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THE EFFECT OF RESPIRATORY INHIBITORS DURING GROWTH AND DEVELOPMENT OF *DICTYOSTELIUM DISCOIDEUM*

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Two major pathways of electron transport to oxygen were identified in intact cells of *Dictyostelium discoideum*, a cyanide-sensitive pathway and a cyanide-insensitive, salicylhydroxamic-acid-sensitive pathway. The extent to which each pathway contributed to the total respiratory activity was shown to change during exponential growth and throughout development. During exponential growth both pathways appear to be utilized to varying degrees dependent on culture age, during late exponential growth the activity of the salicylhydroxamic-acid-sensitive pathway would seem to be almost totally lost. During development the cyanide-sensitive pathway appears to be dominant up to the aggregation stage, but both pathways are active in pseudoplasmodial cells. It is also suggested that the presence of iron in the growth medium may be essential directly or indirectly, for the maintained activity of the salicylhydroxamic-acid-sensitive pathway late in exponential growth.

Introduction

Cyanide-resistant respiration involving the presence of a cyanide resistant cytochrome oxidase is widespread [1,2] and has been reported in a wide range of microbes such as the protozoa *Trypanosoma brucei* [3], *Acanthamoeba castellanii* [4], the bacteria *Azotobacter vinelandii* [5], *Pseudomonas aeruginosa* [6], *Paracoccus denitrificans* [7] and in many plants [8]. The oxidases involved in the cyanide resistant respiration of *T. brucei* [3] and the plant *Arum maculatum* [9,10] have also been extensively characterized.

A major advance in understanding the value of cyanide insensitive respiration was the discovery that the pathway can be inhibited by substituted benzhydroxamates (salicylhydroxamic acid) [11] and this led to a large number of publications which claimed that the cyanide insensitive, salicylhydroxamic acid sensitive alternative oxidase has a role in a variety of intact cells [12].

Limited work with *Dictyostelium discoideum* has also indicated that vegetative cells are not totally inhibited by cyanide [13]. In this organism, the addition of cyanide to both dormant and germinating spores also revealed the presence of a second pathway for oxygen uptake which was inhibited by salicylhydroxamic acid, although the inhibition of the salicylhydroxamic acid sensitive pathway did not result in spore destruction [14].

This paper describes the effect of specific inhibitors of mitochondrial electron transport on the respiration of *D. discoideum* during growth and development.

Methods

Organism and growth conditions. Dictyostelium discoideum AX-2 amoebae were grown as previously described [15]. Cells were counted in a

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Fuchs-Rosenthal haemocytometer slide (Weber and Sons, Lancing, Sussex, U.K.).

Measurement of oxygen uptake rates. Oxygen uptake rates were measured polarographically with a Clark oxygen electrode [16]. Measurements were made on 1 or 2 ml of undiluted suspensions of cells in growth medium at 22°C. Potassium cyanide, sodium arsenite and sodium malonate, all at pH 7.4, were used as aqueous solutions. Antimycin A, rotenone and salicylhydroxamic acid were used as ethanolic solutions and additions were made so that the final ethanol concentration was not more than 1.0% (v/v).

Development conditions. Development was initiated by washing and plating approx. $5 \cdot 10^7$ cells on a Millipore filter (AABP047) supported on a Millipore absorbent pad, which was saturated with a solution containing 50 mM phosphate buffer (pH 6.4) and 1.5 g KCl, 0.5 mg $MgCl_2$ per l. A second method was to spread cells on 2% w/v agar plates. In all cases incubation was at 22°C, which resulted in mature fruiting body formation after a period of between 24 and 30 h.

Results

Sensitivity of cells to respiratory inhibitors

Initial experiments to investigate the effect of the respiratory inhibitors, cyanide, salicylhydroxamic acid and antimycin A on whole cell respiration showed that at different stages of exponential growth cells showed varying responses. The responses of cells to all three inhibitors in the early exponential phase of growth (cell density, $3 \cdot 10^5$ cells/ml) are shown in Fig. 1. Cyanide stimulated respiration up to a concentration of 3 mM with maximum stimulation of 40% at 0.7 mM cyanide. Stimulation also occurred in the presence of salicylhydroxamic acid alone. Antimycin A caused up to 50% inhibition of respiration at a concentration of 80 nmol/10^6 cells, and at no concentration tested did it cause stimulation of respiration. Cyanide and salicylhydroxamic acid added together always resulted in greater inhibitions of respiration than that produced with either inhibitor alone. Antimycin A would appear not be be as effective an inhibitor as cyanide, with or without the presence of salicylhydroxamic acid.

