Terminal Oxidase of *Crithidia fasciculata*. Reactions with Carbon Monoxide and Oxygen at Subzero Temperatures and Photochemical Action Spectra

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Room temperature CO-difference spectra of whole cells of *Crithidia fasciculata* show two CO-reacting haemoproteins. The reaction of cytochrome *a/a*₃ with CO is complete within 1 min of bubbling with CO; that of cytochrome *b* takes longer than 40 min. A non-photodissociable O₂-containing compound of cytochrome *a/a*₃ was formed in whole cell suspensions at –112 °C after photolysis of CO in the presence of 200 µM-O₂. No O₂-cytochrome *b* compound was observed under these conditions. Photochemical action spectra for the relief of CO-inhibited respiration, obtained at different O₂ tensions, indicate cytochrome *a/a*₃ to be the major haemoprotein terminal oxidase; no evidence for a *b*-type cytochrome oxidase has been found.

INTRODUCTION

The trypanosomatid *Crithidia fasciculata* contains *a*-, *b*- and *c*-type cytochromes (Hill & Anderson, 1970; Hill & White, 1968*a, b*; Edwards & Lloyd, 1973) together with a pigment having absorbance maxima at 419, 540 and 570 nm in CO-difference spectra and designated 'cytochrome *o*' (Hill & White, 1968*b*). A partial purification of this CO-binding pigment has been made (Hill & Cross, 1973). Evidence for the presence of cytochrome *o* (from CO-difference spectra) in *Crithidia oncopelti* (Srivastava, 1971) and other *Kinetoplastidae* (Hill & Cross, 1973) has been presented. Respiratory inhibitors have been used to show routes of electron transport alternative to the main respiratory chain in *C. fasciculata* (Hill & Cross, 1973; Edwards & Lloyd, 1973), although the nature of the alternative oxidase(s) is not known. A biphasic cyanide inhibition titration indicated the presence of two oxidases in *C. oncopelti* (Edwards & Chance, 1982). Proof of the presence, and functional activity, of cytochrome *o* in eukaryotes would be of evolutionary significance since cytochrome *o* has not been convincingly demonstrated to be a functional oxidase in unicellular or multicellular eukaryotes (Kusel & Storey, 1973).

A direct method for demonstrating the existence and functionality of CO-binding haemoprotein terminal oxidases, first devised by Warburg & Negelein (1928), is the determination of the photochemical action spectrum for CO dissociation. All known haemoproteins which bind or react with O₂ in the ferrous form also bind CO in this form and are photodissociable. Evidence for the functional activity of cytochrome *o* in *Trypanosoma mega*, *Blastocrithidia culicis* and *Leishmania tarentolae* from photochemical action spectra has been claimed (Kronick & Hill, 1974); evidence for cytochrome *o* from photochemical action spectra was not found in *C. oncopelti*. However, a more recent study by Edwards & Chance (1982), using

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a liquid dye laser instead of a conventional monochromator, showed cytochrome *o* activity in this organism.

Evidence for cytochrome *o* activity in *C. fasciculata* has been presented by Kusel & Kronick (1972) who found a 'shoulder' in the action spectrum at 415–420 nm, corresponding to the cytochrome *o*–CO complex. A component with a maximum at 570 nm in the photochemical action spectrum was observed by Edwards & Lloyd (1973) but no corresponding maximum in the Soret region of the action spectrum was found, leading to the conclusion that cytochrome *o* was not present. The action spectra obtained by Kusel & Storey (1973) led these investigators to a similar conclusion.

In an attempt to resolve the question of the presence and activity of cytochrome *o* in *C. fasciculata* we have re-examined the CO- and O₂-binding properties of haemoproteins in this organism. CO-difference spectra reveal the presence of a *b*-type cytochrome that binds CO slowly. Low-temperature photodissociation spectra show only one O₂-containing intermediate (i.e. that of cytochrome *a/a*₃) and photochemical action spectra at different O₂ tensions using a liquid dye laser indicate only cytochrome *a/a*₃ as terminal oxidase. We conclude that cytochrome *o* does not act as a terminal oxidase in this organism, and that any alternative oxidases are not haemoproteins.

