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Electron Partitioning between the Two Branching Quinol-oxidizing Pathways in *Acanthamoeba castellanii* Mitochondria during Steady-state State 3 Respiration*

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Amoeba mitochondria possess a respiratory chain with two quinol-oxidizing pathways: the cytochrome pathway and the cyanide-resistant alternative oxidase pathway. The ADP/O method, based on the non-phosphorylating property of alternative oxidase, was used to determine contributions of both pathways in overall state 3 respiration in the presence of GMP (an activator of the alternative oxidase in amoeba) and succinate as oxidizable substrate. This method involves pair measurements of ADP/O ratios plus and minus benzohydroxamate (an inhibitor of the alternative oxidase). The requirements of the method are listed and verified. When overall state 3 respiration was decreased by increasing concentrations of *n*-butyl malonate (a non-penetrating inhibitor of succinate uptake), the quinone reduction level declined. At the same time, the alternative pathway contribution decreased sharply and became negligible when quinone redox state was lower than 50%, whereas the cytochrome pathway contribution first increased and then passed through a maximum at a quinone redox state of 58% and sharply decreased at a lower level of quinone reduction. This study is the first attempt to examine the steady-state kinetics of the two quinol-oxidizing pathways when both are active and to describe electron partitioning between them when the steady-state rate of the quinone-reducing pathway is varied.

The mitochondrial respiratory chain of the amoeba *Acanthamoeba castellanii* possesses, like that of plant mitochondria (1), the following: (i) a cyanide- and antimycin-resistant alternative oxidase (AOX)[1] in addition to the conventional cytochrome *c* oxidase (2, 3); (ii) the rotenone-insensitive external NADH dehydrogenase, located on the outer surface of the inner mitochondrial membrane (4, 5); and (iii) two internal NADH dehydrogenases, the rotenone-sensitive complex I and the non-electrogenic rotenone-insensitive dehydrogenase (6).

As in mitochondria from most higher plants, many fungi, and protozoa (7), the alternative pathway of amoeba mitochondria branches from the main respiratory chain at the level of quinone (Q), and electron flux through AOX is not coupled to the generation of protonmotive force and ADP phosphorylation (2). The cyanide-resistant respiration of amoeba mitochondria is strongly stimulated by purine nucleoside 5′-monophosphates: AMP, GMP (with the highest efficiency), and IMP (2, 8, 9). A similar effect of purine mononucleotides on the activity of the alternative pathway was observed in other microorganisms: *Euglena gracilis* (10), *Moniliella tomentosa* (11), *Neurospora crassa* (12), *Paramecium tetraurelia* (13), and *Hansenula anomala* (14). In contrast, the alternative oxidase of higher plant mitochondria is stimulated by α-keto acids, like pyruvate (7, 15, 16), but not by purine mononucleotides, whereas the α-keto acids do not stimulate the alternative respiration in amoeba mitochondria (17). These properties emphasize an important difference at the level of allosteric regulation between both types of AOX. However, monoclonal antibodies developed against *Sauromatum guttatum* cross-react with the AOX protein of amoeba mitochondria (18), as they do in a wide range of thermogenic and non-thermogenic plant species, some fungi, and trypanosomes (7, 16, 19), indicating that this protein is well conserved throughout the species.

Regulation of electron distribution between the energy-conserving cytochrome pathway and the redox potential-dissipating alternative pathway is obviously of the utmost importance for the energy economy of the cell. Several levels of control of AOX activity are known in plant mitochondria (for reviews, see Refs. 7, 16, 19, and 20): (*a*) the amount of the oxidase protein present in the mitochondria (gene expression); (*b*) the redox status of the protein (post-translational modification); (*c*) the presence of allosteric regulators (allostery); and (*d*) Q concentration in the inner mitochondrial membrane and the redox state of quinone (Q_{red}/Q_{tot}) (substrate-product availability). With the exception of the regulation by change in redox state of the protein, all these levels have been found for amoeba AOX (18, 21). Precise understanding of the interplay and quantitative analysis of these various levels of regulation can be reached only by measurements of the actual activities of the two branching oxidases during steady-state respiration. Such a study depends on the ability to measure the respective contributions of both pathways in total respiration. These determinations are hampered by the fact that both oxidative pathways have the same substrate and product and that the use of specific inhibitors must be considered as unsuitable because an

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[1] The abbreviations used are: AOX, alternative oxidase; Q, quinone; Q_{ox}, oxidized quinone; Q_{red} or $Q\text{-}H_2$, reduced quinone (quinol); Q_{tot}, total endogenous pool of quinone in the inner mitochondrial membrane ($Q_{ox} + Q_{red}$); Q_{red}/Q_{tot}, reduction level of quinone; ΔΨ, transmembrane electrical potential difference (external potential minus internal potential); BHAM, benzohydroxamate; v_3, rate of state 3 respiration; v_{cyt}, contribution of the cytochrome pathway; v_{alt}, contribution of the alternative pathway; $(ADP/O)_{cyt}$, ADP/O ratio in the presence of BHAM.

inhibition of one of the pathways inevitably affects electron flux via the other pathway (22, 23).

