The SHAM-sensitive Alternative Oxidase in *Tetrahymena pyriformis*: Activity as a Function of Growth State and Chloramphenicol Treatment

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The SHAM-sensitive alternative oxidase has been studied in *Tetrahymena pyriformis* strain ST as a function both of growth state and of chloramphenicol treatment. In low density cultures the total alternative oxidase activity, as revealed by SHAM titration in the presence of CN\(^-\), is equivalent to 17% of total respiration and is slightly utilized at all times. Stationary phase cells have somewhat less of the oxidase and it is not utilized even in CCCP-uncoupled cells. Respiration in chloramphenicol-treated cells is 100% CN\(^-\)-resistant. Alternative oxidase activity is equivalent to 30-40% of this total and one third of it is active. The remaining 60% residual respiration is due to unknown oxidases. Following CCCP-uncoupling, a CN\(^-\)-sensitive pathway is demonstrable and the alternative oxidase is fully utilized. The higher proportion of alternative oxidase in chloramphenicol-treated cells is brought about by its conservation at a time when the cytochrome chain is becoming non-functional. There is no large scale induction of the alternative oxidase.

INTRODUCTION

Chloramphenicol (CAP) is known to affect mitochondrial translation, blocking the synthesis of mitochondrially-derived components of cytochrome oxidase, cytochrome *b* and oligomycin-sensitive ATPase (Tzagoloff *et al*., 1979). In *Tetrahymena*, treatment of cells with concentrations of CAP known to block mitochondrial translation (Young & Hunter, 1979) results in the arrest of cell growth after several hours, a stimulation of mitochondrial division, increases in the mitochondrial phospholipid:protein ratio and reductions in the level of some cytochromes (Turner & Lloyd, 1971; Gleason *et al*., 1975; Rohatgi & Krawiec, 1973; Ruben & Hooper, 1978; Curgy *et al*., 1980). Somewhat surprisingly, however, the P:O ratio of mitochondria appears to be unchanged (Gleason *et al*., 1975).

Ruben & Hooper (1978) could detect no change in energy charge after CAP treatment in *Tetrahymena* and concluded that cessation of growth was not brought about by limitations in respiratory capacity. They concluded that reductions in O\(_2\) consumption per cell after CAP treatment mirrored similar reductions found in cells approaching stationary phase. In fungi, however, it is known that growth in the presence of inhibitors of mitochondrial translation results in an enhancement and greater utilization of the salicylhydroxamic acid (SHAM)-sensitive alternative oxidase (Hanssens & Verachtert, 1976; Vanderleyden *et al*., 1979; Lambowitz *et al*., 1972a, b; reviewed in Henry & Nyns, 1975). Since *Tetrahymena* has an alternative oxidase (Eichel & Stearns, 1977; Stearns *et al*., 1978; Lloyd *et al*., 1980), it seemed likely that following CAP-treatment this pathway would become more active. Under these conditions, since both sites II and III of oxidative phosphorylation were not utilized (Henry & Nyns, 1975), there would be less ATP production for a given level of O\(_2\) consumption and, although the level of O\(_2\) consumption was similar, this would make for a major distinction between the energetics of the stationary phase and of the CAP-treated cell.

Abbreviations: CAP, chloramphenicol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; SHAM, salicylhydroxamic acid.
The nature of respiration in CAP-inhibited *Tetrahymena* has been investigated. All of the respiration in CAP-inhibited cells was found to be CN$^-$-resistant; however, the level of SHAM-sensitive respiration expressed on a per cell basis remained near the normal level. Most of the respiration was both CN$^-$ and SHAM-resistant. In addition, data are presented about the levels and extent of use of the alternative oxidase in exponentially growing and stationary phase cells.

**METHODS**

**Maintenance and growth of organisms.** *Tetrahymena pyriformis* (strain ST) was maintained in low density exponential growth in Neff's medium (Leick & Plesner, 1968) with shaking at 28 °C. Stationary phase was reached at 5 to 7 × 10$^6$ cells ml$^{-1}$ with gentle shaking and 1-2 to 1.7 × 10$^6$ cells ml$^{-1}$ with more vigorous aeration. Cell counts were made using a Coulter counter (Model ZF, 100 μm aperture). CAP (Sigma) was added as an ethanolic solution (stock 250 mg ml$^{-1}$).