At later stages of exponential growth (Fig. 2) the stimulatory effect of cyanide which had been observed with early exponential cells was lost. At a culture density of $9 \cdot 10^5$ cells/ml slight stimulation occurred at very low concentrations of cyanide but 8% inhibition occurred at high concentrations. With late exponential phase cells, cyanide became an effective inhibitor of respiration with up to 95% inhibition occurring at 2.5 mM. Similarly, Antimycin A became more effective with increasing culture age (Fig. 2). However, the effect of salicylhydroxamic acid was different with a concentration of 1 mM, causing 40% inhibition at a cell density of $9.0 \cdot 10^5$ cells/ml, but showing 20% stimulation when the cell density had increased to $6.0 \cdot 10^6$ cells/ml. Once again cyanide and salicylhydroxamic acid together resulted in greater inhibition than either inhibitor alone.

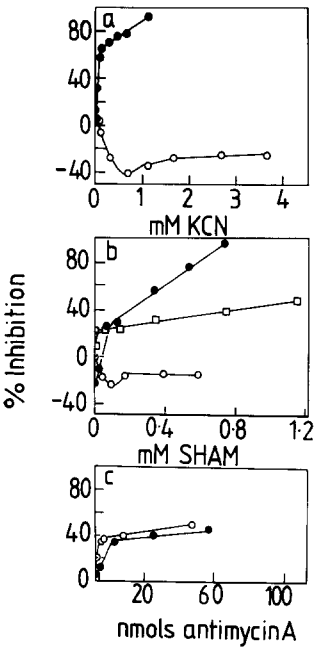

Fig. 1. The effect of respiratory inhibitors salicylhydroxamic acid, cyanide and antimycin on whole-cell respiration of *D. discoideum*. Inhibition or stimulation of respiration expressed as a percentage of inhibitor titre; the controls (0%) were the respiration rates in the absence of inhibitor. Cells were from a culture of cell density $3 \cdot 10^5$ cells/ml. (a) Effect of cyanide alone (○) or with 1 mM salicylhydroxamic acid (●). (b) Effect of salicylhydroxamic acid (SHAM) alone (○) with 1 mM KCN (●) or antimycin A, at a concentration of 50 nmol per 10^6 organisms (□). (c) Effect of antimycin A alone (○) or with 1 mM salicylhydroxamic acid (●).

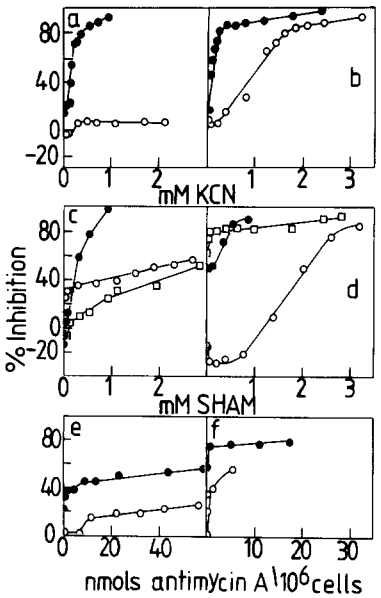

Fig. 2. The effect of respiratory inhibitors on whole-cell respiration during later stages of exponential growth. Cells were from cultures of cell density $9.0 \cdot 10^5$ cells/ml (a, c and e) or $6 \cdot 10^6$ cells/ml (b, d and f). (a, b) Effect of cyanide alone (○) or with 1 mM salicylhydroxamic acid (SHAM) (●). (c, d) Effect of salicylhydroxamic acid alone (○), with 1 mM cyanide (●) or in the presence of antimycin A, at a concentration of 50 nmols per 10^6 organisms (□). (e, f) Effect of antimycin A alone (○) or with 1 mM salicylhydroxamic acid (●).

As salicylhydroxamic acid and antimycin A were administered as ethanolic solutions the effect of ethanol on respiration was studied as a control. In all experiments, the total ethanol concentration was less than 1% which gave rise to slight stimulation of respiration with exponentially growing cells. With late exponential/stationary phase cells, however, slight inhibition up to a maximum of 2% was evident.

