The Effects of Cyanide, Azide, Carbon Monoxide and Salicylhydroxamic Acid on Whole-cell Respiration of *Acanthamoeba castellanii*

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(Received 1 May 1981; revised 18 June 1981)

Both early- and late-exponential phase cells of *Acanthamoeba castellanii* possess a terminal oxidase which is inhibited by 1 mM-cyanide but not by azide, salicylhydroxamic acid or CO. In early-exponential phase organisms this oxidase has a low affinity for O₂, but in late-exponential phase organisms it is modified or replaced by an oxidase which can function rapidly at around 1 µM-O₂.

INTRODUCTION

In many micro-organisms subsidiary terminal oxidases may function as alternatives to the main respiratory chain oxidase (Degn *et al.*, 1978); the existence of these alternative oxidases is demonstrated by studies of the effects of different respiratory inhibitors on mitochondrial and whole-cell respiration. In the soil amoeba *Acanthamoeba castellanii* evidence has been presented for three different mitochondrial terminal oxidases (Edwards & Lloyd 1977, 1978*a, b*; Lloyd *et al.*, 1979): (1) cytochrome *a*₃, inhibited by azide, CO and cyanide; (2) an oxidase inhibited by salicylhydroxamic acid (SHAM); and (3) an oxidase inhibited by cyanide, but not by azide or SHAM, which has a low affinity for O₂ (apparent $K_m > 7 \mu\text{M-O}_2$). The present study confirms the presence of these three distinct oxidases in early-exponential phase organisms and shows that oxidase (3) is not inhibited by CO. The use of mass spectrometry to measure respiration at low O₂ concentrations (<1 µM) indicates either that the affinity of oxidase (3) for oxygen is increased in cells from late-exponential phase cultures, or that it is replaced by a fourth oxidase.

METHODS

Maintenance and growth of organisms. *Acanthamoeba castellanii* was maintained and grown axenically with shaking at 30 °C in a medium containing (% w/v): Difco proteose peptone, 0.75; Oxoid yeast extract, 0.75; glucose, 1.5. Organisms were counted in a Fuchs–Rosenthal haemocytometer slide (Baird & Tatlock, Chadwell Heath, Romford, Essex, U.K.) after suitable dilution in distilled water.

Measurements of O₂ consumption rates. Measurements of respiration were made in stirred open reaction systems at 30 °C by two different methods: (1) in a membrane-covered oxygen electrode (Degn *et al.*, 1980), or (2) using a quadrupole mass spectrometer with a membrane inlet (Lloyd *et al.*, 1981). The latter apparatus allows the simultaneous measurement of O₂ and CO in solution. CO was measured as a minor peak at mass 12 because its major peak at mass 28 coincides with that of N₂.

Inhibitors. Fixed concentrations of CO in reaction mixtures were established by mixing fixed proportions with N₂ and allowing the resulting mixtures to enter the air/N₂ gas stream to the open reaction vessel (Petersen, 1977). KCN and NaN₃ were used as aqueous solutions (pH 7.4). SHAM was dissolved in ethanol. Inhibition (%) is expressed relative to the respiration rate in the absence of any inhibitor.

RESULTS AND DISCUSSION

The effects of inhibitors of respiration on steady-state concentrations of dissolved O₂ in growing cultures of *A. castellanii* are shown in Fig. 1. In early-exponential phase cultures

Fig. 1. Polarographic determination of the effects of respiratory inhibitors on steady-state O₂ concentrations in cultures of *Acanthamoeba castellanii*. (a) An early-exponential phase culture (2.3×10^5 organisms ml⁻¹) concentrated 20-fold. (b) A late-exponential phase culture (1.2×10^6 organisms ml⁻¹) concentrated 4-fold. Measurements were made in the open electrode system on organisms in 4.5 ml growth medium; the results are typical of triplicate experiments. Constant partial pressure of O₂ (160 μM) in the gas phase was monitored with a second electrode.