**Measurement of O$_2$ consumption.** Measurements of O$_2$ consumption were made in a closed 1 ml reaction vessel with a Clarke-type oxygen electrode (Hansatech).

**Inhibitors.** SHAM and carbonyl cyanide m-chlorophenylhydrazone (CCCP) were added in dimethylformamide or dimethylsulphoxide. The solvents had no effect on O$_2$ consumption at the concentrations used. KCN or NaN$_3$ were added as buffered aqueous solutions. All inhibitors were made up immediately before use.

**RESULTS**

**Inhibition of respiration by CN$^-$.**

The level of CN$^-$-resistant respiration has been shown previously to vary with growth conditions (Stearns et al., 1978). CN$^-$-sensitivity of respiration was therefore measured for cells in different growth states and for CAP-treated cultures (Fig. 1). Exponentially growing cells consumed 0.260 ± 0.006 (± s.e., n = 12) pmol O$_2$ min$^{-1}$ per cell of which 28% was CN$^-$-resistant. Maximum inhibition occurred at concentrations of CN$^-$ above 5 × 10$^{-6}$ M. Stationary phase cells had a lower O$_2$ consumption rate of 0.103 ± 0.003 (n = 6) pmol O$_2$ min$^{-1}$ cell$^{-1}$ and were more resistant to CN$^-$. Maximum inhibition to a residual rate of about 25% occurred at CN$^-$ concentrations above 10$^{-5}$ M. Little effect was noted at concentrations below 10$^{-6}$ M. Cells treated with CAP for 24 h respired at a rate per cell similar to that of stationary phase cells. This respiration was, however, 100% resistant to CN$^-$ or N$_3^-$. In the presence of CCCP, respiration increased to 180% of the

**Inhibition of respiration by SHAM.**

The SHAM-sensitivity of the CN$^-$-resistant respiration was determined for untreated, CN$^-$-inhibited and CCCP-uncoupled cells (Fig. 2). In exponentially growing cells in the absence of CN$^-$, 50% inhibition occurred at 5 mM-SHAM (Fig. 2a). The inhibition at 1 mM was 10 to 15%. In the presence of CN$^-$, the residual respiration (27% of the control) was strongly inhibited by SHAM to a final level of 10 to 12% of the control rate at concentrations of 1 mM or less. Little additional inhibition occurred at concentrations up to 5 mM. These data suggest that SHAM has substantial non-specific effects in these cells. The inhibition curve in the presence of CN$^-$ suggests that it is selective for the alternative oxidase at concentrations of 1 mM or less. In the presence of CCCP, respiration was stimulated by 25 to 30%. This stimulated O$_2$ consumption could be completely inhibited by CN$^-$ or N$_3^-$ down to the residual level found in the absence of CCCP.

Stationary phase cells were less sensitive to SHAM at low concentrations; no effect was noted below 1 mM (Fig. 2b). CCCP treatment caused a very strong stimulation (80%) of O$_2$ consumption. Again, however, little if any inhibition by SHAM occurred below 1 mM. Following CN$^-$ inhibition, residual respiration represented 35 to 40% of the control, of which 10 to 15% could be blocked by SHAM at concentrations of 1 mM or less. In the presence of CN$^-$ or N$_3^-$, the respiration in CCCP-treated cells had the same level of residual respiration as in the absence of CCCP.

Following CAP-treatment, the O$_2$ consumption displayed a similar SHAM-sensitivity to that found in control cells with about 15% inhibition occurring at 1 mM (Fig. 2c). The respiration was 100% CN$^-$-and N$_3^-$. In the presence of CN$^-$ or N$_3^-$ increased the SHAM-sensitivity to about 40% at 1 mM. In the presence of CCCP, respiration increased to 180% of the
control activity. This stimulated O$_2$ consumption could be inhibited back to the control level with CN$^-$ or N$_3^-$. It was also very sensitive to SHAM-inhibition with approximately one-third of the total activity being blocked at 1 mM-SHAM.

