The Cytochromes of *Acanthamoeba castellanii*

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1. Low-temperature difference spectra of gradient-purified mitochondria of *Acanthamoeba castellanii* reveal the presence of cytochromes *b*-555, *b*-562 and *c*-549, with *a*-type cytochromes having a broad asymmetrical maximum at 602 nm; these components were also observed in spectra of whole cells. 2. The *a*-type cytochromes are unusual in that they have split Soret absorption maxima (at 442 and 449 nm) and an uncharacteristic CO difference spectrum. 3. CO difference spectra of whole cells and 'microsomal' membranes show large amounts of cytochrome *P*-420 compared with cytochrome *P*-450. 4. Difference spectra in the presence of cyanide indicate the presence of an *a*-type cytochrome and two cyanide-reacting components, one of which may be cytochrome *a*₃. 5. Whole-cell respiration in a N₂/O₂ (19:1) atmosphere was decreased by 50%, suggesting the presence of a low-affinity oxidase. This lowered respiration is inhibited by 50% by CO, and the inhibition is partially light-reversible; photochemical action spectra suggest that cytochrome *a*₃ contributes to this release of inhibition. Other CO-reacting oxidases are also present. 6. The results are discussed with the view that cytochrome *a*₃ is present in *A. castellanii*, but its identification in CO difference spectra is obscured by other component(s).

The recent developments for the isolation of mitochondria from eukaryotic micro-organisms have resulted in the characterization of many components concerned with mitochondrial electron transport (Lloyd, 1974). The observed features have been shown to be sometimes quite different from those reported for mammalian systems (see Lloyd, 1974, for review). Although the respiratory components of yeasts have been extensively studied, investigations of fungi, algae and protozoa are very incomplete. This seems surprising in view of the fact that, of the protozoa studied, many have been shown to possess unusual characteristics in those components mediating terminal oxidation; examples are *Tetrahymena pyriformis* (Turner *et al.*, 1971; Lloyd & Chance, 1972), *Crithidia fasciculata* (Hill, 1972; Edwards & Lloyd, 1973) and *Trypanosoma mega* (Ray & Cross, 1972).

Although the fine structure of mitochondria of the free-living soil amoeba *Acanthamoeba castellanii* has been extensively studied (Bowers & Korn, 1968, 1969), there have been few reports on the nature of the cytochrome components of this organism. The first published account was by Lloyd & Griffiths (1968). Room-temperature difference spectra of mitochondrial preparations indicated the presence of cytochromes *c*-551, *b*-560–563 and *a*-606. Also, Lloyd & Griffiths (1968) suggested the involvement of two different *b*-type cytochromes responsible for the oxidation of exogenous and endogenous NADH, the

α -absorption band for cytochrome *b* being shifted from 565 to 556 nm in difference spectra with malate and NADH respectively as reductants.

The present investigation was undertaken to resolve the mitochondrial and 'microsomal' cytochrome components of *A. castellanii* by low-temperature spectroscopic analysis and to identify CO-reacting pigments that may function as terminal oxidases.

Experimental

Maintenance and growth of the organism

A. castellanii, originally obtained from Dr. K. M. G. Adam, Department of Zoology, University of Edinburgh, was routinely maintained and grown axenically as described by Griffiths & Chagla (1972), in a medium containing (% w/v): Difco proteose peptone, 0.75; Oxoid yeast extract, 0.75; glucose, 1.5. Stock cultures (50 ml in 250 ml conical flasks) were shaken at 30°C and subcultured every 2 days by inoculating with 5 ml of 2-day-old amoebae. Large cultures (2.5 litres) were grown in 5-litre flat-bottomed flasks, with shaking, at 30°C.

Harvesting of the organism

Organisms were counted in a Fuchs–Rosenthal haemocytometer slide (Baird and Tatlock, Chadwell Heath, Essex, U.K.) after a suitable dilution with either fresh growth medium or 50 mM-MgCl₂. Cultures

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containing between 1×10^6 and 3×10^6 cells/ml were harvested, at 4°C , by centrifugation at 3000 rev./min for 3 min in the 6×250 ml rotor of an MSE 18 centrifuge or at 2000 rev./min for 8 min in the 6×1 litre head of an MSE Mistral centrifuge.