A kinetic approach has been developed (24) taking into account the interplay between quinol-oxidizing and quinone-reducing steady-state electron fluxes and the redox poise of the quinone pool. This method is based on the homogeneous quinone pool hypothesis and on a strict specificity of the inhibitors of each oxidative pathway that are used to measure the activity of one pathway when the other is inhibited. It allows the prediction of the real contribution of each pathway at a given total steady-state respiratory rate. Another method, considered to be the only method that exists at present for making quantitative measurements of AOX activity (23), has been developed for measuring relative contributions even *in vivo*. This method is based on the differential oxygen isotope discrimination between the cytochrome and alternative oxidase pathways (25, 26). Calibration used in this method requires the use of inhibitor for each respiratory pathway and assumes that change in the total respiration rate does not change the discrimination factor.

Because the alternative pathway does not contribute to ATP synthesis with succinate (in the presence of rotenone) as oxidizable substrate, comparison of the ADP/O ratio with and without an inhibitor of AOX (*e.g.* hydroxamic acids) allows the estimation of the contribution of this pathway in total mitochondrial respiration. A method based on ADP/O ratio determination proposed a long time ago (27, 28) has not been widely used, although it is one of the best as it avoids the use of the rates of electron transport of both pathways in the presence of inhibitors (cyanide and hydroxamic acids), and it does not require a strict specificity of the inhibitors of both pathways. Moreover, the ADP/O ratio method is not linked to the homogeneous quinone pool hypothesis. This method is claimed to have a limited range of application (29). Of course, it cannot be applied under state 4 conditions and could hardly be used with intact tissues. However, this method could be applied in permeabilized cells and in isolated mitochondria in state 3 respiration, even if an AOX inhibitor does not decrease total state 3 respiration. In this study, we have successfully developed the ADP/O method to determine the actual contributions of both the cytochrome and alternative pathways in isolated amoeba mitochondria and to describe how these contributions change when the steady-state rate of the quinone-reducing pathway is varied.

MATERIALS AND METHODS

Cell Culture and Mitochondrial Isolation—The soil amoeba *A. castellanii* (strain Neff) was cultured as described by Jarmuszkiewicz *et al.* (18). Trophozoites of amoeba were collected 22–24 h following inoculation at the middle exponential phase (at a density of ~2–4 × 10^6 cells/ml). Mitochondria were isolated and purified on a self-generating Percoll gradient (31%) as described before (18).

Assay Procedures—Oxygen uptake was measured polarographically using a Rank Brothers oxygen electrode in 3 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) bovine serum albumin with 1.5–1.8 mg of mitochondrial protein. The membrane potential of mitochondria (ΔΨ) was measured simultaneously with oxygen uptake using a tetraphenylphosphonium-specific electrode according to Kamo *et al.* (30). For calculation of ΔΨ, the matrix volume of amoeba mitochondria was assumed as 2.0 μl × mg^{-1} protein. Measurements of ΔΨ, performed in the presence of 8 μM tetraphenylphosphonium, allowed the fine control of the duration of state 3 respiration. The ADP/O ratio was determined by the ADP pulse method, as illustrated in Fig. 1. The oxidizable substrate was succinate (10 mM) in the presence of rotenone (15 μM) to block electron input from complex I. Increasing concentrations of *n*-butyl malonate from 2 to 20 mM were added to the incubation medium to decrease steady-state 3 respiration. A rather large amount of added ADP (480–520 nmol) was chosen to ensure an accurate determination of the steady-state O_2 consumption in state 3. The total amount of oxygen consumed during state 3 respiration was used to calculate the ADP/O ratio (see Fig. 1). A prepulse of ADP (200 nmol) was always applied before the main pulse to ensure that a true state 4 had been achieved and to activate succinate dehydrogenase by the produced ATP. To fully activate the alternative pathway, GMP, the allosteric effector of AOX in amoeba mitochondria, was added to the incubation medium at a concentration of 0.6 mM ($K_{0.5}$ = 20 μM) (9). Measurements of the ADP/O ratio were performed in the absence or presence of 1.5 mM benzohydroxamate (BHAM), a concentration sufficient to totally inhibit the KCN-insensitive respiration in amoeba mitochondria.

