Evidence for the Presence of Two Terminal Oxidases in the Trypanosomatid *Crithidia oncopelti*

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Increasing concentrations of cyanide inhibited the respiration of whole cells of *Crithidia oncopelti* in a biphasic fashion. Approximately 80% inhibition was attained with 40 μM-KCN. No further inhibition occurred until the concentration of KCN reached approximately 200 μM. Thereafter inhibition rose gradually to 100% at 1500 μM-KCN. Difference spectra revealed the presence of two CO-reacting haemoproteins. These were shown to be two different functional oxidases by photochemical action spectra obtained by using laser light. One oxidase was identified as cytochrome *a+a₃* whilst the other had the properties of cytochrome *o*. Both oxidases could be detected in cells at all stages of growth by the above methods.

INTRODUCTION

Many investigators have reported that the respiratory chains of some eukaryotic micro-organisms are terminated by oxidases other than cytochrome *a+a₃*, the classical terminal oxidase of eukaryotes (Degn *et al.*, 1978; Lloyd *et al.*, 1980). This phenomenon is especially prevalent among protozoa such as the trypanosomes and the amoeba *Acanthamoeba castellanii* (Hill, 1976; Hill & Degn, 1977; Edwards & Lloyd, 1978). Much controversy has been raised regarding the status of cytochrome *o* as a functional oxidase in eukaryotes (for a review see Lloyd & Edwards, 1978). This haemoprotein is common in bacteria (Jurtshuk & Yang, 1980), and a pigment resembling it can readily be detected by CO-difference spectra from a variety of eukaryotes, especially those of the family Trypanosomatidæ. There is little evidence to support a functional role *in vivo* for cytochrome *o* for these organisms. Photochemical action spectra showed that cytochrome *a+a₃* acted as the sole terminal oxidase in the trypanosomatid *Crithidia fasciculata* (Edwards & Lloyd, 1973; Kusel & Storey, 1973). The former investigators also listed five possible candidates for the cytochrome *o*-like component observed in CO-difference spectra. In another study, Kronick & Hill (1974) were able to demonstrate a functional cytochrome *o* by photochemical relief of CO-inhibition of respiration in *Trypanosoma mega*, *Blastocrithidia culicis* and *Leishmania tarentolae*. They were unable to detect any oxidase other than *a+a₃* in the trypanosomatid *Crithidia oncopelti*.

In this paper, we present evidence for the presence of two functional terminal oxidases in *C. oncopelti* which can be resolved by their differential sensitivity to KCN and by photochemical action spectra. One of the oxidases is the conventional cytochrome *a+a₃* whilst the other has similar characteristics to cytochrome *o*.

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METHODS

Organism and growth conditions. *Crithidia oncopelti* was kindly supplied by Dr B. A. Newton, Molteno Institute of Biology and Parasitology, Cambridge, U.K. The protozoa were grown at 25 °C, with shaking at 100 rev. min⁻¹, in a medium that contained (g l⁻¹): bacteriological peptone (Evans Medical, Liverpool, U.K.), 20; glucose, 5; NaCl. Growth was measured by counting in a haemocytometer slide (Thoma B, Hawksley, London).

Polarographic measurements. Oxygen uptake rates of whole cell suspensions were measured at 25 °C using a Rank oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.) linked to an MSE Vitatron chart recorder. Samples (2-4 ml) of undiluted culture were rapidly transferred into the reaction chamber and respiration rates determined in the absence and presence of increasing amounts of cyanide made up as an aqueous solution of glucose,

ethylene glycol

Samples of cell suspensions in growth medium as described by Edwards et al. (1981). Reduction of cell suspensions was achieved by the addition of glucose to a final concentration of 50 mM-Tris/HCl, 50 mM-EGTA (pH 7.4) and resuspended in the same buffer which contained ethylene glycol (30%, v/v), to a cell density of approximately 5 x 10⁹ organisms ml⁻¹. Reduction of the suspension was achieved by the addition of glucose to a final concentration of 50 mM. Oxidation was achieved in the presence of 100 mM-H₂O₂. For CO-difference spectra, a reduced cell suspension was bubbled with CO for 5 min. Treated cell suspensions were transferred to aluminium cuvettes with Perspex windows (path length, 2 mm) and cooled to −20 °C for 5 min. The spectrum of the oxidized suspension was stored in the digital memory of the spectrophotometer and subtracted automatically from that of the reduced spectrum to give a reduced minus oxidized difference spectrum. Similarly, for reduced plus CO minus reduced difference spectra, the reduced spectrum was stored in the memory and subtracted from the reduced plus CO spectrum. All spectra were recorded at a temperature of −80 °C maintained by a flow of cooled nitrogen over the cuvette within the sample compartment of a dual-wavelength scanning spectrophotometer (Chance & Graham, 1971).

