Cytochrome a_{620} in *Tetrahymena pyriformis*

Reactions with carbon monoxide and oxygen at subzero temperatures and photochemical action spectra

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1. Mitochondria-enriched fractions of the ciliate protozoan *Tetrahymena pyriformis* ST contained CO-reacting cytochromes b_{560} and a_{620} . 2. A non-photodissociable oxygen-containing compound of cytochrome a_{620} was formed in whole cell suspensions at -114°C after photolysis of CO in the presence of $200\mu\text{M-O}_2$. 3. Electron transport, indicated by the oxidation of cytochrome a_{620} and cytochrome c , occurred at temperatures higher than -72°C . 4. Photochemical action spectra for the relief of respiratory inhibition of whole cells by CO obtained by using a liquid dye laser indicate that the only CO-reacting terminal oxidase detectable was cytochrome a_{620} . 5. It is concluded that the alternative electron transport chains in this organism utilize non-cytochrome terminal oxidases.

Almost all aerobic eukaryotes possess mitochondrial respiratory chains that terminate at cytochrome $a + a_3$, but the ciliate protozoan *Tetrahymena pyriformis* is exceptional in that its a -type cytochromes (Turner *et al.*, 1971) are different from the classical oxidase in several respects. The unusual spectrum of *Tetrahymena* cytochrome a_{620} , first reported by Baker & Baumberger (1941) has been studied more recently (Turner *et al.*, 1971; Lloyd & Chance, 1972; Kilpatrick & Erecinska, 1977a,b). Mammalian cytochrome c is not oxidized by cell-free homogenates or mitochondria (Ryley, 1952; Keilin & Ryley, 1953; Eichel, 1954; Kobayashi, 1965; Van de Vijver, 1966) as the cytochrome c_{553} of this organism has distinctive electron donor properties (Yamanaka *et al.*, 1968). Earlier reports of lack of sensitivity of substrate oxidations to cyanide, azide or CO probably indicated the presence of pathways of electron transport alternative to the main phosphorylating respiratory chain (Lloyd, 1974; Lloyd & Edwards, 1978; Lloyd *et al.*, 1980). Functional evidence for the role of cytochrome a_{620} as the major terminal oxidase of *T. pyriformis* was provided by the demonstration of its kinetic competence

(Turner *et al.*, 1971). Other CO-binding cytochromes present in this organism include cytochrome b_{560} (Yamanaka *et al.*, 1968; Lloyd & Chance, 1972; Kilpatrick & Erecinska, 1977b) present in mitochondrial fractions, and sometimes referred to as cytochrome o (Perlsh & Eichel, 1971), cytochromes b_5 , P_{450} and P_{420} found in 'microsomal' fractions (Poole *et al.*, 1971), and a haemoprotein purified by Yamanaka *et al.* (1978). The present study shows that cytochrome a_{620} is the only cytochrome which reacts with O_2 in *T. pyriformis* ST to give a detectable oxygen-containing compound at sub-zero temperatures; photochemical action spectra confirm its role as the major functional terminal oxidase in this protozoan. Any alternative electron transport pathways (Lloyd *et al.*, 1980) must therefore utilize non-cytochrome terminal oxidases, e.g. the salicylhydroxamic acid-sensitive oxidase previously described in *T. pyriformis* (Eichel & Stearns, 1977).

Experimental

Maintenance and growth of the organisms

Tetrahymena pyriformis ST was maintained and grown on 2% (w/v) proteose peptone (Difco)/0.1% liver digest (Oxoid) with shaking at 30°C exactly as described previously (Lloyd *et al.*, 1971). Organisms were counted in a Sedgewick–Rafter cell (Graticules Ltd., Tonbridge, Kent, U.K.) after fixa-

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tion in 4% (v/v) aqueous formaldehyde and suitable dilution in water.

Harvesting and breakage of organisms, and preparation of mitochondrial fractions

Organisms were harvested from cultures in the late exponential phase of growth (cell density approx. 10^5 organisms/ml), disrupted by hand homogenization, and mitochondrial fractions were obtained by differential centrifugation as previously described (Turner *et al.*, 1971).

Cytochrome spectra

Difference spectra were measured by using a Hitachi-Perkin Elmer 557 spectrophotometer. Spectra were recorded at room temperature (18°C) 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 various times as indicated.

Low-temperature flash photolysis

The technique used was that of Chance *et al.* (1975b). Washed whole-cell suspensions of *T. pyriformis* containing 30% (v/v) ethylene glycol were incubated at room temperature in the absence of added respiratory substrates in 3 mm light-path cuvettes for 5 min; endogenous respiration ensured attainment of anaerobiosis. After bubbling with CO for 2 min and a further 2 min incubation at room temperature, cuvettes were cooled to -20°C in a bath containing ethanol and solid CO₂ and were allowed to equilibrate in the dark for 5 min. At this stage some suspensions were oxygenated by stirring vigorously with a stainless-steel coil that fitted the cuvette; 200 µM-O₂ was introduced by stirring at 2 Hz for 20 s (Chance *et al.*, 1975a). Cuvettes were then transferred to a bath containing ethanol and solid CO₂ at -80°C in the dark. Spectrophotometric analysis was performed in a dual-wavelength scanning spectrophotometer (Chance *et al.*, 1972); 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 (monitored and maintained by a thermocouple adjacent to the sample); during the 10 min equilibration period precautions were taken to prevent photodissociation by the monitoring light beam. Flash photolysis (six flashes of a 20 J xenon lamp) was followed by repetitive scanning (at 90 s intervals).

