Photochemical action spectra indicate that cytochrome a/a_3 is the predominant haemoprotein terminal oxidase in *Acanthamoeba castellanii*

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1. Room-temperature CO-reduced minus reduced difference spectra of intact cells of *Acanthamoeba castellanii* show the presence of CO-reacting haemoproteins in cells from the early-exponential, late-exponential and stationary phases of growth. 2. The relative rates of reaction with CO of the two haemoproteins differ; that of cytochrome a/a_3 with CO is complete within 1 min of bubbling with CO, whereas that of cytochrome b takes longer than 90 min. 3. Photochemical action spectra reveal cytochrome a/a_3 as the predominant haemoprotein oxidase at all stages of growth. 4. It is concluded that the alternative oxidases known to be present in these organisms are not cytochromes.

Many different eukaryotic micro-organisms contain terminal oxidases in addition to cytochrome a/a_3 (Degn *et al.*, 1978), although the nature and function of these alternative oxidases is not well understood. The soil amoeba *Acanthamoeba castellanii* contains three different mitochondrial terminal oxidases (Edwards & Lloyd, 1977, 1978*a,b*; Lloyd *et al.*, 1979, 1982*a*): (1) cytochrome a/a_3 , inhibited by azide, CO and cyanide (K_m for $O_2 < 1 \mu M$); (2) an oxidase inhibited by salicyl-hydroxamic acid ($K_m < 1 \mu M$); and (3) an oxidase with a low affinity for O_2 ($K_m > 7 \mu M$) that is not inhibited by CO but is inhibited by cyanide. Either the affinity for O_2 of this third oxidase is increased in cells from the late-exponential phase of growth or it is replaced by a fourth oxidase (Lloyd *et al.*, 1982*a*). The respiration of mitochondria isolated from early-exponential-phase cultures is stimulated by up to 50% by 1 mM-cyanide; the respiration of mitochondria from the late-exponential phase is inhibited by 70–80% (Edwards & Lloyd, 1977). It has been suggested (Edwards & Lloyd, 1978*a*) that these changes in cyanide-sensitivities reflect alterations in the proportions of electron fluxes through the pathways leading to different oxidases.

The reaction of CO with some haemoproteins has often been used as an aid to their identification and also as an indication of those components that may react with O_2 (Chance, 1957; Lemberg & Barrett, 1973; Lloyd, 1974). Mitochondria isolated from *A. castellanii* contain four different CO-binding haemoproteins: (i) cytochrome a_3 ; (ii) cytochrome a_{614} ; (iii) a b -type cytochrome; and (iv) a c -type cytochrome (Edwards & Lloyd, 1980). All of these haemoproteins react with O_2 at low temperatures to form

non-photodissociable compounds. However, only cytochrome a_3 has been shown to be a functional terminal oxidase under physiological conditions (Lloyd *et al.*, 1981). Whether these CO-reacting haemoproteins are associated with the alternative oxidases present in *A. castellanii* remains to be ascertained.

The present study was undertaken to determine whether the CO-binding haemoproteins present in *A. castellanii* are functional oxidases. Highly detailed photochemical action spectra for the release of CO-inhibited respiration using a liquid-dye laser (Lloyd & Scott, 1983) reveal that cytochrome a/a_3 is the predominant haemoprotein oxidase in *A. castellanii*. Alternative pathways of electron transport present in this organism must therefore terminate in non-haemoprotein oxidases.

Experimental

Maintenance, growth and harvesting of the organisms

Acanthamoeba castellanii was maintained, grown axenically with shaking at 30°C and counted as described previously (Edwards *et al.*, 1977). Cells were harvested at room temperature by centrifugation at 10 g (r_{av} , 10 cm) for 5 min in the 4 × 50 ml head of an MSE bench centrifuge. Harvesting was carried out in the early-exponential, late-exponential or stationary phase of growth (after 24, 48 or 72 h respectively) (Edwards & Lloyd, 1978*b*).

Cytochrome spectra

Cells were harvested, washed once in a buffer containing 50 mM-MgCl₂, pH 7.4, and resuspended

with 6 ml of buffer containing 0.5% (w/v) methylcellulose and 50 mM-MgCl₂, pH 7.4. Difference spectra were measured by using a Hitachi/Perkin-Elmer 557 spectrophotometer. 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. CO was bubbled into open cuvettes for 60 s, which were scanned at various times after the addition of CO.

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 572–634 nm and Rhodamine 110 over the range 534–572 nm (Lloyd & Scott, 1983; Lloyd *et al.*, 1982b). 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 laser power were made with a Spectra-Physics model-404 power meter. 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 Ltd., Copenhagen, Denmark) and stirred at 1500 rev./min. A CO/O₂ (19:1) gas mixture flowed over the vortex of the stirred suspension. Full details of the method are presented elsewhere (Lloyd & Scott, 1983; Lloyd *et al.*, 1982b).