Disruption of the organism and fractionation of extracts

Cells were washed once with 10 vol. of 50 mM- MgCl_2 , pH 7.4, resedimented and then resuspended in 2 vol. of disruption buffer containing 0.32 M-sucrose, 10 mM-Tris/ H_2SO_4 , 0.2 mM-EGTA, pH 8.0. This pH buffer is necessary to prevent 'gelling' of the homogenate, which may be due to the action of acid hydrolases released by the disrupted cells (Evans, 1973). The suspension was transferred to a chilled hand homogenizer (Jencons Scientific Ltd., Hemel Hempstead, Herts., U.K.) and homogenized. Six passages were usually sufficient for 50% breakage (estimated microscopically). Intact cells and nuclei were sedimented by centrifugation at 3000 rev./min (1000 g; r_{av} 7.6 cm) for 8 min in the 8×50 ml rotor of an MSE 18 centrifuge. The supernatant was carefully decanted, avoiding disturbance of the lower layers, and centrifuged at 10000 rev./min (10000 g; r_{av} 7.6 cm) for 10 min in the same rotor. The supernatant (S_1) was retained, and the crude mitochondrial pellet was washed once with 3 vol. of the disruption buffer, pH 7.4, and then recentrifuged at 10000 rev./min (10000 g; r_{av} 7.6 cm) for 10 min. The washed pellet was resuspended in 2 vol. of the disruption buffer, pH 7.4, and carefully layered on top of a 15–60% (w/w) linear sucrose gradient (45 ml in a 60 ml centrifuge tube) containing 10 mM-Tris/HCl, pH 7.4. The gradient was then centrifuged at 22000 rev./min (48700 g; r_{av} 11.5 cm) for 1 h in the 3×65 ml head of an MSE 65 centrifuge. After centrifugation the mitochondrial fraction ($\rho = 1.22$ g/ml) had been separated from contaminating membranes and was carefully removed. The suspension was diluted with 2 vol. of disruption buffer, pH 7.4, and sedimented at 10000 rev./min (10000 g; r_{av} 7.6 cm) for 10 min. The resultant pellet was suspended in 0.8 M-mannitol (2 vol.) and used as a purified mitochondrial suspension. 'Microsomal' membranes were sedimented by centrifugation of the S_1 supernatant at 40000 rev./min (106000 g; r_{av} 5.98 cm) for 1 h in the 8×10 ml rotor of an MSE 40 centrifuge. The pellet thus produced was resuspended in 2 vol. of 0.8 M-mannitol. All operations were at 4°C .

Analytical methods

Difference spectra (Chance, 1957) were measured by using a Unicam SP.1800 spectrophotometer. Spectra were recorded at room temperature (18°C) in cuvettes of 10 mm path-length, whereas those used at the temperature of liquid N_2 (77 K) had a path-

length of 2 mm, as described in detail by Edwards & Lloyd (1973), in a specially constructed Dewar vessel, 3 cm from the photomultiplier tube. The spectral bandwidth was 1 nm and the scanning speed was 1 nm/s. Manometric measurements of respiration were made in conventional Warburg apparatus (Umbreit *et al.*, 1957); the flasks were shaken at 120 cycles/min and at 2.5 cm amplitude. Reversal by light of CO inhibition was obtained by focusing a 300 W projector lamp 25 cm from the Warburg flask through a 2 cm-path-length filter containing 7% CuSO_4 . Photochemical action spectra were obtained by using Filtraflex-B-40 interference filters (Balzers, Berkhamsted, Herts., U.K.) to obtain light of different wavelengths. The wavelengths of maximal transmission were determined with a Hartridge reversion spectroscope (Ealing Optics, Watford, Herts., U.K.). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Chemicals

Protease peptone was from Difco Laboratories, Detroit, MI, U.S.A., yeast extract from Oxoid, London SE1 9HF, U.K., antimycin A from Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K. Bovine serum albumin was from Armour Pharmaceuticals Co., Eastbourne, Sussex, U.K. All other chemicals were of analytical grade.