**Extent of utilization of the SHAM sensitive pathway**

To determine the activity of the alternative pathway in the absence of inhibitors under various conditions, the curves in Fig. 2 were analysed according to Bahr & Bonner (1973) and Theologis & Laties (1978) (Fig. 3). $V_T$, the total respiration minus the residual CN$^-$ and SHAM-resistant component, was plotted against g(i), the activity of the alternative oxidase at a given SHAM concentration.
Relative activity of alternative oxidase $g(i)$

Fig. 3. $V_i^r$ (total oxygen consumption minus the residual CN$^-$, SHAM-resistant portion), has been plotted against $g(i)$ (the relative activity of the SHAM-sensitive pathway) at various concentrations of SHAM for (a) exponentially growing cells, (b) stationary phase cells and (c) CAP-treated cells. The data are taken from the curves in Fig. 2 and represent controls (○) and CCCP-treated cells (●). The data have been normalized to the control rate (100) before the addition of any inhibitors. The slope of the lines ($p$) indicates the degree to which the alternative oxidase is active.

concentration, as determined from the SHAM titration in the presence of CN$^-$. The slope ($p$) of these lines indicates the degree to which the alternative oxidase is active. Since all *Tetrahymena* respiration was clearly very sensitive to higher concentrations of SHAM, this graph often did not yield a straight line. The break in the curve indicated more than one sensitive component. The lines to determine $p$ have therefore been plotted only through the points representing high $g(i)$ (equivalent to low SHAM concentrations). This is justified, since Fig. 2(a) clearly shows the alternative oxidase responding to SHAM at concentrations below 1 mM. The slopes, $p$, are indicated in Fig. 3.

In exponential growth, the alternative oxidase is used to a limited extent ($p = 0.16$) and even following CCCP-uncoupling the rate only rises to $p = 0.38$ (Fig. 3a). The pathway does not appear to function at all in stationary phase cells ($p = 0$) (Fig. 3b). Following CAP-treatment the alternative pathway is used ($p = 0.3$) and, in the presence of CCCP, this rises to $p = 1$ (Fig. 3c). The marked non-linearity of the curve in the presence of CCCP makes the drawing of the line somewhat arbitrary, nevertheless for high $g(i)$ a value of $p$ close to 1 is indicated. The fact that the CAP-treated cells in the absence of CCCP titrate in a linear fashion to low $g(i)$ values suggests that in the presence of CCCP, where the curve is not linear, an additional pathway with marked SHAM sensitivity is making its presence known. Since all of the CCCP-stimulated activity can be blocked with CN$^-$ or N$$_3^-$, the cytochrome chain is the most likely candidate. It is perhaps in a partially damaged or altered state in these cells and displays enhanced non-specific sensitivity to SHAM under these conditions.

**Kinetics of cell growth and respiration in the presence of CAP**

Cell growth and respiration during CAP-treatment were followed in the presence of several concentrations of CAP (Fig. 4). In the presence of 100 or 500 µg CAP ml$^{-1}$ total O$_2$ consumption in the cultures levelled off after 80 to 100 min. When these data are expressed on a per cell basis, it is seen that total O$_2$ consumption per cell began to decrease at approximately the same time. This is a reflection of some continuing cell division. In CAP-treated cultures, total CN$^-$-resistant respiration per cell rises in parallel with increases in cell number for 100 to 200 min and, eventually, as CN$^-$-sensitive respiration per cell decreases, is similar in level to total O$_2$ consumption per cell. As CN$^-$-resistant respiration rises in CAP-treated cultures, the proportion of SHAM-sensitive respiration does not increase.
DISCUSSION

Respiration in Tetrahymena and sensitivity to inhibitors

The results with respect to general levels of respiration and inhibitor sensitivity for exponentially growing and stationary phase cells are in good agreement with previously published data (Ruben & Hooper, 1978; Lloyd et al., 1980; Skriver & Nilsson, 1978).

It is of note that CCCP allows O₂ consumption in stationary phase cells to increase to a value similar to the level found, per cell, in exponential phase cells. The increase is CN⁻-sensitive. This suggests that the respiratory chain is present in amounts quantitatively similar to the exponential phase cell, but that under these conditions it is under-utilized. This is consistent with measurements of the mitochondrially-specific phospholipid, cardiolipin, in Tetrahymena which shows an approximate doubling in amount per cell in going from exponential to stationary phase growth (Hemmingsen, et al., 1983). These two observations suggest a selective retention of mitochondrial inner membrane; as cell size is reduced going into stationary phase, mitochondria are apparently conserved.