METHODS

Growth and harvesting of the organism. *Crithidia fasciculata* was grown in a medium which contained 2% (w/v) proteose peptone, 0.1% liver digest, 1% (w/v) glycerol, 0.5% Tween 80, 0.6% triethanolamine, 25 mg haemin l⁻¹ and 2.5 mg folic acid l⁻¹, as described by Edwards & Lloyd (1973). Harvesting of cells in the late exponential phase of growth was by centrifugation at 1500 *g* (*r*_{av}, 7.0 cm) at room temperature in the 8 × 50 ml rotor of a Sorvall RC-5B centrifuge.

Cytochrome spectra. Difference spectra were measured using a Hitachi/Perkin–Elmer 557 spectrophotometer (Lloyd *et al.*, 1982; Scott & Lloyd, 1983). Cells were harvested, washed twice in 20 mM-potassium phosphate buffer (pH 8.0) and resuspended in 6 ml 20 mM-phosphate buffer (pH 8.0) containing 0.5% methyl cellulose. Spectra were recorded at room temperature in cuvettes of 10 mm path-length; spectral band-width was 2 nm and the scanning speed 60 nm min⁻¹. CO was bubbled for 30 s into open cuvettes which were scanned at various times after the addition of CO.

Low-temperature flash photolysis. The technique used was that of Chance *et al.* (1975*a*). Washed whole-cell suspensions of *C. fasciculata* were incubated in 30% (v/v) ethylene glycol for 5 min in 3 mm path-length cuvettes at room temperature; endogenous respiration ensured anaerobiosis. Cuvettes were bubbled with CO for 2 min, incubated at room temperature for a further 2 min, cooled to –20 °C and equilibrated in the dark for 5 min. Some suspensions were then oxygenated (Chance *et al.*, 1975*b*) by vigorous stirring; cuvettes were then transferred to a bath containing ethanol and solid CO₂ at –80 °C in the dark. Analyses were performed in a dual-wavelength spectrophotometer; baseline corrections (spectra of CO-liganded anaerobic cell suspensions) were stored in a digital memory. The cuvette was cooled by a stream of cold N₂ gas to the required temperature. Flash photolysis by a 20 J xenon lamp was followed by repetitive scanning at 90 s intervals. Further details are provided by Lloyd *et al.* (1982).

Photochemical action spectra. Reversal by light of CO inhibition of respiration was obtained using a liquid dye laser excited by an 8 W argon ion laser. Rhodamine 6G was used to obtain laser light over the range 568–630 nm and Rhodamine 110 over the range 533–568 nm (Lloyd *et al.*, 1982; Lloyd & Scott, 1983). The power of laser light used for illumination was kept constant for each wavelength used by altering the power of the input argon ion laser. Measurements of the respiration of whole-cell suspensions were made at 30 °C with an open reaction system (5 ml working volume) fitted with an oxygen electrode (Radiometer, Copenhagen, Denmark) and stirred at 1500 r.p.m. Either a CO/O₂ or CO/air (19:1, v/v) gas mixture flowed over the vortex of the cell suspension. Full details of the method are presented elsewhere (Lloyd & Scott, 1983).

RESULTS

Room-temperature CO-difference spectra

Room-temperature CO-difference spectra of washed whole-cells of *C. fasciculata* are shown in Fig. 1. The first scan, 1 min after bubbling with CO (Fig. 1*a, b*), shows in the α -region a maximum at 590 nm (assigned to the cytochrome *a*₃–CO complex) and a shoulder at 570 nm (due to a cytochrome *b*–CO complex); a minimum at 558 nm was observed. The γ -region shows a

Fig. 1. Room-temperature CO-difference spectra of *C. fasciculata* and the kinetics of reaction with CO. Harvested, washed cells were resuspended in 20 mM-phosphate buffer (pH 8.0) containing 0.5% methyl cellulose and allowed to become anaerobic in the presence of 50 mM-glycerol. A reduced minus reduced baseline (not shown) was recorded and the sample cuvette bubbled with CO for 30 s. Spectra (a) and (b) were recorded 1 min after the addition of CO; (c) and (d) were recorded 68 min later. In (e) the absorbance changes at 570–558 nm (●) and 590–603 nm (○) are plotted as a function of time after bubbling with CO. The scan speed was 60 nm min⁻¹, slit width 2 nm, path-length 1 cm and the protein content was 32.4 mg ml⁻¹.

