The Effect of Inhibitors on the Oxygen Kinetics of Terminal Oxidases of *Tetrahymena pyriformis* ST

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Respiration of early-exponential phase cultures of the ciliate protozoon *Tetrahymena pyriformis* is inhibited in two stages with increasing concentrations of cyanide. Up to 40 to 50 % inhibition occurs at low concentrations ($< 15 \mu\text{M}$). Maximal inhibition was obtained with $300 \mu\text{M}$ -cyanide; at this concentration 20 % of the respiration was still unaffected. Competitive inhibition of respiration by CO occurs ($K_i = 4.25 \mu\text{M}$); azide inhibition of oxygen consumption is uncompetitive ($K_i = 4$ to 9 mM). A salicylhydroxamic acid-sensitive oxidase is present which is not inhibited by cyanide, azide, CO or H_2S . Three other pathways of terminal oxidation are present which are insensitive to azide, CO and salicylhydroxamic acid. One of these is cyanide- and sulphide-sensitive, a second is cyanide-insensitive but sulphide-sensitive, and a third is cyanide-sensitive but sulphide-insensitive; these pathways may not possess unique terminal oxidases. No oxidase with a very low affinity for oxygen was detected, but the overall affinity for oxygen of *T. pyriformis* in the absence of inhibitors ($K_m = 2.9 \mu\text{M}$) is lower than that of some other protozoa.

INTRODUCTION

Many organisms possess terminal oxidases other than cytochrome $a+a_3$, the classical oxidase of the phosphorylating respiratory chain of eukaryotes (Degn *et al.*, 1978). Studies of the effects of respiratory inhibitors on the oxygen consumption of various protozoa have demonstrated a number of different alternative terminal oxidases; both trypanosomes and the amoeba *Acanthamoeba castellanii* can have at least two terminal oxidases acting alongside cytochrome c oxidase (Hill, 1976; Hill & Degn, 1977; Edwards & Lloyd, 1978; Lloyd *et al.*, 1979).

The respiration of the ciliate protozoon *Tetrahymena pyriformis* has several unusual features and has been the subject of study since Baker & Baumberger (1941) noted its atypical a -type cytochromes. Subsequent investigators reported that mammalian cytochrome c was not oxidized by cell-free homogenates or mitochondrial preparations, but that substrate oxidations were inhibited by azide, CO and cyanide (Ryley, 1952; Keilin & Ryley, 1953; Eichel, 1954; Kobayashi, 1965; Van de Vijver, 1966). Functional evidence for the role of cytochrome a_{620} as the main terminal oxidase of the mitochondria of *T. pyriformis* was provided by measurement of the kinetics of its reaction with oxygen (Turner *et al.*, 1971; Lloyd & Chance, 1972). A claim for the presence of a mitochondrial cytochrome o (Perlsh & Eichel, 1971) is inconclusive, and there is no evidence in this ciliate for a functional role for any b -type cytochrome as a terminal oxidase (Lloyd & Chance, 1972; Kilpatrick & Erecińska, 1977). An oxidase sensitive to inhibition by salicylhydroxamic acid has recently been demonstrated in *T. pyriformis* by Eichel & Stearns (1977).

In the present paper we describe the effects of cyanide, azide, CO, H₂S and salicylhydroxamic acid on the respiration of intact organisms and suggest that at least three different terminal oxidases may function in *T. pyriformis*.

METHODS

Abbreviations. CCCP, Carbonyl cyanide *m*-chlorophenylhydrazine; SHAM, salicylhydroxamic acid.

Maintenance and growth of organisms. *Tetrahymena pyriformis* ST was maintained and grown axenically on 2% (w/v) proteose peptone (Difco), 0.1% (w/v) liver digest (Oxoid) with shaking at 30 °C exactly as described previously (Lloyd *et al.*, 1971). Dilution of cultures was with cell-free supernatants ('conditioned growth media'). Organisms were counted in a Sedgewick-Rafter cell (Graticules Ltd, Tonbridge, Kent) after fixation in 4% (v/v) aqueous formaldehyde and suitable dilution in water.

Measurements of oxygen-consumption rates. Measurements of respiration were made at 30 °C by three different methods. (1) In a conventional closed reaction vessel (total vol. 2 ml) fitted with an oxygen electrode (Rank Brothers, Bottisham, Cambridge). (2) In an open electrode system at 30 °C (total vol. 4.5 ml) (Degn & Wohlrab, 1971), modified as described by Petersen & Degn (1978) to permit on-line computer control of time-dependent gradients of dissolved oxygen. The details of this method were described by Lloyd *et al.* (1979) and by Degn *et al.* (1980). (3) In an open system at 30 °C (total vol. 4.0 ml) using a mass spectrometer with membrane inlet to measure steady-state concentrations of dissolved oxygen (Lundsgaard *et al.*, 1976). The system was based on a MS10 mass spectrometer fitted with a S10 sorption pump, a P25 ion pump (Kratos Ltd, A.E.I. Scientific Instruments, Manchester) and a peak detector which repeatedly scanned a narrow range around the desired mass-peak (Lundsgaard & Petersen, 1974). The liquid sample was separated from the vacuum by a 12 µm thick Teflon membrane (type D602; Radiometer, Copenhagen). The calibration procedure was as described by Lundsgaard & Petersen (1974).