Changes in the effect of 0.5 mM salicylhydroxamic acid and 1 mM cyanide during exponential growth

Due to the different responses shown in the previous experiments the effect of fixed concentrations of salicylhydroxamic acid and cyanide through the entire growth phase to stationary phase was followed. Cell numbers increased exponentially with a generation time of approx. 9.0 h, along with a parallel increase in respiration rate. The stationary phase population was reached after approx. 80 h.

Addition of 1 mM cyanide initially (at time $t = 0$; cell density, $1.0 \cdot 10^5$ cells/ml) inhibited respiration by about 20%, but this effect was lost as the culture grew and respiration of older cells was stimulated by cyanide with a peak of stimulation occurring at 20 h ($3.5 \cdot 10^5$ cells/ml). After 28 h ($7.0 \cdot 10^5$ cells/ml) whole cell respiration became increasingly more sensitive to cyanide until the maximum inhibitory effect was observed after 60 h ($9.0 \cdot 10^6$ cells/ml) of growth (Fig. 3a). Addition of 0.5 mM salicylhydroxamic acid showed a pattern markedly different from that of cyanide. Respiration was stimulated slightly up until 15 h; then a progressive increase in inhibition occurred reaching a peak at 30 h. This was followed by a decrease in inhibition until stimulation once again

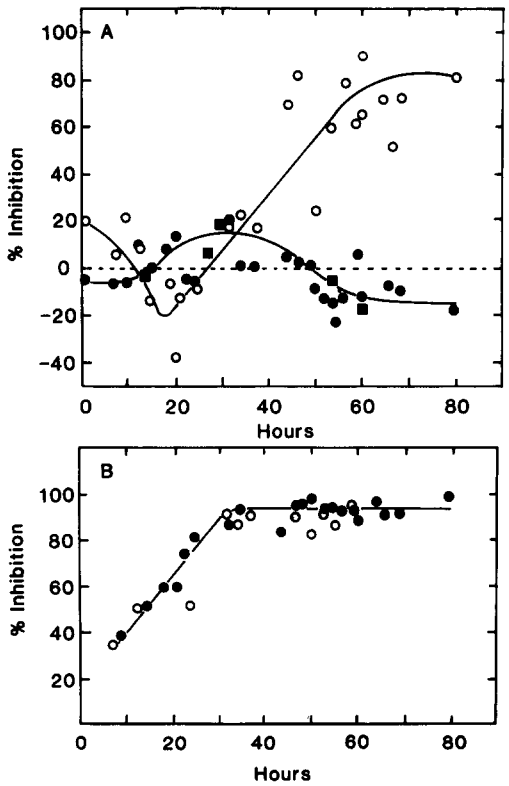

Fig. 3. Changes in sensitivity of whole-cell respiration to salicylhydroxamic acid and cyanide during exponential growth. (a) Effect of 1 mM cyanide alone (○), effect of 0.5 mM salicylhydroxamic acid alone (●) (■) results of two separte experiments). (b – Effect of both 0.5 mM salicylhydroxamic acid and 1 mM cyanide on cell respiration, (○) salicylhydroxamic acid added first, (●) cyanide added first.

occurred after 50 h and persisted up to 60 h of growth of the culture.

Respiration in the presence of 1 mM cyanide and salicylhydroxamic acid together was inhibited by 40% after 10 h growth and, as growth proceeded, the inhibitory effect increased reaching 96% after 35 h (Fig. 3b). No difference was observed when the order of addition of inhibitors was reversed. This would suggest that any differential flow of electrons resulting from blockage of one pathway does not result in any significant change in effectiveness of the inhibitors on the remaining pathway.

The effects of malonate, rotenone and arsenite were also investigated during exponential growth. Malonate and arsenite inhibited respiration by as much as 50% at concentrations of up to 20 mM and 50% inhibition occurred at 0.6 mM rotenone. In all cases there was little difference in inhibitory effect with increasing inhibitor concentration or when cells at various stages of growth were used for the experiments.