broad asymmetric peak with a maximum at 428 nm. A further scan, obtained after a further 68 min (Fig. 1c, d) shows a maximum at 590 nm; the intensity of the 570 nm component is increased and the associated trough has shifted from 558 to 555 nm. The peak in the γ -region of the spectrum is increased in intensity and shifted from 428 to 419 nm, and is assigned to the formation of a cytochrome *b*-CO complex; the cytochrome *a/a*₃-CO complex is no longer clearly visible. The kinetics of the formation of the CO complexes of cytochrome *a/a*₃ and cytochrome *b* are shown in Fig. 1(e). The reaction of cytochrome *a/a*₃ with CO is complete within 1 min, indicated by the constant ΔA (590–603 nm). The cytochrome *b*-CO complex takes longer to form; the reaction is virtually complete after 40 min.

Low-temperature photodissociation spectra

Photodissociation spectra of whole cells of *C. fasciculata* after treatment with CO for 2 min are shown in Fig. 2 and indicate the presence of more than one CO-reacting haemoprotein. The first scan after photolysis (i.e. flash-photolysed minus CO-reduced) is shown. The minima at 595 and 435 nm are due to the CO-liganded forms of cytochrome *a*₃; the maximum at 449 nm is due to the appearance of the reduced form. Photolysis also yielded components with maxima at 428 and 564 nm, due to the reduced form of *b*-type cytochrome. Repeated scans (not shown) indicated that the recombination of cytochrome *a*₃ with CO at this temperature was hardly detectable over 15 min and hardly any photolytic dissociation was obtained by a further flash after 11 scans. Photolysis in the presence of 200 μ M-O₂ at -112 °C (Fig. 3) and successive

Fig. 2. The reaction of haemoproteins of *C. fasciculata* with CO at -112°C . The spectrum of CO-ligated endogenously reduced cells was scanned and stored in the digital memory of a dual-wavelength spectrophotometer. The first scan yielded the baseline (----) and represents a CO-reduced minus CO-reduced spectrum. The next scan (—) followed photolysis at -112°C using three flashes. Scanning was from right to left at 3.5 nm s^{-1} and the reference wavelength was 575 nm . Absorbance increments and the wavelengths (in nm) of distinctive spectral features are shown.

Fig. 3. The reaction of haemoproteins of *C. fasciculata* with O_2 at -112°C . The spectrum of a suspension of CO-ligated, endogenously reduced (anaerobic) organisms into which O_2 had been stirred at -20°C was scanned at -112°C and the spectrum stored in the memory of a dual-wavelength spectrophotometer. The reference wavelength was 575 nm . Subsequent scans are difference spectra with the stored spectrum subtracted. The dashed line is the CO-reduced minus CO-reduced baseline, recorded before initiation of the reaction by three flashes. Subsequent spectra were recorded at 90 s intervals; alternate spectra are shown.

scanning indicated the formation of an O_2 -containing intermediate of cytochrome a_3 which was spectrally distinct from the CO-ligated oxidase. This O_2 -containing complex was not photodissociable by light intensities giving photolysis of the CO-ligated oxidase (results not shown). No O_2 -containing complex of a b -type cytochrome was detectable at this temperature.

Fig. 4. Photochemical action spectra of *C. fasciculata*. Cells were harvested, concentrated 10-fold and resuspended in growth medium. The gas phase passing over the cell suspension in (a) contained CO/air (95:5, v/v), and in (b) it contained CO/O₂ (95:5, v/v), giving O₂ concentrations in the liquid phase of 7.5 and 43.6 μ M, respectively. Laser powers used to illuminate the cell suspensions for Rhodamine 6G were 25 mW and for Rhodamine 110 40 mW. Spectra obtained with Rhodamine 110 (535–568 nm) were normalized to those of Rhodamine 6G (568–630 nm) by assuming the rates obtained at 568 nm with the two dyes to be equal and expressing rates as a function of the maximum rate obtained.