(Fig. 1a), addition of 1 mM-SHAM stimulated respiration: this confirms the earlier observations of Edwards & Lloyd (1977), who have discussed possible mechanisms involved in cyanide- or SHAM-stimulated respiration. In the presence of SHAM, azide effectively blocks the main phosphorylating electron transport chain of mitochondria (Lloyd *et al.*, 1979); in the experiment shown, inhibition of this pathway by 1 mM-azide (together with the inhibited SHAM-sensitive pathway) accounted for only 60% of the total respiration. Equilibration with up to 380 μM-CO (at 120 μM-O₂) gave no additional inhibition of respiration, indicating that any oxidase sensitive to CO had already been inhibited by azide. However, inhibition of the SHAM-sensitive oxidase and cytochrome *a*₃ did not account for all of the cyanide-sensitive respiration; the remaining 40% of the total respiration was almost completely inhibited on further addition of 3 mM-KCN.

The response of the respiration of organisms of late-exponential phase cultures to inhibitors was markedly different (Fig. 1b). As previously demonstrated (Edwards & Lloyd, 1977), addition of 1 mM-SHAM produced no measurable effect; almost all of the electron flux is by way of the major terminal oxidase (cytochrome *a*₃) in these cells. The effect of 180 μM-CO was to give only partial inhibition of this oxidase (25% of total respiration), as would be expected at high O₂ concentrations for a competitive inhibitor. The subsequent addition of azide gave a far greater extent of inhibition of cytochrome *a*₃; only 11% of the total respiration then remained, and this portion was cyanide-sensitive (mediated by oxidase 3). These results were typical of those obtained in 12 experiments in which all possible sequences of inhibitor additions were explored. Similar results were obtained with growing cultures and with washed cell suspensions. It was concluded that in high O₂ concentrations (in the range 80–140 μM), cyanide was the most effective inhibitor, followed by azide, with CO least effective. The presence of SHAM (especially in the case of organisms from early-exponential phase cultures) was necessary to obtain maximal effects of these inhibitors.

Fig. 2. Steady-state concentrations of O_2 (—) and CO concentrations (----) in non-growing suspensions of *Acanthamoeba castellanii* in the open system measured by mass spectrometry. (a) Cells from an early-exponential phase culture (5.6×10^5 organisms ml^{-1}) were washed twice and concentrated 2.5-fold in 0.9% NaCl. (b) Cells were obtained in an identical way from a late-exponential phase culture (1.6×10^6 organisms ml^{-1}) but were diluted by one-third with saline. The gas phase contained 0.125% O_2 in N_2 throughout the experiments; CO (2.5% in N_2) was added as indicated into the gas stream entering the open system. The liquid volume was 4.5 ml. The results are typical of triplicate experiments.

To resolve further the contributions of different terminal oxidases to overall cellular respiration, measurements were made at low O_2 concentrations, in order both to increase the effectiveness of competitive inhibition by CO (by increasing the CO/ O_2 ratio), and to minimize the contribution of any oxidase with a low affinity for O_2 . Respiration of organisms from the early-exponential phase of growth (Fig. 2a) was almost completely inhibited in the presence of 225 μM -CO and 1 mM-SHAM when the O_2 concentration was 0.75 μM . Addition of 1 mM-azide gave an extra 8% inhibition, but subsequent addition of cyanide (2 mM) was without effect. However, in cells from late-exponential phase cultures (Fig. 2b), inhibition by SHAM, CO and azide at low O_2 concentrations left a considerable residual respiration (50%), which was cyanide-sensitive. The use of higher CO concentrations in this experiment gave a similar result. Changing the order of introduction of respiratory inhibitors gave similar results for both kinds of organisms (11 experiments). The inhibitory effect of CO was completely reversible in organisms exposed to this inhibitor for up to 40 min. It is concluded that during the later phases of exponential growth the third terminal oxidase (azide-, CO-, and SHAM-insensitive), which has a low O_2 affinity early in growth, becomes modified in its O_2 -binding properties or is replaced by a fourth oxidase which has a similar spectrum of

inhibitor sensitivities. Oxidases which are cyanide-sensitive but azide- and SHAM-insensitive also occur in trypanosomes (Hill, 1978) and *Tetrahymena pyriformis* (Lloyd *et al.*, 1980).

D.L. thanks members of the Institute of Biochemistry at Odense for their help and hospitality and Mr N. Williams and Mrs J. Williams for technical help.

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