Results

Cytochrome components of whole cells

Excess sodium dithionite, 1% (w/v) glucose or allowing the sample to become anaerobic for 5 min, resulted in the same extent of reduction of cytochromes. Although $\text{K}_3\text{Fe}(\text{CN})_6$, 0.1% H_2O_2 or aeration for 5 min appeared equally effective in oxidizing the contents of the reference cuvette, it was found that with $\text{K}_3\text{Fe}(\text{CN})_6$ as the oxidant, cooling the sample to 77 K, thawing and then refreezing at 77 K produced increased oxidation in the reference cuvette compared with that produced with a single freezing. This phenomenon only occurred in whole cells with $\text{K}_3\text{Fe}(\text{CN})_6$.

Difference spectra obtained at room temperature by using $\text{Na}_2\text{S}_2\text{O}_4$ as reductant with $\text{K}_3\text{Fe}(\text{CN})_6$ to oxidize the contents of the reference cuvette indicated the presence of absorption maxima at 550 and 603–606 nm, the α -absorption maxima of *c*- and *a*-type cytochromes (Fig. 1a). At 77 K, absorption maxima of 1–3 nm shorter wavelength were evident, at 549, 561 and 603 nm. Less-well-defined maxima were also present at 547, 554, 558 and 580 nm (Fig. 1b). It was found that 0.8 M-mannitol produced maximal intensification at 77 K and resulted in intensification

Fig. 1. Difference spectra of *A. castellanii*

Organisms were suspended in 0.8M-mannitol, after washing with 10mM-MgCl₂, pH7.4. Spectra (a) and (b) were recorded after addition of Na₂S₂O₄ to the sample cuvette, with K₃Fe(CN)₆ used to oxidize the reference cuvette. (a) was recorded at room temperature, and (b) at 77 K, after freeze-thawing. For (c) reduction of the sample cuvette was by 10mM-ascorbate+1 mM-NNN'-N'-tetramethyl-*p*-phenylenediamine. For (d) 10mM-sodium succinate was added to the sample cuvette after the addition of 10μM-antimycin A. In (c) and (d) the cell suspension was sonicated for 30s (20kHz, 500W MSE Sonicator, power setting 5). The contents of the reference cuvettes were oxidized by aeration before immersion in liquid N₂. The cell concentration was 10⁸ cells/ml. (a) and (b) ΔA = 0.02; (c) and (d) ΔA = 0.008 as indicated.

Fig. 2. Low-temperature difference spectra of gradient-purified mitochondria of *A. castellanii*. The contents of the reference cuvettes were oxidized by aeration while the sample cuvettes were reduced as follows: (a) $\text{Na}_2\text{S}_2\text{O}_4$, (b) 10mM-sodium succinate, (c) 10mM-sodium succinate after the addition of 10 μM -antimycin A, (d) 10mM-ascorbate+1 mM-*NNN'*-tetramethyl-*p*-phenylenediamine. The mitochondrial suspension contained 5.0mg of protein/ml.

factors of 3.7, 6.1 and 13.3 for the α -absorption bands of *c*- (550–540nm), *b*- (560–570nm) and *a*-type (600–630nm) cytochromes respectively.

Reduction of the contents of the sample cuvette with 10mM-ascorbate+1mM-*NNN'*N'-tetramethyl-*p*-phenylenediamine (Fig. 1c), used to define absorption maxima corresponding to *c*- and *a*-type cytochromes, showed the presence of maxima at 420, 546 and 549 nm [*c*-type cytochrome(s)] and 441, 449, 518 and 602 nm (*a*-type cytochromes). A maximum at 562 nm (*b*-type cytochrome) is also evident. Addition of succinate to a cell suspension containing antimycin A and allowed to become anaerobic revealed the presence of *b*-type cytochromes with maxima at 431, 529, 536, 556 and 563 nm. That little or no *c*- or *a*-type cytochromes have been reduced suggests that there was little electron transport past the antimycin 'block' (Fig. 1d).