Photochemical action spectra. Action spectra for the relief of CO-inhibited respiration were determined directly on cell suspensions in growth medium as described by Edwards et al. (1981). Monochromatic light generated by a Lexel Model 95 Argon Ion Laser was focused through rhodamine 6G (for light between 580 and 610 nm) or rhodamine 560 (for light between 545 and 578 nm). The laser light beam was tuned to the desired wavelength using a tunable dye laser. Light intensity was standardized prior to each determination. A drop of cell suspension (approximately 0.1 ml) was held on the glass stage of a closed Perspex chamber by means of a micro-oxygen electrode in a gas atmosphere of CO/O₂ (60:20, v/v). Relief of CO-inhibited respiration was measured as a rate increase from the steady-state CO-inhibited respiration at each wavelength. Maximal relief occurred at the λₘₚₙ of the CO-bound oxidase. All determinations were made at room temperature.

Chemicals. Rhodamine 560 (chloride) and rhodamine 6G were obtained from Exciton Chemical Co., Dayton, Ohio, U.S.A.

RESULTS

Inhibition of respiration by cyanide

Addition of increasing amounts of KCN to mid-exponential phase cells in growth medium caused an initial rapid inhibition of respiration (Fig. 1). Inhibition rose from 18% at 10 μM-KCN to 80% at 40 μM-KCN. Thereafter, the degree of inhibition remained constant at approximately 84% until the concentration of KCN had reached 200 μM. Further addition resulted in a secondary phase of inhibition, much slower than the first, such that the inhibition of oxygen uptake rose from 84% to 100% over the range 200–1500 μM-KCN. Similar biphasic inhibition curves were obtained for cells at all stages of the growth cycle.

Cytochromes in low-temperature spectra

Further resolution of the cyanide-reacting species was attempted using low-temperature difference spectra of whole cell suspensions. In the reduced minus oxidized spectrum (Fig. 2a) the presence of a + a₃ was indicated by maxima at 444 nm and 607 nm. The presence of b-type cytochromes is suggested by maxima at 427 nm, 432 nm, 526 nm, 532 nm, 560 nm and 567 nm, and that of c-type cytochromes by maxima at 420–423 nm, 516 nm, 550 nm and 554 nm.

For the detection to CO-reacting haemoproteins, a reduced cell suspension was bubbled with a steady stream of CO for 5 min and the difference spectrum versus a reduced suspension determined (Fig. 2b). Two major CO-binding pigments were revealed. Reaction of a₃ with CO resulted in absorption minima at 445 nm and approximately 610 nm whilst the
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Fig. 1. Effect of cyanide on the respiration of whole cells of *Crithidia oncopelti*. Inhibition is expressed as a function of cyanide concentration, the control (0%) being the respiration rate in the absence of the inhibitor. In this experiment organisms were from the mid-exponential phase of growth (3 × 10⁷ cells ml⁻¹).

Fig. 2. Low-temperature difference spectra of *Crithidia oncopelti* from the exponential phase of growth. Cuvettes contained approximately 1 × 10⁹ organisms ml⁻¹. (a) Reduction was achieved in the presence of 50 mM-glucose; the oxidized sample contained 100 mM-H₂O₂. (b) Difference spectrum of a reduced suspension of organisms bubbled with CO for 5 min minus that of a reduced suspension. Base lines for (a) (oxidized minus oxidized) and (b) (reduced minus reduced) are represented by dashed lines.
Fig. 3. Photochemical action spectrum of relief of the CO-inhibited respiration of *Crithidia oncopheli*. The organisms were from the exponential phase of growth \((3 \times 10^7\) organisms ml\(^{-1}\)). The spectrum obtained using rhodamine 6G (583–605 nm) was normalized with that obtained with rhodamine 560 by standardizing relief of CO-inhibited respiration at 591 nm to that at 576 nm.

reduced CO-liganded \(a_3\) showed maxima at 433 nm and 592 nm. The second CO-reacting compound resulted in absorption minima at 562 nm and possibly 529 nm and 413 nm, whilst the reduced CO-liganded form had maxima at 423 nm, 546 nm and 576 nm.