Growth in the presence of salicylhydroxamic acid and Antimycin A for prolonged periods

The effects of respiratory inhibitors on growth were also studied. Increasing the concentration of antimycin A in the growth medium from 20–40 to 80 nmol antimycin A per ml resulted in up to a 48% decrease in the exponential growth rate, followed by a reduction of at least 50% in the final cell density reached as compared with non antimycin A treated cells. With antimycin A at a concentration of 80 nmol/ml, a lag period for up to 35 h was observed before exponential growth was initiated. Growth in the presence of 2 mM salicylhydroxamic acid resulted in an exponential growth rate similar to that of the control (Generation time 9.0 h), but as with antimycin-A-treated cultures, the final stationary phase population was greatly reduced. The morphological appearances of cells grown with antimycin A were unchanged compared with the control cells, but cells grown with salicylhydroxamic acid appeared to be smaller than cells which had not been exposed to the inhibitor. Cells incubated in the presence of both inhibitors (2 mM salicylhydroxamic acid 40 nmol/ml antimycin A) showed no increase in cell numbers after 140 h.

Changes in sensitivity to salicylhydroxamic acid and cyanide through development

Cells at various stages of development were harvested from Millipore filters or agar plates and resuspended in distilled water at cell density of $6 \cdot 10^6$ cells/ml. Aggregates were then gently disrupted and samples quickly transferred to the oxygen electrode. The cell suspension was stirred sufficiently vigorously to maintain an aggregate free suspension and the effects of cyanide and salicylhydroxamic acid evaluated (Fig. 4). With preaggregating cells (approx. 3 h into development) and aggregating cells, cyanide caused the highest inhibition of respiration (95%) at a concentration of 0.7 mM. With pseudoplasmodial cells only 60% inhibition occurred at this concentration, and an increase in inhibitor concentration had little effect. With salicylhydroxamic acid (6 mM) the respiration of pseudoplasmodial cells was inhibited by 50% while preaggregating cells showed only 20% inhibition, respiration of aggregating cells was stimulated by up to 12%. The concentration of cyanide required to cause 95% inhibition of respiration in the presence of 4 mM salicylhydroxamic acid also varied during development with pseudoplasmodial cells requiring 25 mM, whereas preaggregating cells required only between 0.3 and 0.6 mM.

Prolonged exposure to salicylhydroxamic acid and antimycin A during development

Cultures were grown in the absence of inhibitors until they attained a density of $5 \cdot 10^6$ cells per ml when they were transfered to Millipore filters resting on support pads which were saturated with up to 80 nmol/ml antimycin A and/or up to 4 mM salicylhydroxamic acid. In all cases, fruiting-body formation occurred but in the presence of salicylhydroxamic acid abnormally small fruiting bodies, about one-fifth of the normal size, were obtained. Salicylhydroxamic acid-treated cells formed fruiting bodies after the same time as untreated cells but antimycin-A-treated cells took up to 3 days to form fruiting bodies. Cells incubated in the presence of both inhibitors, however, also eventually completed fruiting body formation, indicative of the failure of the inhibitor(s) to gain access to the cells from the support medium effectively.

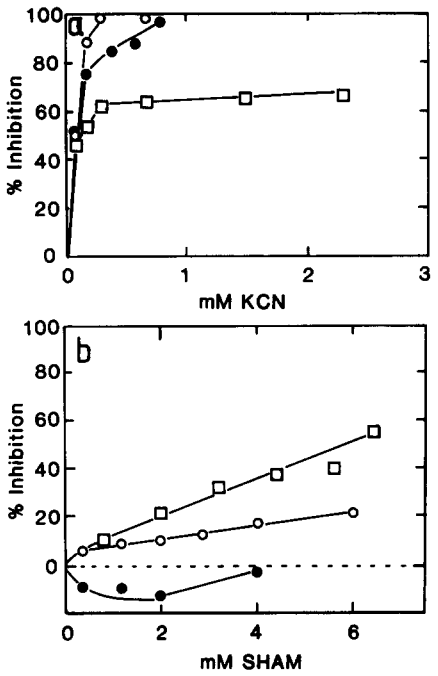

Fig. 4. Changes in the effects of salicylhydroxamic acid (SHAM) and cyanide at different times in development of *D. discoideum*. Inhibition expressed as a percentage of inhibitor titre, the control (0%) was the respiration rate in the absence of inhibitor. Amoeba were collected at various stages of development, gently disrupted, and resuspended at cell density of $6.0 \cdot 10^6$ cells/ml in distilled water in the oxygen electrode reaction vessel. The cell suspension was stirred sufficiently vigorously to maintain an aggregate-free suspension. (a) Effect of cyanide alone, (b) effect of salicylhydroxamic acid alone. Cells types were (○) preaggregating amoeba; (●) aggregating amoeba and (□) amoebae which had reached the pseudoplasmodial stage.