Difference spectra of purified mitochondria

Use of $\text{Na}_2\text{S}_2\text{O}_4$ as reductant of a gradient-purified mitochondrial suspension, the contents of the reference cuvette being oxidized by vigorous aeration, produced a difference spectrum, at 77K, with absorption maxima not dissimilar to those obtained with whole cells. As well as showing discrete maxima at 429, 442, 448, 549, 562 and 602nm, less-well-defined maxima were evident at 545, 590 and 595 nm (Fig. 2a). With succinate as reductant (Fig. 2b) the overall pattern was similar (maxima at 442, 447, 510, 518, 524, 529, 545, 548, 589 and 602nm), but less *b*-types were reduced (at 427 and 562nm). Reduction of the sample cuvette in the presence of antimycin A shows absorption maxima at 428, 530, 555 and 562 nm, corresponding to *b*-type cytochrome(s) (Fig. 2c). Use of 10mM-ascorbate+1mM-*NNN'*N'-tetramethyl-*p*-phenylenediamine as reductant showed maxima at 418, 524, 545 and 549 nm, corresponding to *c*-type cytochromes and *a*-type cytochromes with maxima at 518 and 603 nm, again with two discrete Soret absorption maxima at 442 and 449 nm (Fig. 2d).

CO difference spectra

A suspension of intact organisms was reduced in both the sample and reference cuvettes by $\text{Na}_2\text{S}_2\text{O}_4$ and a baseline recorded. The sample cuvette was then sparged with CO for 30s. Absorption minima were evident at 444 and about 560nm with maxima at 418, 452, 542 and 570 nm. The absorption minimum at 444 nm may be due to the reaction of cytochrome a_3 with CO (Chance, 1953), but no CO-reacting components were evident at 590–605 nm (Fig. 3a). A CO difference spectrum of a 'microsomal' membrane fraction indicated that the spectrum obtained with intact cells is largely due to the contribution of these

Fig. 3. CO difference spectra of *A. castellanii*

Both sample and reference cuvettes were reduced with $\text{Na}_2\text{S}_2\text{O}_4$ and a baseline was recorded (not shown); all spectra are redrawn after subtraction of these baselines. (a) Spectrum obtained after sparging the sample cuvette, containing whole cells (10^8 cells/ml) for 30s with CO. (b) Spectrum obtained after sparging 'microsomal' fraction (30mg of protein/ml) with CO for 30s. (c) Spectrum obtained after sparging a gradient-purified mitochondrial suspension (5.12mg of protein/ml) with CO for 2min. Spectra were recorded at room temperature; the path-length was 10mm.

membranes (Fig. 3b). A CO difference spectrum of purified mitochondria (Fig. 3c) is unusual, with a minimum at 436 nm and an asymmetrical maximum at 425 nm, which may contain more than one component.

Difference spectra in the presence of CN^-

Spectrophotometric separation of mammalian cytochromes *a* and a_3 has been reported by Yonetani (1960) depending on the difference in the reaction of these two components with CN^- , although other interpretations are possible (Wanio *et al.*, 1968). Reduced or oxidized mammalian cytochrome a_3 reacts with CN^- ; the reduced CN^- complex is easily autooxidized, whereas the oxidized CN^- complex is not easily reduced (Keilin & Hartree, 1939). Cytochrome *a* does not react with CN^- . With 1mM- CN^- initially present in both cuvettes, the sample then being reduced with 10mM-ascorbate+1mM-*NNN'*N'-

