus Q redox state has already been observed for a lower value of Q_{red}/Q_{tot} (Jarmuszkiewicz *et al.*, 1998). However, these measurements were performed in the presence of GMP and in the absence of BHAM with succinate as oxidizable substrate. However, as demonstrated in Fig. 4, the presence of GMP has no significant influence on the kinetics of the BHAM-resistant cytochrome pathway. Then, in order to check a possible effect of BHAM on the cytochrome pathway kinetics, the approach including determination of the two oxidizing pathway contributions was tested with external NADH as oxidizable substrate.

The Cytochrome and Alternative Pathway Contributions in State 3 Respiration With External NADH: ADP/O Measurements

Results obtained in the absence of GMP, the amoeba AOX activator, are shown in Fig. 5. In this case, BHAM has no effect on the ADP/O ratio at any Q reduction level (Fig. 5(A)) suggesting that AOX is not engaged. In the presence or absence of BHAM, a constant ADP/O value (1.333 ± 0.006, SD, $n = 33$) is observed for Q reduction levels above 30%. The respiratory rate in state 3 (V_3) versus Q redox state (Fig. 5(B)) as well as the phosphorylation rate (ADP/O $\times V_3$) versus Q redox state (Fig. 5(C)) are very similar with and without BHAM. Therefore, it can be concluded that BHAM is not a direct effector of the phosphorylating cytochrome pathway.

In the presence of GMP and BHAM, the ADP/O values (Fig. 6(A), ●) are the same as in the absence of GMP (Fig. 5(A), ●). Taking into account ADP/O values for Q reduction level above 33%, a mean value of 1.328 ± 0.014 (SD, $n = 32$) is found. Lower ADP/O values observed with GMP in the absence of BHAM (Fig. 6(A), □) suggest that engagement of the nonphosphorylating alternative pathway starts near 35% of the Q reduction level.