Photochemical action spectra

In order to determine whether the slowly CO-reacting *b*-type pigment observed in difference spectra (Fig. 1) acted as a functional terminal oxidase, photochemical action spectra for the release of CO-inhibited respiration using a liquid dye laser (Lloyd & Scott, 1983) were obtained. Rhodamine 110 was used for laser light from 533–568 nm and Rhodamine 6G from 568–630 nm. The action spectrum of *C. fasciculata* obtained under a gas phase of CO/air (95:5, v/v) (O₂ concn in the liquid = 7.5 μ M) shows maxima at 592 and 543 nm corresponding to the α - and β -bands, respectively, of the CO-liganded form of cytochrome *a*₃ (Fig. 4a). When a gas phase of CO/O₂ (95:5, v/v) (O₂ concn 43.6 μ M) was employed (Fig. 4b), similar maxima were obtained, indicating the presence of a single oxidase. No evidence for cytochrome *o* activity (indicated by a maximum at about 570 nm) was found in either action spectrum.

DISCUSSION

The present paper confirms the role of cytochrome *a/a*₃ as the major haemoprotein terminal oxidase in *C. fasciculata*. The CO-binding *b*-type cytochrome, present in intact cells and previously referred to as cytochrome *o* does not function as an oxidase. Other slowly CO-reacting *b*-type cytochromes that do not act as oxidases have been found in *Acanthamoeba castellanii* (Scott & Lloyd, 1983) and *Tetrahymena pyriformis* (Lloyd *et al.*, 1982). The time-course of the slow formation of the cytochrome *b*-CO complex in *C. fasciculata* (Fig. 1e) is different from the monotonic increase observed in *A. castellanii* (Scott & Lloyd, 1983) and may be due to the presence of ingested haemin, or to two or more haemoproteins that differ in their rates of reaction with CO. Other components that yield CO-difference spectra and resemble cytochrome *o* have been suggested by Edwards & Lloyd (1973) and include crithidial haemoglobin, a degradation product of cytochrome P₄₅₀ or cytochrome *b*, and cytochrome *c* peroxidase.

Low-temperature photodissociation spectra also indicate the presence of two CO-binding haemoproteins, but only one of these (cytochrome a_3) reacts to form an O₂-containing intermediate. The presence of more than one photodissociable CO-binding haemoprotein has been shown in *Tetrahymena pyriformis*, although cytochrome a_{620} is the sole haemoprotein oxidase (Lloyd *et al.*, 1982). *Acanthamoeba castellanii* has four CO- (and O₂-) binding haemoproteins (Lloyd *et al.*, 1981) but only one of these (cytochrome a_3) acts as an oxidase (Scott & Lloyd, 1983).

The action spectra obtained by Kronick & Hill (1974) failed to reveal cytochrome o in *C. oncopelti*, although this oxidase was detected by Edwards & Chance (1982) using high light-intensities from a liquid dye laser. The action spectra in the present paper are different from the action spectrum of *C. oncopelti* (Edwards & Chance, 1982) where a maximum at 576 nm was clearly visible. The cellular location of this cytochrome oxidase is unknown and may be associated with the bacterial endosymbiont known to be present (Edwards & Chance, 1982). Action spectra of *C. fasciculata* obtained at different O₂ tensions failed to reveal cytochrome o ; this oxidase has been detected in *Leishmania tarentolae*, although only at lowered O₂ concentrations (Kronick & Hill, 1974).

Previously, action spectra of *C. fasciculata* have revealed the presence of cytochrome o (Kusel & Kronick, 1972), although this finding was refuted by Kusel & Storey (1973) who could find no cytochrome o activity even after growth of cells in the presence of acriflavin, or after obtaining the action spectrum in the presence of antimycin or azide. Edwards & Lloyd (1973) found a component in the action spectrum at 570 nm but with no corresponding maximum in the Soret region. All of these studies used light from conventional monochromators of relatively broad band-width, did not correct for energy content through the spectrum and used the 'hanging drop' method. All of these factors in determining the action spectrum may be criticized (Lloyd & Scott, 1983) and may have contributed to the different results so far obtained. The use of a liquid dye laser and a stirred cell suspension (Lloyd & Scott, 1983) eliminate these problems. Our findings, that the b -type cytochrome present in *C. fasciculata* reacts slowly with CO in difference spectra, does not form an O₂-containing intermediate in photodissociation spectra and does not contribute to the photochemical action spectrum, strongly indicate that it does not function as an oxidase.

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