Inhibitors. Fixed concentrations of CO or H₂S in cell suspensions 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; salicylhydroxamic acid (SHAM) was dissolved in ethanol.

RESULTS

Inhibition of respiration by cyanide and azide in a closed electrode system

Successive additions of cyanide to early-exponential phase cells in growth medium (pH 7.5) gave a complex inhibitory response (Fig. 1*a*). At 13 µM-cyanide 50% inhibition of respiration was produced, and further inhibition was not observed until more than 30 µM-cyanide was added. Maximal inhibition was obtained with 300 µM-cyanide and the residual oxygen consumption accounted for 20% of the total respiration. Addition of 1 mM-SHAM before the cyanide titration did not alter the biphasic nature of the response to cyanide but increased the degree of inhibition by cyanide by about 10% at all concentrations. Azide (25 mM) added after cyanide (550 µM) gave no further inhibition. SHAM (1 mM) added after cyanide (550 µM) and azide (25 mM) gave further inhibition (10% of the uninhibited rate of oxygen consumption). The biphasic titration curve for cyanide inhibition was still observed when the respiration was uncoupled from energy conservation by adding 0.5 µM-carbonyl cyanide *m*-chlorophenylhydrazine (CCCP) but was not evident with late-exponential phase organisms (Fig. 1*a*).

Inhibition by azide was less than 50% even at high concentrations (30 mM) (Fig. 1*b*); addition of 1 mM-cyanide at this high azide concentration gave almost 90% inhibition, and addition of 1 mM-SHAM then inhibited about half of the residual respiration. Similar results were obtained with uncoupled cell suspensions (in the presence of 0.5 µM-CCCP). When azide (up to 30 mM) was added in the presence of 20 µM-cyanide, there was only a small increase of inhibition (< 5%) above that initially caused by cyanide alone (Fig. 1*b*).

Similar results were obtained for all three respiratory inhibitors with cells washed and re-suspended in 67 mM-potassium phosphate buffer pH 7.5.

Fig. 1. Effects of cyanide and azide on the respiration of *Tetrahymena pyriformis*. Inhibition of respiration is expressed as a function of cyanide or azide concentration, the control (0%) being the respiration rate in the absence of any inhibitor. (a) Effect of cyanide on the respiration of an early-exponential phase culture (6.4×10^4 organisms ml^{-1}) in the absence ($\circ$) or presence (Δ) of $0.5 \mu\text{M}$ CCCP, and of a late-exponential phase culture (8×10^5 organisms ml^{-1}) ($\square$). (b) Effect of azide in the absence ($\bullet$) or presence of $20 \mu\text{M}$ cyanide ($\blacktriangle$) on the respiration of an early-exponential phase culture. Measurements were made in the closed electrode system on organisms in growth medium; the results are typical of triplicate experiments.

Fig. 2. Effects of respiratory inhibitors on steady-state oxygen concentrations in cultures of *Tetrahymena pyriformis*. (a) An early-exponential phase culture (3.7×10^4 organisms ml^{-1}) concentrated 20-fold. (b, c) Late-exponential phase cultures (4×10^5 organisms ml^{-1}) concentrated 5-fold. Measurements were made in the open electrode system on organisms in growth medium; the results are typical of triplicate experiments.

Fig. 3. Steady-state measurements of oxygen concentration in non-growing suspensions of *Tetrahymena pyriformis* in the open system measured by mass spectrometry. In (a) a late-exponential phase culture (8×10^8 organisms ml^{-1}) was harvested and the organisms were washed once and resuspended in 67 mM-potassium phosphate buffer pH 7.5 at a density of 10^4 organisms ml^{-1} . In (b) and (c), organisms were obtained in an identical way from an early-exponential phase culture (7.8×10^4 organisms ml^{-1}). The gas phase contained 2.5% O_2 in N_2 throughout the experiments; H_2S (0.25% in N_2) was added as indicated into the gas stream entering the open system. Results are typical of duplicate experiments.