Assuming that the ADP phosphorylated per atom of oxygen consumed by the cytochrome pathway (($ADP/O)_{cyt}$) is independent of AOX activity, the cytochrome pathway contribution (V_{cyt}) and the alternative pathway contribution (V_{alt}) can be calculated using the ADP/O values in the absence (ADP/O) or presence (($ADP/O)_{+BHAM}$) of BHAM (Jarmuszkiewicz *et al.*, 1998). Indeed, as V_{cyt} is identical to $V_3 \times (ADP/O)/(ADP/O)_{cyt}$ where V_3 is the total respiratory rate ($V_{cyt} + V_{alt}$), the calculated contributions (V_{cyt}^c and V_{alt}^c)
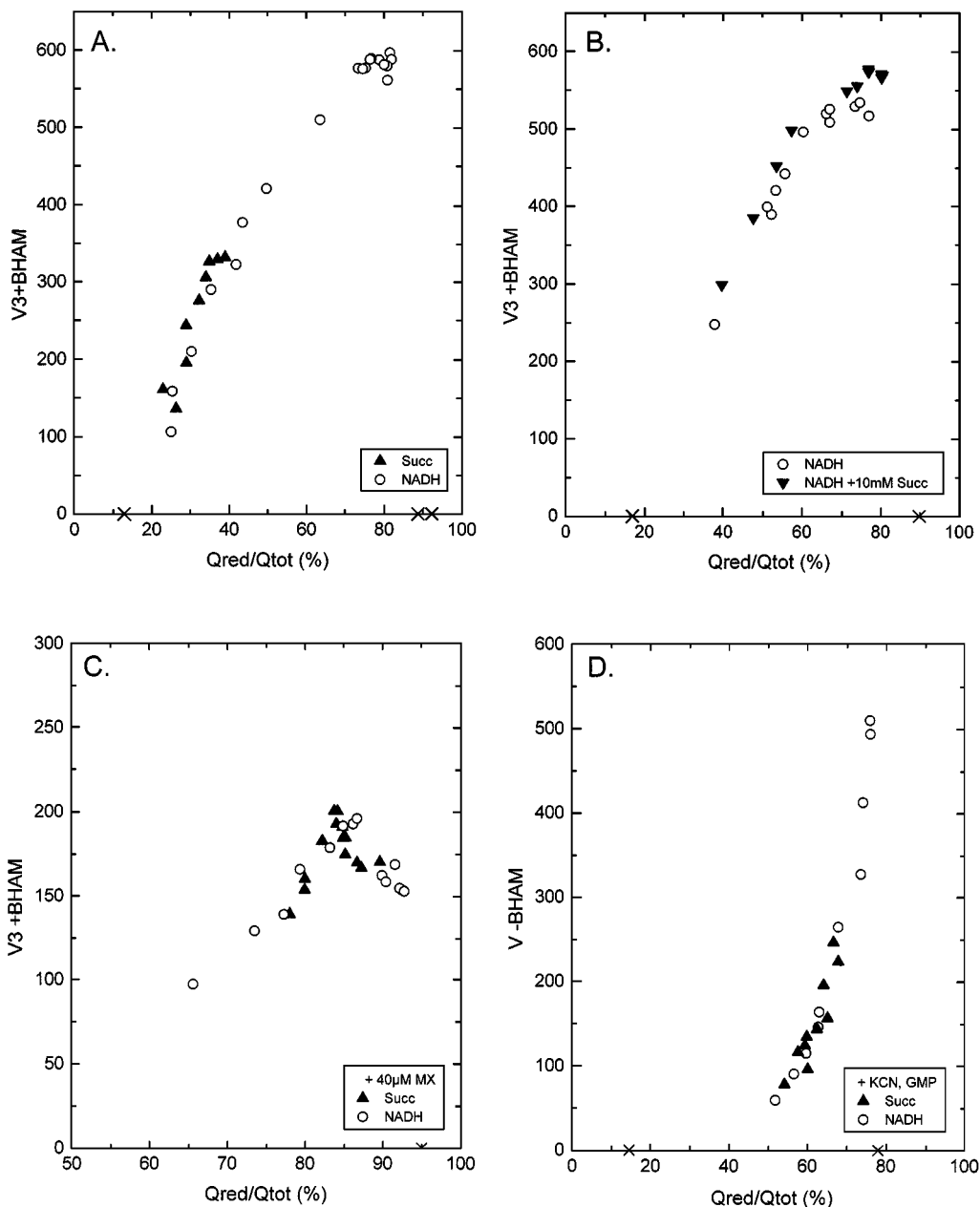
Fig. 3. Respiration as a function of quinone reduction level with succinate and/or external NADH. State 3 respiration (1.6 mM ADP) in the presence of 1.5 mM BHAM, in the absence (A), (B) or presence (C) of 40 μM myxothiazol (MX). (D) cyanide-resistant respiration in the presence of 1.5 mM KCN and 0.6 mM GMP. Level of Q reduction was varied by titrations of succinate and NADH oxidation as described under "Materials and Methods." For each comparison, shown data come from a single representative mitochondrial preparation. The asterisks correspond to values of completely oxidized and reduced Q_{red}/Q_{tot}. Respiratory rates (V) are in nanomoles of O per minute per milligram of protein.

will be obtained as follows:

$$V_{cyt}^c = V_3 \times \frac{ADP/O}{(ADP/O)_{+BHAM}} \quad \text{and} \quad V_{alt}^c = V_3 - V_{cyt}^c$$