Fig. 4. Effect of cyanide on difference spectra of gradient-purified mitochondria of *A. castellanii* (a) Mitochondrial suspensions were aerated in the presence of 1 mM-CN⁻ in both cuvettes. The spectrum was recorded after the addition of 10 mM-ascorbate + 1 mM-NNN'N'-tetramethyl-*p*-phenylenediamine to the sample cuvette. (b) The mitochondrial suspensions in both cuvettes were reduced by 10 mM-ascorbate + 1 mM-NNN'N'-tetramethyl-*p*-phenylenediamine, after aeration of the reference cuvette in the presence of 1 mM-CN⁻. The mitochondrial suspension contained 5.12 mg of protein/ml and spectra were recorded at 77 K.

tetramethyl-*p*-phenylenediamine, whereas the reference cuvette was aerated before both cuvettes were rapidly immersed in liquid N₂, the difference spectrum shown in Fig. 4(a) was obtained. Since *a*₃-type cytochromes when complexed with CN⁻ are not reduced, they are effectively excluded from the difference spectrum. Absorption maxima are evident at 416 (*γ*-band of cytochrome *c*), 582 and 596 nm, with minima tentatively attributed to CN⁻-reacting components at 588 and 602 nm. Fig. 4(b) shows the difference spectrum obtained after reduction of the

sample and reference cuvettes with 10 mM-ascorbate + 1 mM-NNN'N'-tetramethyl-*p*-phenylenediamine, after the addition of CN⁻ to the reference cuvette. This shows a broad asymmetrical absorption maximum at 590–603 nm. Additional maxima are evident at 443 and 450 nm.

Difference spectra of 'microsomal' membranes

With Na₂S₂O₄ as reductant of the sample cuvette, the contents of the reference cuvette being oxidized

Fig. 5. Difference spectra recorded at 77 K of 'microsomal' membranes of *A. castellanii*

A 'microsomal' suspension (18.5 mg of protein/ml) was reduced by (a) $\text{Na}_2\text{S}_2\text{O}_4$, (b) NADPH, (c) NADH. The contents of the reference cuvettes were oxidized by $\text{K}_3\text{Fe}(\text{CN})_6$.

by $\text{K}_3\text{Fe}(\text{CN})_6$, absorption maxima are evident at 526, 532, 553, 558 and 580 nm (Fig. 5a). With NADH and NADPH as reductants, the overall pattern is similar, although the contributions of components

Fig. 6. Inhibition of respiration of *A. castellanii* by CO. Each manometer flask contained 0.5 mmol of KOH in the centre well and 1.21×10^7 organisms in growth medium in the main compartment. The gas phases were (a) air, (b) N_2/O_2 (19:1) and (c) CO/O_2 (19:1). Illumination of flask (c) was with white light as shown. Temperature of incubation was 30°C.

at 574 and 580 nm show different degrees of reduction (Figs. 5b and 5c).

Inhibition of whole-cell respiration by CO

The respiration rate of whole cells, measured manometrically, was decreased by approx. 50% when N_2/O_2 (19:1) replaced air (Fig. 6). A CO/O_2 (19:1) gas mixture inhibited this lowered respiration rate by about 50%. This inhibition by CO was partially reversible by illumination with white light. In the Soret region a single maximum of respiration occurred in the photochemical action spectrum at 432 nm (Fig. 7a). In the α -region this spectrum was more complex, with maxima at 548, 563, 583 and 595–604 nm (Fig. 7b).

Discussion

Difference spectra obtained by reduction of whole cells of *Acanthamoeba castellanii* reveal the presence of *c*-, *b*- and *a*-type cytochromes, results which are not dissimilar to those reported for mammalian systems. The observed cytochromes appear to be mainly due to the contribution of mitochondrial components; the only possible contribution from 'microsomal' cytochromes is in the *b* region. The *a*-type cytochromes appear unusual with two discrete maxima at 441 and 449 nm (Fig. 2). Spectra that show two distinct maxima in the region expected for an *a*-type cytochrome (438–453 nm) have been reported in several micro-organisms, e.g. *Euglena gracilis* (Sharpless & Butow, 1970), *Astasia longa* (Webster & Hackett, 1965), *Crithidia fasciculata* (Edwards & Lloyd, 1973; Kusel & Storey, 1973) and *Neurospora*