Steady-state oxygen measurements in the presence of respiratory inhibitors

A second method of investigating the effects of respiratory inhibitors confirmed the diversity of terminal oxidases in *T. pyriformis*. Inhibition of respiration increases the steady-state oxygen concentration in the liquid phase in the open electrode system and gives an upward deflection on the traces. Figure 2 shows that, as is the case for many organisms (Degn *et al.*, 1978), the effect of SHAM was observable only when this inhibitor was added after cyanide or azide. Figure 2(a) shows that $180 \mu\text{M}$ -CO gave no further inhibition after 5.8 mM-azide and 0.9 mM-SHAM had been added. Cyanide (2 mM) added after low (0.56 mM) concentrations of azide in the presence of 1 mM-SHAM gave extensive but not quite complete inhibition of cell respiration (Fig. 2b).

Studies of respiratory inhibition by H_2S require the use of a mass spectrometer as this compound interferes with electrode-based oxygen assays; reaction of H_2S with constituents of the growth medium necessitated the use of washed cell suspensions. Inhibition of the endogenous respiration of organisms (suspended in 67 mM-potassium phosphate buffer pH

Fig. 4

Fig. 5

Fig. 4. Reciprocal plots of respiration rate against oxygen concentration for an early-exponential phase culture of *Tetrahymena pyriformis*. The culture was harvested at a density of 7.7×10^4 organisms ml^{-1} and resuspended at ten times this concentration in conditioned growth medium. Arrows indicate increasing (top trace) and decreasing (lower trace) time gradients of dissolved oxygen. Similar results were obtained in 15 experiments.

Fig. 5. (a) Reciprocal plots of respiration rate against decreasing oxygen concentration in the presence of various fixed CO concentrations for an early-exponential phase culture of *Tetrahymena pyriformis*: curve 1, uninhibited culture; 2, $4.7 \mu\text{M}$ -CO; 3, $9.4 \mu\text{M}$ -CO; 4, $14.1 \mu\text{M}$ -CO. The culture was harvested at a density of 6.0×10^4 organisms ml^{-1} and resuspended at five times this concentration in conditioned growth medium. Similar results were obtained in three experiments.

(b) Plot of the reciprocal rate of oxygen uptake against CO concentration at various fixed oxygen concentrations. Data from (a): ●, $4.0 \mu\text{M}$ - O_2 ; △, $4.6 \mu\text{M}$ - O_2 ; ▲, $5.33 \mu\text{M}$ - O_2 .

7.5) by $104 \mu\text{M}$ - H_2S completely removed the azide-sensitive portion of respiration. Subsequent addition of 1 mM-SHAM gave further inhibition, as did 2 mM-cyanide (Fig. 3a). Changing the order of additions confirmed that H_2S gave more complete inhibition than 10 mM-azide and that the respiration which persisted in the presence of these two inhibitors had separate cyanide-sensitive and SHAM-sensitive components (Fig. 3b). A sulphide-sensitive, cyanide-insensitive component of respiration was also detected (Fig. 3c).

Steady-state oxygen kinetics of terminal oxidases

Time gradients over a concentration range 0.5 to $3 \mu\text{M}$ -oxygen were used to investigate the dependence of the respiration rate of organisms on oxygen tension in the growth medium. In the absence of inhibitors the Lineweaver-Burk plots of reciprocal respiration rate against reciprocal oxygen concentration were linear when the oxygen tension was decreased with time (Fig. 4). Plots produced by increasing oxygen tension from anaerobiosis were always convex downwards. Values for the apparent K_m for oxygen were therefore obtained by extrapolation of plots from experiments in which the steady-state level of dissolved oxygen was decreased with time. The apparent K_m for oxygen was $2.9 \pm 0.9 \mu\text{M}$ (mean $\pm$ s.d., 10

Fig. 6. (a, c) Reciprocal plots of respiration rate against decreasing oxygen concentration in the presence of various fixed azide concentrations for early-exponential phase (a) and late-exponential phase (c) cultures of *Tetrahymena pyriformis*. (a) Curve 1, uninhibited culture; 2, 0.5 mM-azide; 3, 2 mM-azide; 4, 10 mM-azide; 5, 50 mM-azide; 6, 50 mM-azide plus 1 mM-SHAM. The culture was harvested at a density of 7.8×10^4 organisms ml^{-1} and resuspended at seven times this concentration in conditioned growth medium. Similar results were obtained in three experiments. (c) Curve 1, uninhibited culture; 2, 5 mM-azide; 3, 10 mM-azide; 4, 20 mM-azide; 5, 40 mM-azide. The culture was harvested at a density of 8×10^5 organisms ml^{-1} and resuspended at three times this concentration in conditioned growth medium.

(b, d) Plots of the corresponding reciprocals of maximal respiration rates at various azide concentrations. Data are taken from (a) and (c), respectively.

determinations) and was not significantly different for organisms at the early- or late-exponential phases of growth ($< 10^5$ or $> 3 \times 10^5$ organisms ml^{-1} , respectively).