Results

CO-difference spectra of whole cells

Fig. 1 shows CO-reduced minus reduced difference spectra of an intact cell suspension of

Fig. 1. Room-temperature CO difference spectra of whole cells of *A. castellanii* harvested from the late-exponential phase of growth

A washed suspension of whole cells in 0.5% (w/v) methylcellulose/50 mM-MgCl₂, pH 7.4, was allowed to become anaerobic in the presence of 16.6 mM-glucose by incubation for 5 min at room temperature. A reduced - reduced baseline (not shown) was recorded and the sample cuvette bubbled slowly with CO for 60 s. Spectrum (a) was recorded 1 min after the addition of CO, (b) after 30 min and (c) after 90 min. The scan speed was 60 nm/min, slit width 2 nm, path-length 1 cm and the protein content was 12.2 mg/ml.

Fig. 2. Kinetics of the reactions of cytochrome a/a_3 and b -type cytochrome with CO

Intact cells of *A. castellanii* from the early-exponential (a), late-exponential (b) or stationary (c) phase of growth were harvested, suspended in buffer and bubbled with CO as described in Fig. 1. Spectra were recorded at various times up to 90 min after the end of bubbling. The absorbance changes at 570–558 nm (●) and 587–604 nm (○) are plotted as a function of time. The protein concentrations of the cell suspensions in (a), (b) and (c) were 13.1, 12.2 and 13.7 mg/ml respectively.

late-exponential-phase *A. castellanii*. The first scan (Fig. 1a) obtained 1 min after the end of bubbling with CO shows a maximum at 587 nm and a shoulder at 570 nm (assigned to the CO complexes of cytochrome a/a_3 and b -type cytochrome respectively). The β -region of the spectrum is broad and is centred around 538 nm. Minima in the spectrum (at 604 and 558 nm) are due to the reduced forms of cytochrome a/a_3 and b -type cytochrome. The

minimum at 440 nm is broad and may be due to a mixture of these cytochromes; cytochrome a_3 absorbs at 446 nm and the b -type cytochrome at 430 nm (Edwards & Lloyd, 1980). Distinct maxima in the γ -region due to the CO complexes of cytochrome a_3 and the b -type cytochrome were not observed; presumably the maximum at 421 nm contains both of these components.

A further scan of the spectrum, after 30 min (Fig. 1b), shows the 570 and 538 nm peaks to be increased in intensity; the trough at 558 nm is also deeper and moved to 555 nm. In the γ -region the 440 nm trough is increased in intensity; the maximum is also increased and moved to about 418 nm. After a further 60 min these effects are more pronounced (Fig. 1c). Similar spectra (not shown) were obtained with cells from both the early-exponential and stationary phases of growth. Binding of CO to cytochrome a_{614} or to a c -type cytochrome was not observed, although these cytochromes can be detected in mitochondrial preparations (Edwards & Lloyd, 1980). The contribution of the microsomal fraction to the CO difference spectra (judged by the presence of cytochrome P-450) appears small.

The rates of formation of CO complexes with b -type cytochrome and cytochrome a/a_3 in early-exponential phase cells are shown in Fig. 2(a). Cytochrome a/a_3 shows rapid reaction with CO; the CO complex is fully formed before the first scan is completed. In contrast, the b -type cytochrome shows a much slower reaction with CO; the CO complex is not fully formed even after 80 min. Similar responses were observed with both late-exponential and stationary phase cells (Figs. 2b and 2c) and with cells in which the time of bubbling with CO was decreased to 15 s (results not shown).

Photochemical action spectra

Photochemical action spectra for the reversal of CO inhibited respiration were obtained to determine if any of the CO-reacting haemoproteins detected in difference spectra could function as terminal oxidases. Early-exponential-phase cells (at an O_2 concentration of $1.5 \mu\text{M}$) showed maxima (Fig. 3a) at 543 and 591 nm, corresponding to the CO complex of cytochrome a/a_3 . No component with a maximum around 570 nm (corresponding to the cytochrome b -CO complex) was observed; the CO-binding cytochromes c and a_{614} were also undetectable in the action spectrum. Similar results were obtained with late-exponential- and stationary-phase cells (at O_2 concentrations of 3.7 and $3.8 \mu\text{M}$ respectively) (Figs. 3b and 3c). The action spectrum of late-exponential-phase *A. castellanii* in the presence of 1 mM-azide is shown in Fig. 3(d). Illumination of the cell suspension resulted in responses that were decreased by about 50% when compared with those in the absence of azide (Fig. 3b). The action

Fig. 3. Photochemical action spectra of intact cells of *A. castellanii*

Cells were harvested during (a) the early-exponential, (b) the late-exponential or (c) the stationary phases of growth and resuspended with 5 ml of culture supernatant. (d) Shows the action spectrum of the late-exponential-phase cells in the presence of 1 mM-azide. Laser powers used to illuminate the cell suspensions for Rhodamine 6G were 30 mW for each of (a), (b), (c) and (d); for Rhodamine 110, powers were (a) 40 mW, (b) 20 mW and (c) 40 mW. Spectra obtained with Rhodamine 110 (532–572 nm) were normalized to those of Rhodamine 6G (572–633 nm) by assuming the rates obtained at 572 nm with the two dyes to be equal; respiration rates were then expressed as a function of the maximum rate obtained. The O_2 concentration in the liquid phase was (a) $1.5 \mu M$, (b) $3.7 \mu M$, (c) $3.8 \mu M$ and (d) $4.5 \mu M$. The gas phase passing over the cell suspension was CO/O_2 (19:1), which is equivalent to concentrations of $814 \mu M$ -CO and $56 \mu M$ - O_2 .