$$(1)$$

The validity of these relationships does not require homogeneity within the Q pool as far as $(ADP/O)_{+BHAM}$ is independent of the Q redox state. However, if the Q pool is homogenous, as suggested by the results reported in Fig. 3, the actual V_{cyt} and V_{alt} contributions should also be equal
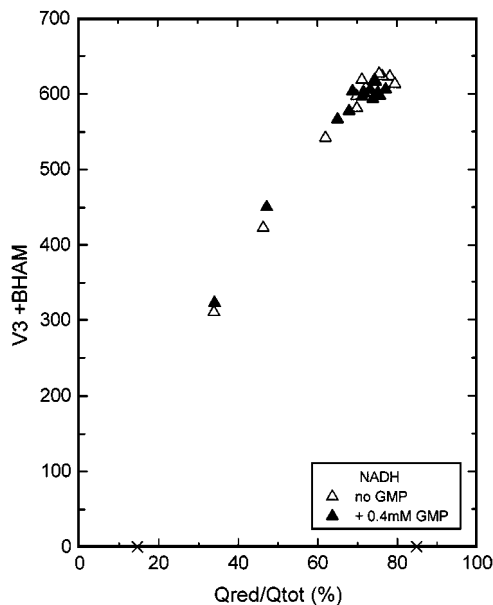
Fig. 4. Effect of GMP on the cytochrome pathway activity as a function of quinone reduction level with external NADH as a substrate. Mitochondria were respiring in the presence of 1.6 mM ADP (state 3 respiration), 1.5 mM BHAM and the absence or presence of 0.6 mM GMP. To vary Q reduction level titration of NADH oxidation was performed as described under "Materials and Methods." Data deal with a single representative mitochondrial preparation. The asterisks correspond to values of completely oxidized and reduced Q_{red}/Q_{tot}. State 3 respiratory rates (V_{3+BHAM}) are in nanomoles of O per minute per milligram of protein.

shown that BHAM enhances the ADP phosphorylation in the 60–70% Q reduction range (Fig. 6(C)) while it had no such effect in the absence of GMP (Fig. 5(C)). Therefore, it appears that for this high Q reduction level (without BHAM, with GMP, Fig. 6) the increase in AOX activity is accompanied by a decrease in the cytochrome pathway activity or in the $(ADP/O)_{cyt}$ ratio (i.e. intrinsic ratio of the cytochrome pathway). It must be stressed that if $(ADP/O)_{+BHAM}$ is higher than $(ADP/O)_{cyt}$, the calculated AOX contribution (Eq. 1) would be an overestimate of the true one. Then, the difference between the true AOX contribution and the KCN-resistant respiration would be higher than the already important difference shown in Fig. 6(C) (i.e. the difference between calculated AOX contribution and the KCN-resistant respiration). Moreover, KCN does not activate respiration when the cytochrome pathway is fully inhibited by a mixture of antimycin and myxothiazol (data not shown) excluding a stimulation of AOX by KCN.

Therefore, as KCN is not a direct effector of AOX and as BHAM is not a direct effector of the cytochrome pathway, but as nevertheless the inhibitors have an effect through the inhibition of one pathway on the kinetics of the other pathway, the kinetic parameters determining the steady-state activities at a given Q reduction level, for the ADP phosphorylation sustained by the cytochrome pathway on the one hand, and for the alternative oxidase on the other hand, are not fully independent of each other.

to V_{+BHAM} and V_{+KCN} respectively, when taken at the same Q_{red}/Q_{tot} ratio. Furthermore, the ADP phosphorylation rate at fixed Q_{red}/Q_{tot} should be BHAM-insensitive.

Figure 6(B) shows the calculated contributions of cytochrome and alternative pathway ($\circ$, V_{cyt}^c and $\triangle$, V_{alt}^c) together with the total respiration ($\square$), the BHAM-resistant respiration ($\bullet$) and the KCN-resistant respiration ($\blacktriangle$) for NADH as a substrate, in the presence of GMP. Unexpected differences are clearly visible at Q reduction levels higher than 60%. Namely, for both QH$_2$-oxidizing pathways, at a given Q_{red}/Q_{tot} value, the calculated activities are below the activities measured when the other pathway is inhibited (i.e. $V_{cyt}^c < V_{+BHAM}$ and $V_{alt}^c < V_{+KCN}$) and the total respiration (V_{-BHAM}) is lower than the sum of the two inhibitor-resistant respiration ($V_{+BHAM} + V_{+KCN}$) indicating that at least one of them must be different from the contribution it should represent. On the other hand, the calculated cytochrome pathway contribution (V_{cyt}^c) decreases with Q_{red}/Q_{tot} increasing from 70 to 80% (Fig. 6(B)). As shown in Fig. 6(C), a decrease occurs in the unquestionable phosphorylation rate in the absence of BHAM when the alternative pathway is activated by GMP. It is also