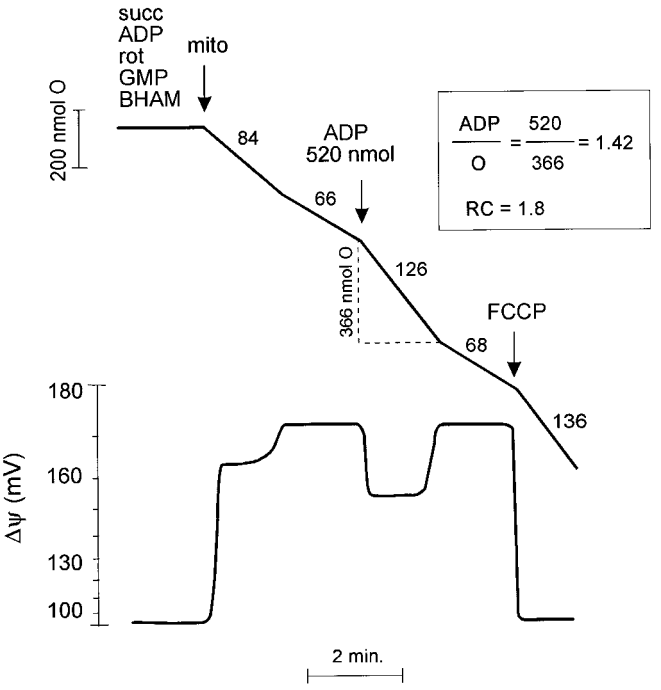

FIG. 1. **ADP/O ratio determination.** Assay conditions were as described under "Materials and Methods." Mitochondria (*mito*) were incubated in the presence of 10 mM succinate (*succ*), 200 nmol of ADP (prepulse), 15 μM rotenone (*rot*), 0.6 mM GMP, and 1.5 mM BHAM. The ADP pulse amounted to 520 nmol. Afterward, respiration was uncoupled by 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. *Numbers* on the trace refer to O_2 consumption rates in nmol of oxygen × min^{-1} × mg^{-1} protein. Membrane potential changes are presented in mV. *RC*, respiratory control.

The redox state of quinone in steady-state respiration was determined by an extraction technique according to Van den Bergen *et al.* (24). For calibration of the peaks, commercial Q-9 (Sigma) was used. Protein was estimated by the biuret method (31) with bovine serum albumin (fraction V) as a standard.

Chemicals—Tetraphenylphosphonium was obtained from Fluka, and *n*-butyl malonate was from Aldrich. All other chemicals were purchased from Sigma.

RESULTS

The method proposed in this paper is based on the ADP/O ratio determination for complexes III and IV in the presence and absence of BHAM, a specific inhibitor of the alternative oxidase, taking into account that the alternative pathway, when supplied with electrons by succinate as oxidizable substrate (+rotenone), is not energy-conserving. The method consists in measuring the ADP/O ratios and the rate of state 3 respiration during ADP pulses (Fig. 1). To describe how the contribution of each pathway changes with variations in the overall state 3 respiration (without BHAM), *n*-butyl malonate, a non-penetrating competitive inhibitor of succinate uptake, is used to decrease the rate of the quinone-reducing pathway. If $v_3 = v_{cyt} + v_{alt}$, where v_3 is the rate of state 3 respiration, v_{cyt} is the contribution of the cytochrome pathway in electron flux,

and v_{alt} is the contribution of the alternative pathway in electron flux, and if

$$\alpha = \frac{\mathrm{ADP/O}}{(\mathrm{ADP/O})_{cyt}} = \frac{\frac{\mathrm{ADP}}{O_{cyt}+O_{alt}}}{\frac{\mathrm{ADP}}{O_{cyt}}} = \frac{O_{cyt}}{O_{cyt}+O_{alt}} = \frac{v_{cyt}}{v_{cyt}+v_{alt}} \qquad \text{(Eq. 1)}$$

where ADP/O is the ADP/O ratio in the absence of BHAM (when both respiratory pathways are active), $(ADP/O)_{cyt}$ is the ADP/O ratio in the presence of BHAM (when only the cytochrome pathway is active), and O_{cyt} and O_{alt} are the amounts of oxygen taken up related to the activities of the cytochrome and alternative pathways, respectively, then Equations 2 and 3 follow.

$$v_3 \times \alpha = v_{cyt} = \text{contribution of the cytochrome pathway} \qquad \text{(Eq. 2)}$$

$$v_3 - v_{cyt} = v_{alt} = \text{contribution of the alternative pathway} \qquad \text{(Eq. 3)}$$

Requirements—The proposed ADP/O method is valid only if (i) the ADP/O ratio in the presence of cyanide is equal to zero; (ii) BHAM does not induce a proton leak (has no uncoupling effect) and thereby does not affect $\Delta\Psi$; (iii) the ADP/O ratio in the presence of BHAM is independent of the state 3 respiratory rates (within the applied range); and (iv) isolated mitochondria are well coupled and stable during the experimental procedure.

It is well known that the electron transport from ubiquinol to O_2 via the alternative pathway does not lead to the synthesis of ATP (for review, see Ref. 32). The non-electrogenic character of the alternative pathway, when supplied with electrons from complex II, was confirmed under conditions applied in this study (*i.e.* in the presence of the allosteric activator of the pathway (GMP) and rotenone). After the addition of KCN that excluded the activity of the cytochrome pathway (entire collapse of $\Delta\Psi$), no effect of either ADP or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone on the respiratory rate is observed (data not shown). Acceleration of the respiration caused by GMP, entirely sensitive to BHAM, is due exclusively to the stimulation of the electron flux through the alternative pathway. Thus, the ADP/O ratio in the presence of cyanide is certainly equal to zero.

Among the tested inhibitors of the alternative oxidase, only BHAM meets the second requirement of the method and, at the maximal concentration used (1.5 mM), does not decrease $\Delta\Psi$ generated by the cytochrome pathway during oxidation of succinate and also decreases the O_2 uptake in both state 4 and state 3 respiration (data not shown). In amoeba mitochondria, the other inhibitors (1.5 mM salicylhydroxamate and 100 μM *n*-propyl gallate) display a slight uncoupling effect on $\Delta\Psi$, decreasing it by ~2–5 mV.[2] Therefore, for further investigations, BHAM was chosen as the most suitable inhibitor of AOX. Moreover, there is no residual respiration in amoeba mitochondria in the presence of 1.5 mM BHAM and 1 mM KCN.