The non-linear Lineweaver-Burk plots obtained when the oxygen tension was increased with time may result from a control mechanism which causes a retardation in the response of the respiratory system to an increased oxygen concentration. Thus each small stepwise increase of the oxygen tension in the gas phase leads to an overshoot of the oxygen concentration in the culture before the attainment of the new steady-state. A similar phenomenon has been observed in other protozoa and with yeasts (H. Degn, unpublished observations).

Figure 5(a) shows that the inhibition of respiration by various fixed partial pressures of CO at low oxygen concentrations was strictly competitive. Secondary plots gave a K_i value of $4.25 \mu\text{M}$ for inhibition by CO (Fig. 5b). Similar results were obtained with early- or late-exponential phase organisms.

In the presence of various fixed concentrations of azide (up to 10 mM) a series of parallel Lineweaver-Burk plots were obtained with organisms from both early- and late-exponential phase cultures (Fig. 6a, c). At the highest azide concentrations (40 to 50 mM) departures from linearity were observed. Secondary plots of intercepts against inhibitor concentration gave a non-linear relationship for early-exponential phase organisms with a K_i value of about 4 mM (Fig. 6b) and a linear dependence for late-exponential phase organisms ($K_i = 9.5$

Fig. 7. Inhibitor-sensitivities of pathways of terminal oxidation in *Tetrahymena pyriformis*.

mm; Fig. 6d). Addition of 1 mM-SHAM to early-exponential phase cultures containing 50 mM-azide (Fig. 6a) gave further inhibition of respiration over the range of oxygen concentrations studied (1 to 4 μ M).

DISCUSSION

Studies of the effects of respiratory inhibitors on the respiration of intact *T. pyriformis* confirm that the process of terminal oxidation in this protozoon can be resolved into five distinct pathways (terminating in at least three oxidases); the simplest hypothetical representation of these (neglecting the possibilities for confluence and branching) is shown in Fig. 7. Pathway 1 is inhibited by azide ($K_i = 4$ to 9 mM), CO ($K_i = 4.25$ μ M), cyanide ($K_i \approx 15$ μ M) and H_2S . A second major pathway is inhibited by much higher cyanide concentrations and by H_2S . Pathway 3 is H_2S -sensitive but cyanide-insensitive, while a fourth pathway is inhibited only by high concentrations of cyanide (1 to 2 mM). Pathway 5 is unaffected by any of these inhibitors but is sensitive to inhibition by SHAM. Under the growth conditions employed pathways 3, 4 and 5 account for only a small proportion of the total electron flux, and further evidence is required (e.g. from other specific inhibitors) that pathways 3 and 4 possess unique terminal oxidases.

Linear Lineweaver-Burk plots of reciprocal respiration rate against reciprocal oxygen concentration are not to be expected for a mixture of oxidases with different kinetic properties. That linear plots are nevertheless obtained suggests either that these properties are very similar for the individual oxidases, or that one pathway of electron transport is dominant under the conditions of measurement.

No indication of the presence of an oxidase with a low affinity for oxygen was observed; the azide-insensitive (but cyanide-sensitive) component observed here is thus different from the low-affinity oxidase of *Acanthamoeba castellanii* (Lloyd *et al.*, 1979). The overall affinity of the respiratory system of *T. pyriformis* for oxygen is, however, lower than those measured for several other eukaryotes. The value of 2.9 μ M-oxygen for the apparent K_m of this ciliated protozoon compares with <1 μ M for *A. castellanii* (Lloyd *et al.*, 1979), 0.1 μ M for the tsetse fly vector form of a trypanosome (Hill, 1978) and <1.5 μ M for baker's yeast (H. Degn, unpublished results).

Further work is necessary to decide whether each of the five pathways of terminal respiration, identified by successive inhibitor additions, represent five different terminal

oxidases. It has been shown that cytochrome a_{620} is kinetically competent as a terminal oxidase (Turner *et al.*, 1971), and spectral data indicate that this cytochrome reacts with CO and cyanide (Lloyd & Chance, 1972) and with azide (D. Lloyd, unpublished results). The present work suggests that this oxidase (pathway 1) is also inhibited uncompetitively by azide and by H_2S . The high concentrations of azide required for inhibition may reflect the presence of a permeability barrier to this inhibitor in whole organisms: inhibition in the presence of CCCP shows that azide is not acting as an inhibitor of ATP synthetase. Component 5 has previously been identified in *T. pyriformis* as a SHAM-sensitive oxidase (Eichel & Stearns, 1977), highly active in organisms grown in media supplemented with metal ions (Stearns *et al.*, 1978). Components 2, 3 and 4 may represent novel alternative terminal oxidases or modified forms of component 1, possibly in specialized membrane microenvironments. Further investigations are necessary to identify the alternative pathways of electron transport in *T. pyriformis* and the changes in apportionment of electron flux between these during growth in batch cultures.

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