DISCUSSION

According to the protonmotive Q cycle mechanism of the cytochrome bc_1 complex (complex III), reduction of cytochrome c by QH$_2$ requires the reduction of one Q at center i (inner side of the membrane) for two QH$_2$ oxidized at center o (outer side of the membrane) (Trumpower, 1990). Owing to the autocatalytic character of this process, the reaction rate should pass through a maximum and decrease when the Q pool becomes highly reduced. However, this may be difficult to evidence if the affinity of center i for Q is very high. Moreover, as the crystal structure of the dimeric bc_1 complex exhibits two large cavities each connecting the center o of one monomer to the center i of the other (Yu *et al.*, 1998), a single ubiquinone may cycle within the complex decreasing the need for ubiquinone in the membranous bulk pool. Strong evidence in favor of the Q cycle mechanism is reported here for the first time in experiments where steady-state respiration exhibits an optimum in the presence of BHAM (Fig. 1(B), 2, 3(C)). In order to reach a range of Q_{red}/Q_{tot} ratio high enough (>80%) to observe a decrease in the respiratory rate in the
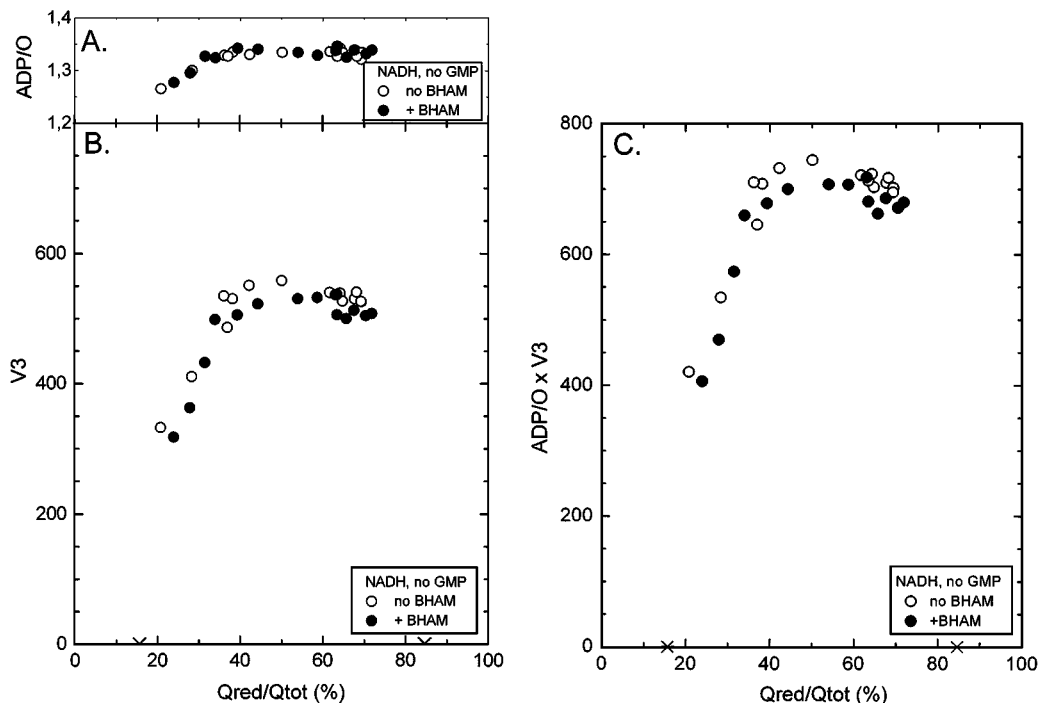
Fig. 5. ADP/O ratio (A), respiratory rate (B), and phosphorylation rate (C) as functions of quinone reduction level. Effect of BHAM in the absence of GMP. State 3 respiration measurements were made with pulse (210 μM) ADP, in the absence or presence of 1.5 mM BHAM with NADH as substrate. Level of Q reduction was varied by titration of NADH oxidation as described under "Materials and Methods." Data deal with a single representative mitochondrial preparation. The asterisks correspond to values of completely oxidized and reduced Q_{red}/Q_{tot}. State 3 respiratory rates (V_3) are in nanomoles of O per minute per milligram of protein.