Necessity of iron for the alternative pathway

As it has been suggested that iron may be a necessary factor for cyanide-insensitive respiration of *Acanthamoeba castellanii* [17] addition of iron to the normal growth medium of *D. discoideum* was investigated. Addition of 30 μM $FeSO_4$ or $FeCl_3$ to the growth medium resulted in an increase of between 50–80% in the final cell population compared with the control cultures. Addition of 30 μM Na_2SO_4 under the same conditions did not produce this effect, indicating that it was $Fe^{2+(3+)}$ and not SO_4^{2-} which produced the stimulating effect on growth.

The effect of cyanide and salicylhydroxamic acid on iron-grown cells

Cells grown with additional iron (30 μM $FeSO_4$) again showed the stimulatory effect of cyanide which was apparent with early exponential phase cells. With iron-grown cells, however, the stimulation of respiration only occurred at very low cyanide concentrations (up to 0.2 mM) and was followed by inhibition (up to 60% at 4 mM cyanide) which was not seen in cells grown without additional iron (Fig. 5a).

In older cultures ($(6–8) \cdot 10^6$ per ml) cyanide was less inhibitory to respiration (Fig. 5b) with 50% inhibition at 2 mM cyanide compared with 88% inhibition with non-iron grown cells. With salicylhydroxamic acid there was a change from inhibition to stimulation in older cultures and at low salicylhydroxamic acid concentrations. In all cases the effect of both inhibitors together was similar with both cell types.

A significant difference in the responses to

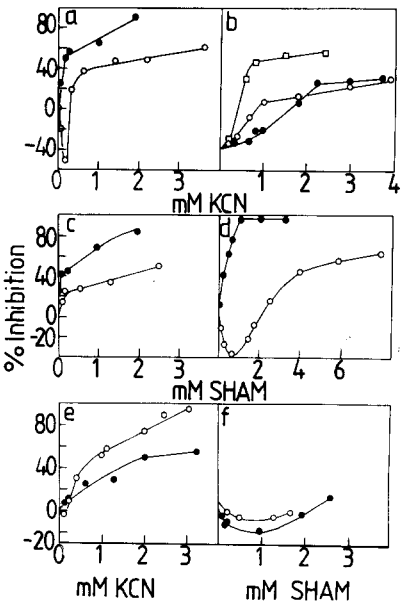

Fig. 5. The effects of cyanide and salicylhydroxamic acid (SHAM) on respiration of cells grown in the presence of 30 μM $FeSO_4$. Inhibition of respiration is expressed as a percentage of inhibitor titre. (a and c) cell density $6.0 \cdot 10^5$ cells/ml; (b and d) $6.0 \cdot 10^6$ cells/ml; (e and f) $1.0 \cdot 10^7$ cells/ml. (a) Cyanide alone (○) or in the presence of 1 mM salicylhydroxamic acid (●). (b) Cyanide alone (○), cyanide alone at cell density $8.0 \cdot 10^6$ cells/ml (●) or in the presence of 1 mM salicylhydroxamic acid (■). (c and d) salicylhydroxamic acid alone (○); or in the presence of 1 mM cyanide (●). (e) Cyanide alone on cells grown without 30 μM $FeSO_4$ (○); cyanide alone (●). (f) salicylhydroxamic acid alone on cells grown without $FeSO_4$ (○); salicylhydroxamic acid alone (●).

cyanide was found with stationary phase cells. Iron-grown cells showed a decrease in inhibition by cyanide (Fig. 5e) with 3 mM cyanide showing 52% inhibition, whereas control cells showed practically complete inhibition at this concentration of cyanide. In order to investigate whether the addition of iron can restore this insensitivity to cyanide 30 μM $FeSO_4$ was added to two cultures growing without iron, one in late log growth, A ($8 \cdot 10^6$ cells per ml) and one in the stationary phase of growth, B ($1.8 \cdot 10^7$ cells per ml). Immediately after the addition of 30 μM $FeSO_4$ (time, 0.1 h) 1 mM KCN inhibited cellular respiration of culture A by 75% and of B by 94%, thereafter a steady decrease is the effectiveness of cyanide on respiration was observed in both cultures. After 4 h of incubation, the lowest level of inhibition by cyanide was reached with 45% for culture A, and 79% for B. Further incubation up to 6.0 h showed no further reduction in the effectiveness of cyanide.