spectrum shows a maximum at 592 nm; the diminished response due to cytochrome a/a_3 does not unmask any other putative haemoprotein oxidase. Action spectra of early-exponential- and stationary-phase cells in the presence of azide also indicated no other components to be present (results

not shown). These results show that cytochrome a/a_3 is the predominant haemoprotein terminal oxidase in *A. castellanii* during the exponential and stationary phases of growth, and that the alternative oxidases of this organism are not haemoproteins.

An oxidase with a low affinity for O_2 (such as that found in *A. castellanii*; Lloyd *et al.*, 1979) can also be detected in photochemical action spectra at O_2 concentration greater than the K_m for the oxidase. Attempts to obtain action spectra at O_2 concentrations greater than $7 \mu M$ proved unsuccessful. Below this concentration, illumination of the cell suspension with laser light resulted in a smooth monotonic change to a new, lower, steady-state O_2 concentration (for typical traces, see Lloyd & Scott, 1983). At higher O_2 concentrations, illumination of *A. castellanii* resulted in an initial increased respiration rate followed by a transient increase to a new, higher, steady state (results not shown). These changes in the steady-state O_2 concentration did not allow the action spectrum to be obtained. This phenomenon was noted in exponentially growing and stationary-phase *A. castellanii*, but has not been observed in a range of other organisms examined (bacteria, yeasts and protozoa; R. I. Scott & D. Lloyd, unpublished work).

Discussion

The present paper has shown that cytochrome a/a_3 is the predominant haemoprotein terminal oxidase in *A. castellanii* harvested from the early-exponential, late-exponential and stationary phases of growth. Other CO-reacting haemoproteins found to be present in isolated mitochondria (Edwards & Lloyd, 1980; Lloyd *et al.*, 1981) do not have a physiological function as terminal oxidases. The CO-binding cytochromes detectable in whole cells are cytochrome a/a_3 and a b -type cytochrome; possibly the CO-binding cytochromes a_{614} and the c -type cytochrome (Lloyd *et al.*, 1981) are at a low concentration and hence are undetectable in intact cells. Even though four CO- and O_2 -binding cytochromes are present in mitochondria isolated from *A. castellanii*, their rates of reaction with O_2 are unknown. The contribution of these other haemoproteins to measurements of O_2 uptake may be small compared with cytochrome a/a_3 .

A slowly CO-reacting b -type cytochrome that does not function as a terminal oxidase has also been demonstrated in *Tetrahymena pyriformis* (Lloyd *et al.*, 1982b). The slow reaction of b -type cytochromes with CO may indicate that denaturation of the cytochrome is occurring (Edwards & Lloyd, 1980). Photochemical action spectra (Lloyd *et al.*, 1982b; Unitt *et al.*, 1983) have revealed that cytochrome a_{620} is the sole haemoprotein oxidase in

T. pyriformis and that the alternative pathways of electron transport known to be present (Lloyd *et al.*, 1980) are not terminated by cytochromes. CO-binding b -type cytochromes have also been found in *Crithidia fasciculata* (Edwards & Lloyd, 1973; Kusel & Storey, 1973); photochemical action spectra (R. I. Scott & D. Lloyd, unpublished work) reveal this cytochrome not to be an oxidase. A b -type cytochrome has been shown to be an oxidase in *Crithidia oncopelti* (Edwards & Chance, 1982), although it is unclear if the oxidase is associated with the bacterial endosymbiont known to be present (McGhee & Cosgrove, 1980). Only in some trypanosomes (Hill, 1976) has there been any evidence for the existence of a functional eukaryotic cytochrome o .

Previous action spectra of *A. castellanii* (Edwards *et al.*, 1977), using manometric measurements of O_2 uptake and light of 7–12 nm bandwidth, have revealed at least three possible components in the α -region of the spectrum, including cytochrome a/a_3 . These discrepancies with the results of the present paper may be due to no correction being made for variation of energy content through the spectrum by Edwards *et al.* (1977). Although the characteristics of respiration with respect to inhibitor sensitivities changes markedly at different stages of growth (Edwards & Lloyd, 1977, 1978a) leading to the synthesis of various terminal oxidases (Edwards & Lloyd, 1977, 1978a,b; Lloyd *et al.*, 1979, 1982a) the present paper has shown cytochrome a/a_3 to be the predominant haemoprotein terminal oxidase of *A. castellanii*. The alternative electron-transport chains of this organism are terminated by non-cytochrome oxidases; further work is necessary to determine their structure and function.

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