The constancy of the $(ADP/O)_{cyt}$ ratio (third requirement), when the operation of the alternative pathway was excluded by BHAM, was checked for state 3 respiration, ranging from 65 to 230 nmol of oxygen $\times$ min^{-1} $\times$ mg^{-1} protein (Fig. 2*A*). This range comprises data from 15 mitochondrial preparations with various rates of state 3 respiration gradually decreased by increasing concentrations of *n*-butyl malonate. The $(ADP/O)_{cyt}$ ratios obtained (1.41 ± 0.06 (S.D., n = 22) in the absence of GMP and 1.41 ± 0.07 (S.D., n = 76) in the presence of GMP) display the constancy required by the method, as they fluctuate randomly without any correlation with either v_3 or the respiratory control ratio (Fig. 2, *A* and *B*). This result is of the utmost importance regarding the validity of the calculation of α proposed above (Equation 1) and suggests that native proton leak has a negligible influence on the ADP/O ratios within the

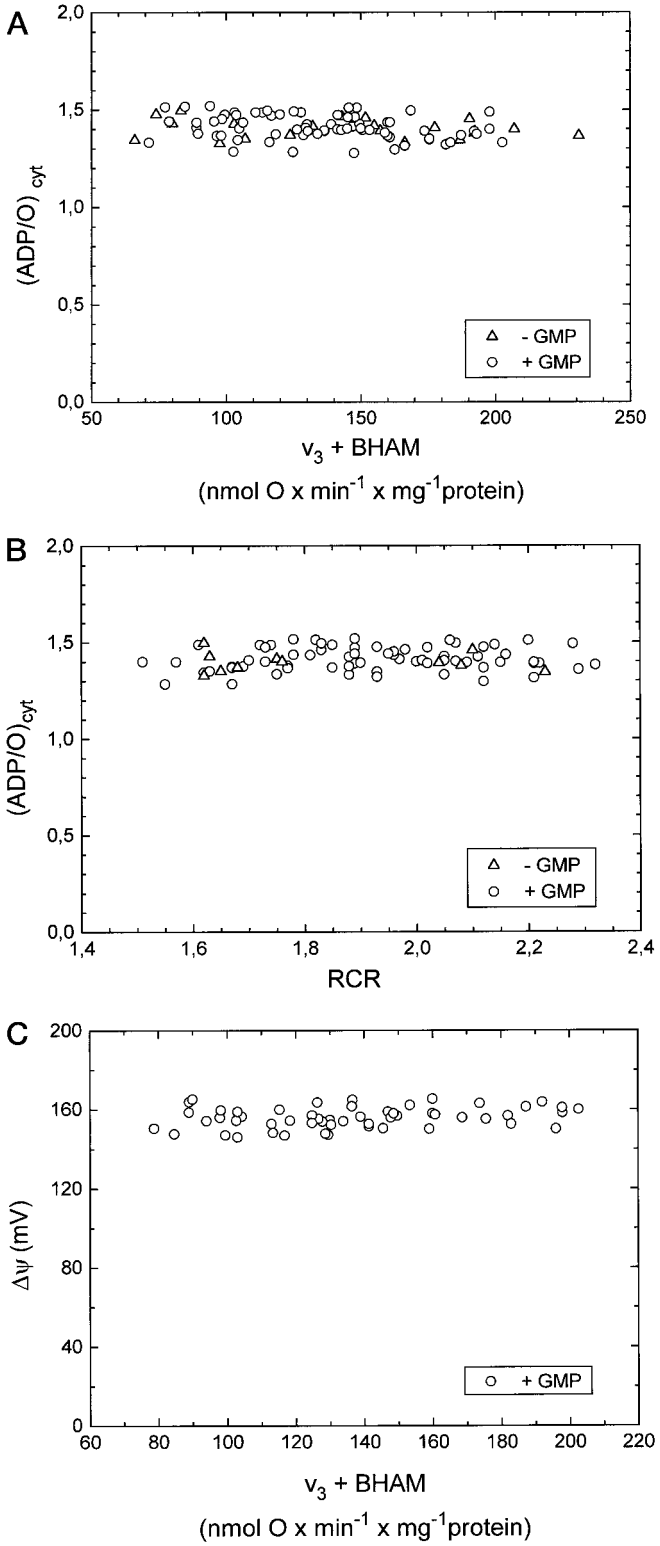

FIG. 2. **$(ADP/O)_{cyt}$ ratio *versus* state 3 respiration (*A*) and *versus* respiratory control ratio (*B*) and membrane potential *versus* state 3 respiration (*C*).** Assay conditions were as described for Fig. 1. Oxidation of succinate in the presence of BHAM was gradually decreased by increasing concentrations of *n*-butyl malonate (0.5–18 mM). *A* and *B*, plus and minus GMP; *C*, plus GMP. Data deal with 15 experiments with different rates of uninhibited state 3 respiration. The mean value of $(ADP/O)_{cyt}$ in the presence of BHAM (±GMP) is 1.41 ± 0.07 (S.D., n = 98). The mean value of $\Delta\Psi$ is 156 ± 6 mV (S.D., n = 56).