Photochemical action spectra

Reversal by light of CO inhibition of respiration was obtained by using a liquid dye laser (model 375, Spectra Physics, Brick Knoll Park, St. Albans, Herts., U.K.) excited by an argon ion laser (8 W, Lexel Corporation, Lambda Photometrics, Lambda

House, Batford Mill, Harpenden, Herts., U.K.); Rhodamine 6G gave an output of >100 mW over the range 572–634 nm and Rhodamine 110 > 50 mW over the range 534–572 nm (Edwards *et al.*, 1981; Lloyd & Scott, 1982). Measurements of the respiration of whole cell suspensions were made at 30°C with an open reactor system (5 ml working volume) fitted with an oxygen electrode (Radiometer, Copenhagen, Denmark) and stirred at 1500 rev./min. A CO/O₂ gas mixture, produced by an accurate digital gas mixer (Degn *et al.*, 1980) flowed at 210 ml/min over the vortex of the stirred suspension. A Johnson Foundation O₂ electrode amplifier connected to a two-channel recorder enabled continuous readout of both steady state O₂ concentrations and the first order derivative of the output signal. Full details of the method are presented elsewhere (Lloyd & Scott, 1982).

Results

CO-difference spectra of mitochondrial fractions at room temperature

Fig. 1 shows difference spectra (CO-reduced minus reduced) of a mitochondria-enriched fraction from *T. pyriformis*. The predominant features of the spectrum obtained after brief exposure to CO were those of cytochrome *a*₆₂₀ (minima at 618 and 448 nm); corresponding maxima (assigned to the CO-liganded form of this cytochrome) were observed at 597 and 433 nm. An absorption minimum in the region 550–560 nm and maximum at 418.5 nm were also evident; these became more pronounced in a repeat scan of the same cuvette contents 16 min later (Fig. 1b), by which time the Soret region was dominated by the CO-liganded cytochrome *b*. After a further 7 min the α -absorption maximum at 572 nm together with the strong γ -absorption at 418.5 nm became clearly evident (Fig. 1c). Similar sequences of changes were observed with succinate as electron donor; in all cases both *a* and *b*-type cytochromes reacting with CO were demonstrated, but whereas reaction with cytochrome *a*₆₂₀ was rapid, that with cytochrome *b* was much slower.

Reaction of whole-cell suspensions with CO at sub-zero temperatures

Low temperature photodissociation spectra of endogenously reduced whole-cell suspensions after treatment with CO for 2 min also indicated the presence of several CO-reacting haemoproteins. At -112.5°C progressive photolysis was produced by five flashes of the xenon lamp (Fig. 2) and gave maxima at 620 nm and 448 nm; corresponding minima due to disappearance of CO-liganded species were observed at 597 and 433 nm. Photolysis of a minor component was also evident at 418 nm. Repeated scans (not shown) indicated that the re-

Fig. 1. Room-temperature CO difference spectra of mitochondrial fraction of *T. pyriformis*

A suspension of mitochondria was allowed to become anaerobic in the presence of 16.6 mM-sodium 2-oxoglutarate in both sample and reference cuvettes. A baseline (reduced-reduced, not shown) was recorded before bubbling the contents of the sample cuvette with CO for 30 s. Spectrum (a) was recorded 1 min later, (b) after 17 min. and (c) after 24 min. Scan speed was $60 \text{ nm} \cdot \text{min}^{-1}$, slit width 2 nm, path length 1 cm and the protein content was 14.0 mg/ml.

combination of cytochrome a_{620} with CO at this temperature was hardly detectable over 30 min and hardly any photolytic dissociation was obtained by further flashes after 23 scans. Photolysis in the presence of $200 \mu\text{M-O}_2$ at -113.5°C gave a spectrum which was identical initially with that obtained in the absence of O_2 (Fig. 3), but successive scans indicated the formation of an oxygen-containing intermediate of cytochrome a_{620} . Several features distinguish the reaction with O_2 from that with CO: (a) the distinctive absorption spectrum (O_2 -containing complex minus CO-liganded reduced complex), (b) the velocity of combination with O_2 is much greater than that with CO, and (c) the non-photo-dissociability of the oxygen-containing complex by light intensities giving photolysis of the CO-liganded oxidase [due to lower quantum yield for photolysis of O_2 -containing compounds (Gibson & Ainsworth, 1957)]. No oxygen-containing complex of a b-type

cytochrome was detectable at any of the temperatures employed (-120 to -80°C).