Amount and utilization of the alternative oxidase

In the absence of CN⁻, there was a marked sensitivity of whole cell respiration to SHAM in exponentially growing cells. At concentrations of 1 mM or less, the inhibitor appears to have
some specificity for the alternative oxidase, as judged by its strong effect on CN⁻-resistant respiration. At higher concentrations, the degree of inhibition attainable (approximately 50% at 4 to 5 mM) suggests a non-specific effect presumably due to the chelating properties of the inhibitor. Lloyd et al. (1980) found no effect of SHAM at 1 mM in whole cells in the absence of CN⁻; however, their cultures were at high density (4 x 10⁶ cells ml⁻¹, concentrated to 2 x 10⁶ cells ml⁻¹ for measurement) and therefore appear to be more typical of the stationary phase cells used here. Data on the absolute level of O₂ consumption per cell were not presented by these authors so that the physiological growth state can not be assessed. Similarly, Perasso et al. (1982) failed to find a SHAM effect; however, the data were not shown and again the growth state cannot be determined from their paper. In addition it is not made clear whether or not the SHAM was tested in the presence or absence of CN⁻.

Metal ions such as Fe³⁺ strongly influence the expression of the alternative oxidase in Tetrahymena (Eichel & Stearns, 1977; Stearns et al., 1978). In the present case, the Neff's medium used contains 100 μM-Fe³⁺, well above the level reported to strongly induce the oxidase (Eichel & Stearns, 1977), however, the level of SHAM-sensitive respiration found is similar to that reported from media lacking added Fe³⁺ (Lloyd et al., 1980). It is likely that factors other than ion content may also influence the level of this oxidase.

Functioning of the oxidase in the absence of CN⁻ in exponentially growing cells is similar to the situation in some plants (for review see Laties, 1982) where it also functions under some normal conditions. In the present case it appears that it may be partially utilized due to the very low levels of excess capacity for electron transport under maximal growth rates (approximately 25%, as shown by CCCP-treatment). Presumably the cytochrome pathway is virtually saturated during exponential growth. In stationary phase cells with a large excess of electron transport capacity the alternative oxidase is unnecessary. These observations seem in keeping with the theory that the alternative path exists for overflow (Lammers, 1980). The stationary phase cells are probably O₂- or glucose-limited, in which case there would not be an excess flux.

**Effect of chloramphenicol**

In CAP-inhibited cells, respiration is 100% insensitive to CN⁻. In this respect it is quite different from the respiration of stationary phase cells. Although CCCP-uncoupling shows that a substantial CN⁻- or N₅⁻-sensitive respiratory chain is present, it appears to be non-functional. Quantitatively, the level of SHAM-sensitive alternative oxidase per cell remains about the same as in exponentially growing cells. Most of the remainder (about 60%) appears to be by CN⁻- and N₅⁻-insensitive pathways. It is inhibited by high SHAM concentrations; however, this appears to have little specificity and is perhaps a reflection of a more general inhibition of metabolism.

From the kinetic experiments, it appears that the residual O₂ consumption per cell continues to increase for about 80 to 100 min following CAP-treatment. Since cell division is continuing, the rates per cell drop. This reduction in O₂ consumption is due to the functional loss of the main respiratory chain. Within 5 to 10 h, virtually all respiration in the cell is CN⁻-resistant. There is apparently no induction of additional alternative oxidase. Based on the graphs of g(i) against Vᵣ (Bahr & Bonner, 1973), it appears that the alternative oxidase is used at low levels in exponentially growing cells with the utilization rising markedly in CAP-treated cells. This situation is quite different from that found in some other organisms. In Neurospora, following CAP-treatment or in poky mutants, the alternative oxidase is derepressed and accounts for a major portion of electron transport (Lambowitz et al., 1972a; b; Slayman, 1977). Similar results have been found in another fungus, Montiellla (Hanssens & Verachtert, 1976).

Given the results presented, the ability of the cell to synthesize ATP must be severely impaired following chloramphenicol-treatment. Although measurements of adenylates and energy charge show they are little changed (Ruben & Hooper, 1978), the available flux must be minimal. It may be coincidental that the level of O₂ consumption in inhibited cells is similar to the level in stationary phase cells; the oxidases operating appear to be quite different. Various other parameters such as protein : phospholipid ratios and morphology of the inner membrane are consistent with this view of a rapid deterioration of the electron transport chain (Gleason et al., 1975; Curgy et al., 1980). It is likely that a direct sensing of limitations in the energy supply
provides the signal which causes these cells to stimulate mitochondrial proliferation following CAP-treatment (Turner & Lloyd, 1971; Gleason et al., 1975).

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REFERENCES