[2] L. Hryniewiecka, unpublished results.

TABLE I
Evolution of the ADP/O ratio (±BHAM) when state 3 respiration is decreased by n-butyl malonate

Assay conditions were as described for Fig. 1. Oxidation of succinate (in the presence of rotenone and GMP) was decreased by increasing concentrations of *n*-butyl malonate (0.6–16 mM). ADP/O ratios were determined in the presence and absence of 1.5 mM BHAM for the given concentrations of *n*-butyl malonate. Values of O_2 uptake in state 3 respiration are in nmol of oxygen × min^{-1} × mg^{-1} protein.

Conc of *n*-butyl malonate	v_3 − BHAM	ADP/O − BHAM	$(ADP/O)_{cyt}$ + BHAM
mM			
0	179	0.93	1.39
0	173	0.94	1.39
0.7	165	1.06	1.48
1.3	163	1.18	1.47
5.4	147	1.29	1.41
7.8	134	1.36	1.47
15.6	104	1.43	1.48

investigated range of v_3. Moreover, membrane potential measured during state 3 respiration triggered by ADP pulse in the presence of BHAM reveals a constant level of ΔΨ for the whole range of state 3 respiration (155.5 ± 5.5 mV, S.D., $n = 56$) (Fig. 2*C*). This constancy is also important in the interpretation of proton leak contribution in state 3 respiration (see "Discussion").

The ATPase activity has also been proven not to interfere with state 4 respiration in the presence of BHAM, as the addition of oligomycin does not affect the respiratory rates (as an example, 68 ± 4 and 70 ± 2 nmol of oxygen × min^{-1} × mg^{-1} protein without and with oligomycin, respectively). On the other hand, the addition of oligomycin in state 3 decreases the respiratory rate exactly to that observed in state 4 (from 144 ± 6 to 68 ± 3 nmol of oxygen × min^{-1} × mg^{-1} protein). Finally, mitochondria isolated from amoeba are characterized by a considerable stability. They exhibit a good coupling of the oxidative phosphorylation, which is not significantly affected within the duration of an experiment (up to 10 h). Thus, all considered conditions required to determine the respective contributions of both respiratory pathways in total respiration were positively verified for amoeba mitochondria.

Contribution of Both Respiratory Pathways in Total Respiration—Table I shows an example of the ADP/O ratios obtained from a single mitochondrial preparation in the presence and absence of BHAM, used for calculation of the contributions of both pathways in total respiration. The rates of state 3 respiration with succinate (in the presence of rotenone and GMP and in the absence of BHAM) are decreased with increasing concentrations of *n*-butyl malonate from the control value of 179–173 to 103 nmol of oxygen × min^{-1} × mg^{-1} mitochondrial protein. At the same time, the ADP/O ratios increase gradually from 0.93–0.94 to 1.43, approaching the value observed in the presence of BHAM ($(ADP/O)_{cyt}$). It must be noted that the $(ADP/O)_{cyt}$ ratio is almost constant whatever v_3 is (mean ± S.D. for this experiment = 1.44 ± 0.04, $n = 7$). These results indicate that the changes in the ADP/O ratios determined in the absence of BHAM are due to the change in the activity of the alternative pathway, which gradually decreases together with the lowering of electron transfer from complex II to quinone.

From the pair measurements of ADP/O ratios in the presence and absence of BHAM, at various state 3 respiration rates, the contributions of both pathways have been calculated (according to Equations 1–3) for series of mitochondrial preparations, which, in the absence of *n*-butyl malonate, present two different rates of state 3 respiration: 180 and 212 nmol of oxygen × min^{-1} × mg^{-1} mitochondrial protein (Fig. 3*A*). When the calculated v_{cyt} and v_{alt} contributions are plotted as a function of v_3,