At temperatures higher than -72°C photolysis in the presence of $200 \mu\text{M-O}_2$ initiated electron transport (Fig. 4). Minima at 618, at about 560 and 553 nm corresponded to the α -absorption maxima of cytochromes a_{620} , b_{560} and c_{553} (Lloyd & Chance, 1972); corresponding minima were observed in the Soret region at 447, 423 and 413 nm (shoulder).

Photochemical action spectra

In order to determine whether both CO-binding pigments detected in difference spectra represent functional terminal oxidases, photochemical action spectra for the reversal of CO inhibition of respiration of growing cell suspensions were obtained. Fig. 5 shows that illumination of organisms under a gas phase of CO/O_2 (19:1) at 597 nm by means

Fig. 2. Repetitive wavelength scanning of the reaction of haemoproteins of *T. pyriformis* with CO at -112.5°C . The spectrum of CO-liganded endogenously reduced (anaerobic) organisms to which O_2 had not been added was scanned and stored in the digital memory of a dual wavelength spectrophotometer. The reference wavelength was 575 nm. Subsequent scans are difference spectra with the stored spectrum subtracted. The first scan, before photolysis, yielded the baseline (—) which represents a CO-reduced-minus-CO-reduced spectrum. Scan (1) followed photolysis at -112.5°C using a single flash, and after four more flashes at 90 s intervals, scan (5) was recorded. Scanning was from right to left at 3.5 nm/s. Absorbance increments and the wavelengths (in nm) of distinctive spectral features are shown.

Fig. 3. Reaction of haemoproteins of *T. pyriformis* with O_2 at -113.5°C .

The spectrum of a suspension of CO-liganded endogenously reduced (anaerobic) organisms into which O_2 had been stirred at -20°C was scanned at -113.5°C and the spectrum was stored in the memory of the dual wavelength spectrophotometer. The reference wavelength was 575 nm. Subsequent scans are difference spectra with the stored spectrum subtracted. The dotted line is the CO-re-

duced-minus-CO-reduced baseline, recorded before initiation of the reaction by flash photolysis. Thirteen spectra (1–13) were subsequently recorded at intervals of 90 s. Absorbance increments and the wavelengths (in nm) of distinctive features of the spectra are shown.

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Fig. 4. Electron transport in *T. pyriformis* at -72°C

The methods used and presentation of results are identical with those of Fig. 3, except that the reaction was initiated and observed at -72°C in the presence of $200\text{ }\mu\text{M-O}_2$.

Fig. 5. Photochemical action spectrum of *T. pyriformis*

(a) Irradiation of a cell suspension at 597 nm (○) and at 550 nm (●); output of Rhodamine 6G and Rhodamine 110 was varied by adjustment of power output of the argon-ion pump laser. (b) Irradiation from 634 to 572 nm used Rhodamine 6G at a power of 20 mW, that from 572 to 534 nm, Rhodamine 110 at 45 mW power; spectra were normalized by comparison of data obtained at 572 nm with both dyes.

Discussion

These results confirm that cytochrome a_{620} is the major terminal oxidase of *T. pyriformis* and the only mitochondrial haemoprotein in this role. This conclusion agrees with the previous observations of kinetic competence (Turner *et al.*, 1971; Lloyd & Chance, 1972) and favourable redox potential (Kilpatrick & Erecińska, 1977a, 1979). It should be stressed, however, that it is premature to suggest (Kilpatrick & Erecińska 1977a,b, 1979) that this cytochrome is a bacterial-type cytochrome *d* (formerly called a_2); its spectral characteristics make it unique and quite different from the cytochromes *d* of *Escherichia coli* (Pudek & Bragg, 1976), *Azotobacter vinelandii* (Kauffman & van Gelder, 1973) or the cytochrome *d*-containing oxidases of *Pseudomonas aeruginosa* (Yamanaka & Okunuki, 1963; Greenwood *et al.*, 1978). The second, more slowly CO-reacting haemoprotein of the mitochondrial fraction is only one of several *b*-type cytochromes present (Yamanaka *et al.*, 1968; Lloyd & Chance, 1972; Kilpatrick & Erecińska, 1977a,b; Yamanaka *et al.*, 1978). It has been suggested that the slowly auto-oxidizable CO-reacting cytochrome *b* in *T. pyriformis* is a bacterial-type cytochrome *o* (Perlish & Eichel, 1971), but its slow rates of oxidation (Lloyd & Chance, 1972), and unfavourable redox potential (Kilpatrick & Erecińska, 1977a) have argued against this function. Failure to detect an O_2 -containing intermediate at low temperatures or any contribution at around 575 nm in the photochemical action spectrum finally divests the *b*-type cytochrome of any possible role as a terminal oxidase. If we can assume that all cytochromes which bind O_2 also bind CO (Horio & Kamen, 1970), the terminal oxidases known to function in this organism as alternatives to cytochrome a_{620} (Lloyd *et al.*, 1980) are therefore not cytochromes.

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