Fig. 7. Photochemical action spectrum for the release of CO inhibition of respiration of *A. castellanii*. O_2 uptake of organisms in growth medium was measured manometrically under light of different wavelengths with a CO/O_2 (19:1) gas mixture. In (a) 1.19×10^7 organisms/flask gave a respiration rate of $2.2 \mu\text{mol/h}$ in the dark and $4.0 \mu\text{mol/h}$ in white light. In (b) 1.36×10^7 organisms/flask gave a respiration rate of $2.4 \mu\text{mol/h}$ in the dark and $4.5 \mu\text{mol/h}$ in white light. No correction was made for variation of energy content through the spectrum. The points shown are calibrated values of maximal transmission for the interference filters used (see the Experimental section). The half-bandwidths were in the range 7–12 nm. The intensity variation of the different interference filters did not contribute appreciably to the total variation in respiration rates; it accounted for less than 7%.

crassa (Lambowitz *et al.*, 1972). Also, the large absorption maximum at 585–605 nm does not appear symmetrical and may contain more than one component (Fig. 2). That the α -type cytochromes in *A. castellanii* are somewhat unusual is also apparent from CO difference spectra of purified mitochondria. Reaction of mammalian cytochrome a_3 with CO results in absorption maxima at 430 and 590 nm and minima at 444 and 600 nm (Chance, 1953). In the present study absorption maxima are evident at 405 and 425 nm, with a minimum at 436 nm for purified mitochondria (Fig. 3). No contribution is visible in the α -region. Unusual CO difference spectra have been reported in *E. gracilis* (Raison & Smillie, 1969). Difference spectra of purified mitochondria in the presence of CN^- suggest the presence of CN^- -reacting components at 590 and 602 nm (Fig. 4); Yonetani (1960) has shown that ferrous cytochrome a_3 - CN^- has an absorption maximum at 589–590 nm. A component that does not react with CN^- is present at 596 nm, and this may be attributed to cytochrome a . It appears therefore that there is an additional component present in *A. castellanii* which has an absorption maximum of 602 nm when complexed with CN^- . It may be that cytochrome a_3 has little absorption in the α -region similar to that reported for skunk-cabbage mitochondria (Bendall & Bonner, 1971).

Further evidence for the presence of cytochrome a_3 in *A. castellanii* comes from the photochemical

action spectrum for the release of CO inhibition of whole-cell respiration (Fig. 7). The single maximum at 432 nm is typical of cytochrome a_3 (Warburg & Negelein, 1928; Castor & Chance, 1959). This suggests that cytochromes $a+a_3$ may be present in *A. castellanii*, but their identity in CO difference spectra is masked by some other component(s) which are CO-reacting; these components contribute to the photochemical action spectrum in the α -region.

Whole-cell respiration at low O_2 partial pressure (N_2/O_2 , 19:1) is decreased by 50% (Fig. 6), which suggests the presence of an oxidase, alternative to cytochrome a_3 , with a lower affinity for O_2 . A CO difference spectrum of whole cells has a profile similar to that reported for cytochrome o (Lemburg & Barrett, 1973). However, the location of this component in the 'microsomal' fraction and the absence of significant maxima at 418 and 570 nm from photochemical action spectra make it unlikely that cytochrome o is present in *A. castellanii*. A maximum at 450 nm in CO difference spectra may be attributed to cytochrome $P-450$ (Fig. 3). Kusel (1970) found that cytochrome $P-450$ in *C. fasciculata* was unstable and rapidly converted into cytochrome $P-420$ by dithionite. It seems likely that this is the case in the present investigation, which would account for such a large proportion of cytochrome $P-420$ compared with $P-450$. Further work is therefore necessary to establish the nature of the low-affinity oxidase and to

resolve fully the components with absorption maxima at 585–605 nm present in difference spectra.

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