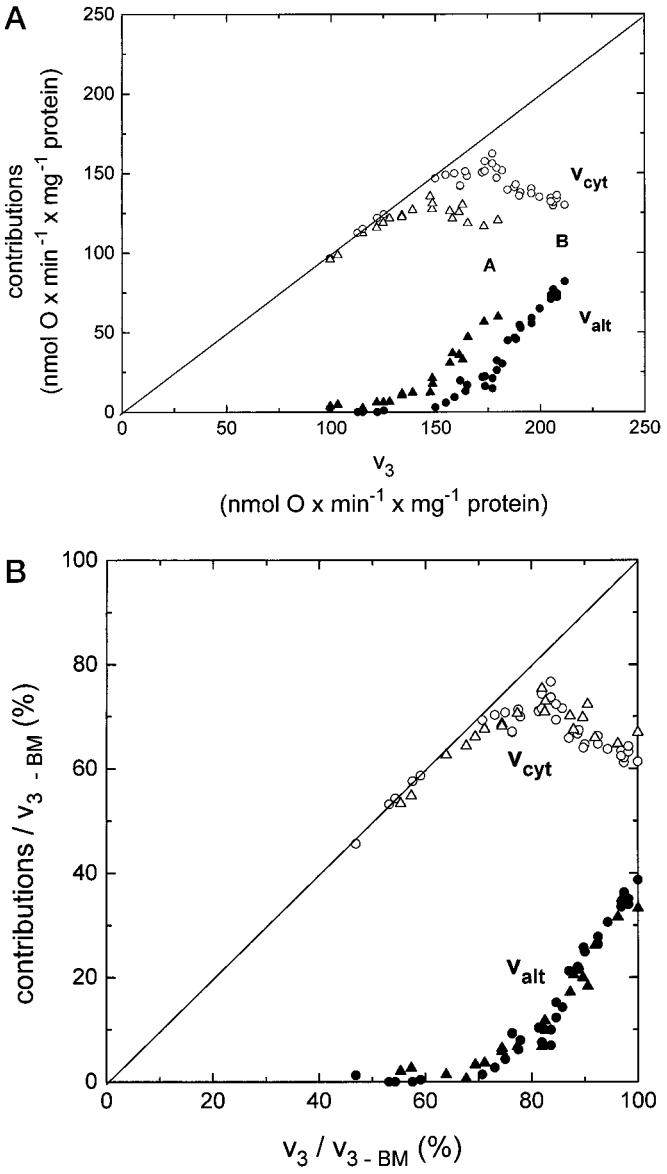

FIG. 3. **Contributions of the cytochrome pathway (v_{cyt}) and of the alternative pathway (v_{alt}) in state 3 respiration (*A*) and normalized contributions of the cytochrome and alternative pathways *versus* normalized state 3 respiration (*B*).** *A*, assay conditions were as described for Fig. 1. Calculations of v_{cyt} and v_{alt} were performed according to Equations 1–3. Oxidation of succinate (in the presence of rotenone and GMP) was decreased by increasing concentrations of *n*-butyl malonate (0.5–18 mM). Contributions (v_{cyt} (△ and ○) and v_{alt} (▲ and ●)) and state 3 respiration are expressed in nmol of oxygen × min^{-1} × mg^{-1} protein. Points of groups A (△ and ▲) and B (○ and ●) were obtained from two sets of experiments exhibiting similar respiratory rates in the absence of *n*-butyl malonate (180 and 212 nmol of oxygen × min^{-1} × mg^{-1} protein, respectively). *B*, normalized contributions of both pathways are expressed in percent and were obtained from the data in *A* as described under "Results." *Triangles* correspond to group A, and *circles* correspond to group B in *A*. *BM*, *n*-butyl malonate.

two groups of results are observed (groups A and B in Fig. 3*A*). Each group exhibits a similar behavior. Inhibition of v_3 by increasing concentrations of *n*-butyl malonate is accompanied by a sharp decrease in the v_{alt} contribution, whereas v_{cyt} first slightly increases, passes through a maximum, and then clearly decreases when v_{alt} becomes very small. When results of groups A and B are divided by the respective values of v_3 in the absence of *n*-butyl malonate (contributions and v_3 are then expressed in percent), both groups belong to the same v_{cyt} and v_{alt} curves (Fig. 3*B*) that exhibit the same profiles as curves in Fig. 3*A*.

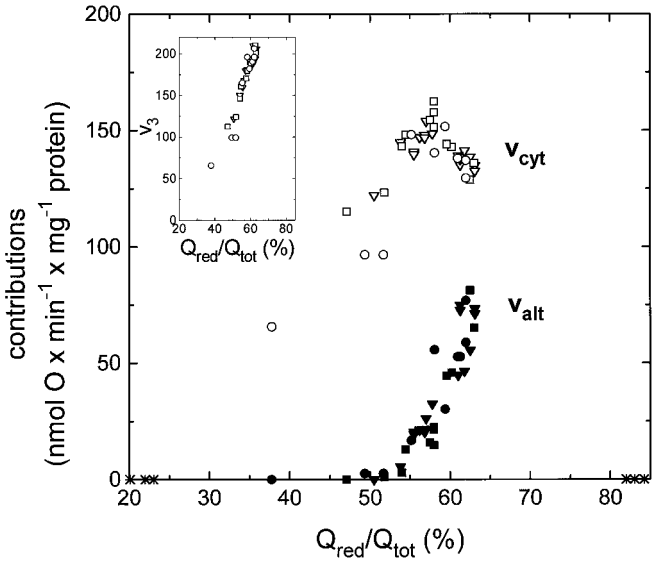

FIG. 4. **Dependence of v_{cyt}, v_{alt}, and v_3 (*inset*) on quinone reduction level.** Assay conditions were as described under "Materials and Methods." Calculations of v_{cyt} and v_{alt} were performed according to Equations 1–3. The three symbols (○/●, ▽/▲, and □/■) correspond to the three experiments of group B in Fig. 3*A*. The *asterisks* correspond to values of Q_{red}/Q_{tot} obtained in the absence of substrate (mean value = 22%) or upon anaerobiosis and in the presence of 1 mM KCN and 1.5 mM BHAM (mean value = 83%).