The results reported above were not due to the $FeSO_4$ merely neutralizing the inhibitory effect of cyanide, as cyanide in solution with 80 μM $FeSO_4$ still inhibited the respiration of cyanide-sensitive cells which had not been grown with additional iron.

Discussion

The results indicate the existence of a salicylhydroxamic acid-sensitive, cyanide-insensitive alternative electron transport system to oxygen in *D. discoideum*. The changes in the responses to inhibitors suggest that the control, or availability, of the alternative system changes during growth and development. These changes may be due to the loss of the alternative salicylhydroxamic acid sensitive oxidase or a change in the control mechanism involved in determining the route of electron transport.

Throughout exponential growth both the cyanide-sensitive and salicylhydroxamic acid-sensitive pathways appear to be utilized to varying degrees which are dependent on culture age (Fig. 3). During development the cyanide sensitive pathway appears to be dominant up to the aggregation stage, but both pathways are functional in pseudoplasmodial cells. The interpretation of these changes are complicated by the fact that two cell types, those destined to become stalk or spore cells, may be present in the population, so that the observed changes may be due to a change in one particular cell type or a change in the whole population. Previous work [14] with germinating spores also indicated that both electron transport pathways were functional during germination.

We have considered the possibility that the permeability properties of the plasma membranes may have controlled the effects of the inhibitors on the amoebae, however, in the cases of the inhibitors cyanide and salicylhydroxamic acid the response upon their addition was immediate (i.e., within 15 s) suggesting no permeability effects are involved.

The cyanide-stimulated respiration of early log-phase cells does not appear to be common but has been found in *A. castellanii*, an organism whose cytochrome components are similar to *D. discoideum* [15]. Cyanide insensitivity, however, has been found to be present in many organisms such as *T. brucei* [3], *Euglena* [18], Trypanosomes [19] and in *A. castellanii* [4].

The presence of iron on the alternative salicylhydroxamic acid-sensitive pathway during late exponential growth may partially explain why the pathways' activity is greatly reduced. It appears that iron is growth-limiting in the later stages of growth and that the presence of iron, either directly or indirectly, is essential for the function of the alternative electron transport system. Alternatively, the presence of iron may merely have stimulated a final round of cell division, by some unknown mechanism and not be involved directly with the electron transport system; however, this appears unlikely.

It has been reported [20] that for the yeast *Saccharomycopsis lypolitica*, Fe^{III} was necessary for the appearance of the alternative respiratory pathway, while Fe^{II} was not effective. This is not the case in *D. discoideum* or *A. castellanii* [17] in which stimulation of the alternative pathway in late growth was obtained with the addition of Fe^{II} or Fe^{III}. Iron deficiency in yeasts has also been found to result in a 20-fold decrease in the iron-sulphur content of the cells [21]. If a similar limitation occurs in *Dictyostelium* this may interfere with systems requiring FeS centres, such as electron transport. The action of cyanide with cytochrome oxidase is well studied [22–25], it is also

known, however, to inhibit other enzymes such as catalase [26] alkaline phosphalase [27] and other metallo enzymes [28,29]. Likewise hydroxamates also inhibit other enzymes such as peroxidase [30] and tyrosinase [9]. These unexamined effects may contribute to the cell's inability to undergo normal development in the presence of inhibitors and the observed reduction in cell population attained during growth.

The inhibition of exponentially growing cells by rotenone is indicative of the presence of site 1 of oxidative phosphorylation. This contrasts with work with *A. castellanii*, in which an absence of sensitivity to rotenone has been suggested as indicating the absence of site 1 [31] or a by-pass mechanism of site 1 which is thought to operate when cells are growing under optimal conditions [4,33]. The insensitivity to antimycin A also suggests that if the alternative oxidase branches from the main respiratory chain, it must occur before cytochrome *b*.

The nature of the alternative oxidase in *D. discoideum* has not yet been identified but non-haem, iron-sulphur proteins have been implicated in the cyanide resistant pathways in other organisms, especially plants [11,33]. There is also increasing evidence that ubiquinone and flavin molecules may be involved in supplying electrons to the oxidase [8,10,34,35].

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