presence of BHAM (when AOX activity is blocked) a partial inhibition of complex III by myxothiazol is necessary.

In the absence of myxothiazol, a decrease in the phosphorylation rate is observed when Q_{red}/Q_{tot} exceeds 70% in the presence of GMP and in the absence of BHAM i.e. when the alternative pathway can contribute to respiration (Fig. 6(C)). This decrease might be attributed to the Q cycle mechanism of complex III. However, the kinetic behavior of the cytochrome pathway appears to be influenced by the alternative pathway activity and *vice versa*. Indeed, for a given Q_{red}/Q_{tot} value, (i) the total respiratory rate is lower (instead to be equal) than the sum of the BHAM-resistant respiration and KCN-resistant respiration (Fig. 6(B)) and (ii) the phosphorylation rate in the absence of BHAM is lower (instead to be equal) than that in the presence of BHAM (for Q reduction level between 60 and 70%, Fig. 6(C)). The cytochrome and alternative pathway inhibitors (KCN and BHAM, respectively) are not directly responsible for these unexpected inequalities which appear to be related to the engagement of the alternative pathway as suggested by the comparison of ADP/O values in the presence and absence of BHAM (Fig. 6(A)).

Following the currently used kinetic approach described by Van den Bergen *et al.* (1994), the cyanide-resistant respiration at a given Q redox state could be tentatively considered as the AOX contribution in uninhibited respiration. Accordingly, the cytochrome pathway contribution would be the difference between the uninhibited respiration and the cyanide-inhibited respiration both measured at the same reduction of the Q pool. As reported in Fig. 7(B), this putative cytochrome pathway contribution starts decreasing dramatically at 60% Q reduction level. The ADP/O ratio of the phosphorylating pathway (the phosphorylation rate divided by the rate of putative O consumption of the cytochrome pathway, $Jp/(V-V_{+KCN})$) would increase with the Q_{red}/Q_{tot} ratio as shown in Fig. 7(A). However, the unlikely high values reached by this putative $(ADP/O)_{cyt}$ ratio invalidate the kinetic approach of Van den Bergen *et al.* (1994) in the case of amoeba mitochondria, demonstrating that the true AOX-contribution cannot be equal to (or higher than) the cyanide-resistant respiration. Consequently and unexpectedly according to Van den Bergen *et al.* (1994), inhibition of the cytochrome pathway by cyanide is