The three experiments belonging to group B (Fig. 3*A*) include also determination of Q reduction level. It was previously found that the endogenous quinone in amoeba mitochondria is Q-9 (21). As shown in Fig. 4, reproducible results are obtained in the three experiments when v_3 is decreased by *n*-butyl malonate from 212 to 100 nmol of oxygen × min^{-1} × mg^{-1} protein (see Fig. 3*A*). When the two contributions (v_{cyt} and v_{alt}) calculated for the decreasing rate of state 3 respiration are presented as a function of Q_{red}/Q_{tot} (Fig. 4), it can be observed that 1) the Q reduction level decreases from 65 to 38%; 2) v_{alt} decreases sharply and vanishes when Q_{red}/Q_{tot} is lower than 53%; and 3) v_{cyt} first slightly increases, reaching a maximum (~160 nmol of oxygen × min^{-1} × mg^{-1} protein) when the Q_{red}/Q_{tot} value is ~58%, and then decreases. The quinone reduction level obtained in the absence of substrate (mean value of 22%) and upon anaerobiosis and complete inhibition of the quinol-oxidizing pathways (mean value 83%) suggests the presence of the inactive Q-H_2 and Q pools as observed with some plant mitochondria (24). In amoeba mitochondria, the inactive pools would constitute 22% (Q-H_2) and 17% (Q) of the total quinone pool size, and the active pool could be ~60% of the total membrane quinone.

DISCUSSION

The $(ADP/O)_{cyt}$ Ratio and the ADP/O Method—It is obvious that determination of the cytochrome and alternative pathway contributions using the ADP/O ratios requires an $(ADP/O)_{cyt}$ value independent of v_{cyt} (*i.e.* electron flux through the cytochrome pathway) when the electron supply by dehydrogenase is modified. Such independence was not a priori expected because it has been discredited by several examples in the literature. In yeast mitochondria where complex I is missing, the ADP/O ratio increases when the respiratory rate decreases below 100 nmol of oxygen × min^{-1} × mg^{-1} protein at a constant protonmotive force, indicating a change in the mechanistic stoichiometry of one of the proton pumps (33). However, when in these mitochondria the respiratory rates measured with various substrates of internal dehydrogenases are higher than 100 nmol of oxygen × min^{-1} × mg^{-1} protein, a constant ADP/O ratio of 1.5 is observed. Other results (34) suggest similar flux efficiency dependence in potato tuber mitochondria (where the alternative oxidase activity is missing). Nevertheless, when in these mitochondria the succinate-dependent state 3 respiratory rate (in the absence of rotenone) is decreased by malonate, an inhibition by 75% does not change the ADP/O ratio (~1.4). Thus, it seems possible that no flux efficiency dependence occurs for succinate-sustained respiration through the cytochrome pathway when electron input is sufficient.

The results reported here show that the $(ADP/O)_{cyt}$ value determined for succinate oxidation (plus rotenone) by the ADP pulse method in the presence of BHAM is constant for respiratory rates between 65 and 230 nmol of oxygen × min^{-1} × mg^{-1} of mitochondrial protein (Fig. 2*A*). Therefore, there is no flux efficiency dependence within this range of respiratory rates. The v_{cyt} values (Fig. 3) were obtained within the respiratory rate range characterized by $(ADP/O)_{cyt}$ constancy as required for the ADP/O method. Of course, to calculate v_{cyt}, one must assume that the $(ADP/O)_{cyt}$ value that characterizes the efficiency of the energy-conserving system of the cytochrome pathway is not modified by the AOX activity and is independent of the presence or absence of BHAM. Such a hypothesis is unavoidable regardless of the method used (including the one based on oxygen isotope discrimination (25, 26, 35) in which discrimination values are used instead of ADP/O ratios to estimate the partitioning of electron flow into both quinol-oxidizing pathways).

The constancy of $(ADP/O)_{cyt}$ observed with isolated amoeba mitochondria (Fig. 2, *A* and *B*) indicates that the proton pumps' stoichiometries remain essentially constant within the range of respiratory rates used and that the influence of the proton leak is either negligible or constant. However, a constant influence of the proton leak on the $(ADP/O)_{cyt}$ value would require that the proton leak remain in a constant ratio with v_{cyt}, and consequently, $\Delta\Psi$ is expected to vary in the same direction as v_{cyt}. It has been observed that in state 3 respiration in the presence of BHAM, $\Delta\Psi$ does not correlate significantly with this respiratory rate (Fig. 2*C*), but remains almost constant (156 ± 6 mV, S.D., n = 56). It can be concluded that the proton leak is likely to be negligible in state 3 respiration. In relation with this conclusion, it should be noted that the $(ADP/O)_{cyt}$ value (1.41 ± 0.07, S.D., n = 8) is very close to the theoretical value (1.46 ± 0.04, S.E.) calculated for zero proton leak in rat liver mitochondria by Van Dam *et al.* (36) (see also Ref. 37).

It has also been observed that the state 4 respiratory rates in the presence of BHAM, sustained mainly by the proton leak (as they are insensitive to oligomycin), are not small compared with the respiratory rates in state 3 (respiratory control ratio ranges from 2.3 to 1.5; Fig. 2*B*) and that $\Delta\Psi$ in state 4 (174 ± 5 mV, S.D., n = 42) is only 18 mV higher than in state 3 respiration. If the proton leak is negligible in state 3 respiration, these observations suggest the existence of an acute "non-ohmic" apparent conductance of the inner mitochondrial membrane of amoeba.