Similar spectra of reduced minus oxidized and reduced plus CO minus reduced were obtained from cell suspensions prepared from all stages of the growth cycle.

**Photochemical relief of CO-inhibited respiration by laser light**

The intensity of emitted light varied with changing wavelength for each dye. For rhodamine 6G a maximum emission of 300 mW was recorded at 590 nm. The intensity fell to 100 mW when the wavelength was 580 nm and to 180 mW at 600 nm. For rhodamine 560 a maximum emission of 100 mW was observed at 560 nm; emission fell to 10 mW at 546 nm and 576 nm. Therefore, prior to each determination of relief of CO-inhibition at a selected wavelength the light intensity was standardized to 10 mW for rhodamine 560 and 100 mW for rhodamine 6G. This limited the range at which measurements could be made to 546–576 nm when using rhodamine 560 and 583–610 nm when using rhodamine 6G.

Rates of \(O_2\) uptake of cells under a 60:20 \((v/v)\) CO/O\(_2\) ratio were measured during exposure to the laser light at known wavelengths (Fig. 3). Peaks of relief from CO-inhibition were apparent at 591 nm and 576 nm, with a definite rise at wavelengths approaching 546 nm. Minima occurred at 561 nm and 580 nm, a further fall occurring towards 610 nm. Similar action spectra were obtained with organisms at different stages of growth.

**DISCUSSION**

The evidence presented here indicates that there are two functional terminal oxidases in the trypanosomatid *Crithidia oncopheli*. They can be distinguished from each other by their different sensitivities to cyanide, which results in a biphasic curve for inhibition of respiration by increasing amounts of cyanide. Lloyd *et al.* (1980) also obtained biphasic inhibition curves for the effects of cyanide and azide on the respiration of the protozoon *Tetrahymena pyriformis*, which provided evidence for alternative routes of electron flow terminated by at least three terminal oxidases.

One of the terminal oxidases of *Crithidia oncopheli* is the conventional cytochrome \(a+a_3\). It can readily be detected in difference spectra and the photochemical action spectrum. The other oxidase identified has similar properties to cytochrome \(a\). Both oxidases are present at all
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stages of growth, a finding which contrasts with previous observations made by Srivastava (1971), who proposed that cytochrome $a+a_3$ was absent during the early stages of growth, the cytochrome $o$-like component functioning as the sole oxidase. The synthesis of $a+a_3$ occurred during the late exponential phase of growth. These findings were based solely on difference spectra, which are not conclusive in the identification of functional oxidases.

A number of possibilities exist for the cytochrome $o$-like component described here. It may be a degradation product of cytochrome P$_{450}$, a crithidial haemoglobin, a cytochrome precursor, or it may be cytochrome c peroxidase as outlined for a similar haemoprotein observed in C. fasciculata (Edwards & Lloyd, 1973). A contribution from haemin in the growth medium can be ruled out because C. oncopelti, unlike other members of the Trypanosomatidae, does not require an added source of haem for growth. This has been ascribed to the presence of a bacterial endosymbiont which is proposed either to supply the trypanosomatid with haem directly, or to possess the missing enzymes for haem biosynthesis (Newton & Horne, 1957; Gill & Vogel, 1962; Gutteridge & Macadam, 1971; Camargo & Freymuller, 1977). A more likely explanation is that the alternative oxidase detected here in difference spectra and action spectra is in fact a cytochrome $o$ oxidase. Other trypanosomes are known to possess such an oxidase, e.g. Trypanosoma mega (Ray & Cross, 1972), Blastocrithidia culicis and Leishmania tarentolae (Kronick & Hill, 1974). Although the latter authors failed to detect cytochrome $o$ in C. oncopelti, a slight peak at approximately 540 nm and 570 nm is apparent on close examination of their action spectrum for this organism. The difference between their results and those presented here may be due to the more powerful laser light source used for the determination of action spectra. Further work is necessary in order to assign a cellular location for the alternative oxidase of C. oncopelti. One interesting possibility is that it is located within the endosymbiont. Little is known of the degree of metabolic autonomy possessed by the endosymbiont but it has been reported that (i) it possesses a double membrane; (ii) it contains DNA of characteristic buoyant density; and (iii) it has a chloramphenicol-sensitive protein-synthesizing system (McGhee & Cosgrove, 1980). We are now directing our efforts to characterizing the cytochrome $o$-like oxidase by kinetic analysis and determining its cellular function by isolation of subcellular organelles.

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REFERENCES