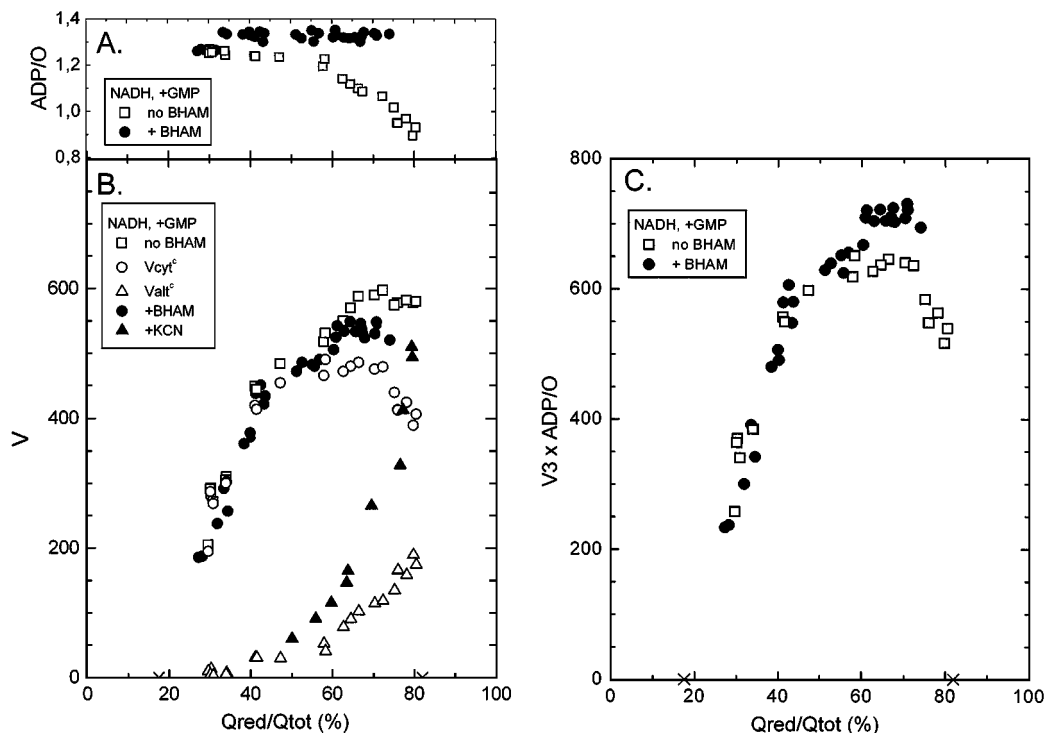
Fig. 6. ADP/O ratio (A), respiratory rate (B), and phosphorylation rate (C) as functions of quinone reduction level in the presence of GMP. State 3 respiration measurements were made with pulse ADP (210 μM), in the presence of 0.6 mM GMP, in the absence or presence of 1.5 mM BHAM, in the absence or presence of 1.5 mM KCN with NADH as substrate. To vary Q reduction level, titration of NADH oxidation was performed as described under "Materials and Methods." Calculations of V^c_{cyt} and V^c_{alt} were performed according to Eq. 1. Data deal with three similar mitochondrial preparations. The asterisks correspond to values of completely oxidized and reduced Q_{red}/Q_{tot}. Respiratory rates (V) are in nanomoles of O per minute per milligram of protein.

accompanied by an increase in the alternative pathway activity at a given Q_{red}/Q_{tot}. This AOX activation could suggest a local increase in the ubiquinol poise, but this cannot be the case. Indeed, site of QH_2 oxidation in the cytochrome pathway is localized near the external face of the mitochondrial inner-membrane (center *o* of complex III). Therefore, the cytochrome pathway inhibition might be accompanied by an increase in the Q reduction level on the external face of the membrane but also, as the measured overall Q reduction level is kept constant, by a decrease of the Q reduction level on the matricial face that could decrease the AOX activity because the QH_2 oxidation site of AOX is localized on the matricial side (Moore *et al.*, 1995). For the same topological reasons, kinetic compartmentation within the Q pool (due to a slow transmembrane diffusion) cannot cause an increase in the cytochrome pathway activity when AOX is inhibited by BHAM and consequently cannot explain the increase observed in the phosphorylation rate with BHAM addition (Fig 6(C)). Furthermore, the results shown in Fig. 3 are in favor of an homogenous reduction level in the active Q pool.