Contributions of the Two Pathways—This study is the first direct approach that has examined the steady-state kinetics of the two quinol-oxidizing pathways when both are active. Indeed, ADP/O ratio determination in the presence and absence of BHAM has made possible the description of the behavior of the steady-state contribution of both quinol-oxidizing pathways under state 3 conditions when the rate of succinate transport is inhibited by different concentrations of *n*-butyl malonate. Furthermore, it has been verified that the total respiratory rate and the quinone reduction level evolve in the same direction (Fig. 4, *inset*). When the two contributions are presented as a function of the total respiratory rate (Fig. 3) or as a function of

Q_{red}/Q_{tot} (Fig. 4), similar curves are obtained. The mitochondrial preparations behave reproducibly despite quantitative differences when contributions are plotted *versus* v_3 (Fig. 3*A*); these differences disappear when normalized rates are considered (Fig. 3*B*).

In amoeba mitochondria, the AOX activity stimulated by GMP is significant in the absence of *n*-butyl malonate (equal to 40% of the total respiratory rate), but decreases sharply and becomes negligible at 40% inhibition of the total respiratory rate (Fig. 3*B*). In contrast to the cytochrome pathway, the alternative pathway is not engaged when the reduction level of the active pool of quinone is lower than 50% (Fig. 4). In plant mitochondria, when the cytochrome pathway is blocked by a specific inhibitor, the respiratory rate mediated by AOX exhibits, in most cases, a threshold-like relationship with the Q redox state (for review, see Ref. 32). A typical example is that of soybean cotyledon mitochondria (38), for which a mechanism with a two-step reduction of the alternative oxidase by quinol has been proposed (39, 40). In conclusion, it appears that in isolated amoeba mitochondria, the kinetic behavior of AOX (when both pathways are active) is similar to that observed with plant mitochondria when the cytochrome pathway is blocked.

The steady-state kinetics of the cytochrome pathway presents an unexpected soft maximum (Figs. 3 and 4). The cytochrome pathway activity has been reported to be proportional to the steady-state reduction level of Q (for a discussion of such kinetics, see Ref. 41), first in uncoupled submitochondrial particles from beef heart mitochondria by Kröger and Klingenberg (42) and then also under state 3 and state 4 conditions in plant mitochondria (for reviews, see Refs. 20 and 32). The peculiar behavior of the cytochrome pathway in the amoeba *A. castellanii* (*i.e.* the observed maximum in v_{cyt}) may be due to 1) a special feature of the cytochrome pathway activity when Q_{red}/Q_{tot} is varied together with a possible modification of the cytochrome *c* redox state imposed by the steady-state conditions within the cytochrome pathway or 2) variation in the concentration of a putative inner effector of the cytochrome pathway antagonizing the substrate effect of Q_{red}/Q_{tot} variations.

The first proposal might be met in the following way. The protonmotive Q cycle (43) is generally accepted as the mechanism by which complex III (cytochrome bc_1 complex) links the redox reaction between Q-H_2 and oxidized cytochrome *c* to the proton translocation from the matrix to the cytosol. In this mechanism, the catalytic cycle involves oxidation of two Q-H_2 molecules at an enzymatic site localized on the cytosolic side of the complex (center *o*) and the reduction of one Q molecule on the matrix side (center *i*). It can be supposed that for both reactions, Q and Q-H_2 belong to the active Q pool of the mitochondrial membrane (44). Then, the steady-state activity requires the presence of the oxidized form of quinone in the bulk pool. A situation in which the steady-state Q_{red}/Q_{tot} ratio should be exactly equal to 1 (fully reduced quinone) would lead to zero activity, and this implies that the activity of complex III as a function of Q_{red}/Q_{tot} exhibits a maximum. However, if the Q-binding site of center *i* is saturated even at a low Q level (binding site with a very high affinity for Q), the maximum will be situated at a Q_{red}/Q_{tot} value very close to 1, and it could be not detectable. Indeed, in such a case, the reaction occurring at site *i* does not need to be explicitly taken into account (*e.g.* Ref. 45), so a steady-state kinetic analysis could be performed considering the global reaction Q_{red} + cyt c_{ox} + $E \rightarrow Q_{ox}$ + cyt c_{red} + E (where cyt *c* is oxidized or reduced cytochrome *c* and *E* is complex III), which follows a ping-pong mechanism (46, 47). It can be speculated that the kinetic constants of complex III in amoeba mitochondria may be such that the mechanistic scheme does not simplify in this way (*i.e.* that the quinone reduction at site *i* is interfering), so that a maximum of activity can be observed in the steady-state kinetics of the cytochrome pathway. If this explanation is correct, the maximum in v_{cyt} must exist in whatever way the electrons are supplied to the Q pool. Disappearance of the maximum with another reducing substrate would favor the second proposal, which assumes the existence of a new factor of regulation of the cytochrome pathway in amoeba mitochondria. Therefore, further studies are needed to for a straightforward interpretation of this peculiar behavior of the cytochrome pathway in amoeba.

It can be concluded that the ADP/O method is valid for direct determination of activities of the cytochrome and alternative pathways in isolated mitochondria during steady-state phosphorylating respiration. Moreover, the approach described in this paper has led to the first detailed examination of the steady-state kinetics of the two quinol-oxidizing pathways when both are active and to the description of electron partitioning between them when the steady-state rate of the quinone-reducing pathway is decreased.

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