This new type of interplay described above between the two QH_2-oxidizing pathways at fixed QH_2 poise may be explained either by an indirect connection or by a direct interaction between the two pathways. Considering indirect connection, two complementary hypotheses should be made as follows: (i) activation of the alternative oxidase in the KCN-inhibited mitochondria (i.e. higher V_{+KCN} compared to V_{alt} at the same given Q_{red}/Q_{tot} value, Fig. 6(C)) is due to a high sensitivity of AOX to the matricial pH which is lower when the cytochrome pathway proton pumps are inactive and (ii) activation of the oxidative phosphorylation in the BHAM-inhibited mitochondria (observed as higher phosphorylating rate in the presence of BHAM compared to that in the absence of BHAM at the same given Q_{red}/Q_{tot} value, Fig. 6(C)) is due to the decrease in a H^+ leak pathway which is linked to the AOX activity. Then, the AOX contribution to the total respiration calculated using ADP/O values (V^c_{alt} in Fig. 6(B)) would be a sum of a leak-sustained contribution of the cytochrome pathway and the actual AOX contribution to respiration. A decrease in the actual cytochrome pathway contribution
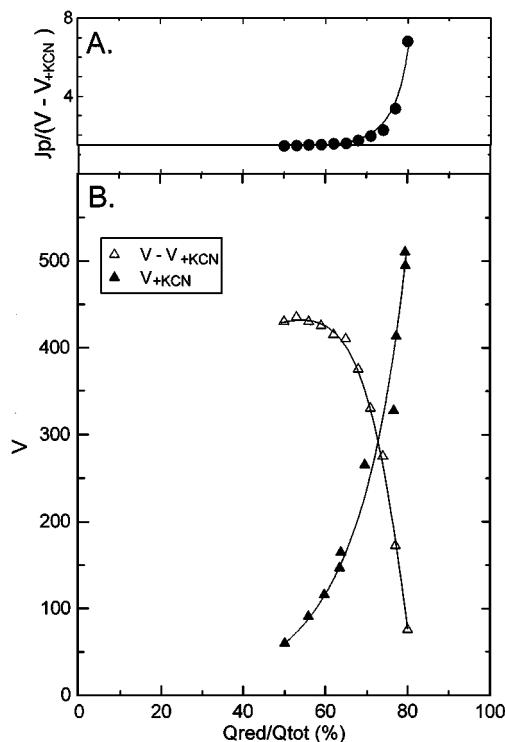
Fig. 7. Calculated phosphorylation yield of the cyanide-sensitive respiration (A), cyanide-resistant and sensitive respiration (B) as a function of quinone reduction level. Experimental conditions as in Fig. 6. V, respiration in the absence of inhibitors; V_{+KCN}, respiration in the presence of 1.5 mM KCN (experimental data from Fig. 6); $Jp/(V-V_{+KCN})$, calculated phosphorylation yield, where Jp is phosphorylation rate in the presence of 1.5 mM BHAM ((ADP/O) $\times V_3$); $V-V_{+KCN}$ and $Jp/(V-V_{+KCN})$ calculated using values interpolated from the data of Fig. 6 for the given Q reduction level. Respiratory rates (V) are in nanomoles of O per minute per milligram of protein.

when the Q pool becomes highly reduced (that is suggested by the constancy of the total respiration rate in the Fig. 6(B), while the AOX activity may be supposed to increase) should be due to the Q cycle mechanism when such indirect connection is assumed. In the case of the direct interaction between the two QH_2-oxidizing pathways, an association must be considered between AOX and one of the complexes of the phosphorylating pathway through protein–protein interaction, together with conformational changes of the proteins according to their ligand binding sites occupancy and the state of their redox centers. This would explain a variable influence of one protein on the enzymatic behavior of the other. The stoichiometry of proton translocation by the complex associated to AOX should remain unchanged and therefore the electron partitioning between the two ubiquinol-oxidizing pathways should be that determined from the ADP/O values.

The main conclusions emerging from this work are the following: (i) The cytochrome bc_1 complex of *Acanthamoeba castellanii* mitochondria seems to follow a protonmotive Q cycle mechanism in which Q cycling occurs, at least in part, via the membranous Q pool. (ii) The membranous active Q pool appears to be homogenous. (iii) The cytochrome and alternative pathways do not simply share the same substrate (ubiquinol) because other links between the two pathways result in diminished individual activities (i.e. respiration through AOX and phosphorylation sustained by the cytochrome pathway) if compared to those obtained at the same Q reduction level when the other pathway is blocked. Direct or indirect interactions can be responsible for this phenomenon.

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