Carbon Monoxide-Reacting Haemoproteins in the Mitochondrial Fraction of *Acanthamoeba castellanii*

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1. Room-temperature (18°C) CO difference spectra of mitochondrial fractions from the amoeba *Acanthamoeba castellanii* reveal the presence of at least four CO-reacting haemoproteins. As well as cytochrome a_3 , other components reacting with CO are: (i) a c -type cytochrome; (ii) a b -type cytochrome; and (iii) another a -type cytochrome. 2. The same components can be identified in low-temperature photodissociation experiments with intact cells or mitochondria. 3. The time of exposure to CO and the nature of the reductant are both important in identifying all the components present, in that the b -type cytochrome is more readily distinguished after longer exposure to CO and more of the c -type cytochrome is detectable when NADH is the reductant. 4. Treatment of mitochondria with ultrasound releases two components, identifiable in low-temperature difference spectra as a c -type and a b -type cytochrome; only the latter appears to have any reaction with CO, and the CO-reacting c -type cytochrome is retained in submitochondrial particles. 5. The complexity of the CO-reacting haemoproteins in this organism is compared with the simpler systems found in other eukaryotic organisms.

The reaction of some haemoproteins with CO to give spectrophotometrically distinct complexes is an invaluable aid to their identification. It also provides an indication of the presence of those species that may react with O₂ (Chance, 1957; Lemberg & Barrett, 1973; Lloyd, 1974). In eukaryotic organisms, cytochrome $a + a_3$ is almost always the major terminal oxidase of the mitochondrial respiratory chain; exceptionally other mitochondrial CO-binding cytochromes are present, e.g. cytochrome a_{620} in the protozoan *Tetrahymena pyriformis* (Turner *et al.*, 1971; Lloyd & Chance, 1972) and cytochrome o in several trypanosomes (Kronick & Hill, 1974), although the mitochondrial location of this latter pigment has not been ascertained. Several other haemoproteins with cytochrome o -like CO difference spectra have been described; these all contain protohaem in a high-spin state and include haemin, cytochrome $P-420$, haemoglobin, cytochrome c peroxidase, and precursors of cytochromes or products of cytochrome degradation (Lloyd & Edwards, 1978).

The soil amoeba *Acanthamoeba castellanii* has at least three distinct mitochondrial terminal oxidases (Edwards & Lloyd, 1978; Lloyd *et al.*, 1979), and the identification of cytochrome a_3 in CO difference spectra is obscured by other CO-binding component(s) (Edwards *et al.*, 1977). Other CO-reacting haemoproteins present in this organism include

microsomal cytochromes $P-420$ and $P-450$ (Edwards *et al.*, 1977). The present study describes the further characterization of CO-binding haemoproteins in a washed mitochondrial fraction from *A. castellanii*; in addition to cytochrome a_3 , three other such components are present: (i) another a -type cytochrome; (ii) a b -type cytochrome; and (iii) a c -type cytochrome.

Experimental

Maintenance and growth of the organisms

Acanthamoeba castellanii was maintained, grown axenically with shaking at 30°C, and counted as described previously (Edwards *et al.*, 1977).

Harvesting and disruption of the organism, and isolation of mitochondria

Procedures for harvesting organisms and for the preparation of mitochondria-enriched subcellular fractions were as detailed by Edwards & Lloyd (1978).

Preparation of submitochondrial fractions

The crude mitochondrial pellet was resuspended in 5 vol. of disruption buffer (0.32 M-sucrose/10 mM-Tris/H₂SO₄/0.2 mM-EGTA, pH 8.0) and resedi-

mented at 10000 rev./min (10000 g; r_{av} , 7.6 cm). The washing was repeated and the pellet finally resuspended in 5 vol. of disruption buffer. Disruption was by treatment for 30 s with ultrasound (20 kHz, 500 W MSE sonicator, power setting 5, 4°C). Unbroken mitochondria were removed by centrifugation at 10000 rev./min (10000 g; r_{av} , 7.6 cm) for 10 min. The resulting supernatant containing submitochondrial particles was centrifuged at 40000 rev./min (100000 g; r_{av} , 6.4 cm) for 1 h in the 10 × 10 ml rotor of an MSE 65 centrifuge to give a sedimentable fraction (which was resuspended in 2 vol. of disruption buffer) and a supernatant (high-speed supernatant).

Cytochrome spectra

Difference spectra 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 N₂ (77 K) had a path-length of 2 mm in a specially constructed Dewar vessel, 3 cm from the photomultiplier tube. The spectral band

width was 1 nm and the scanning speed 1 nm/s. CO was bubbled into open cuvettes for various times as indicated.

Low-temperature flash photolysis

The technique used was that of Chance *et al.* (1975). Washed whole-cell suspensions of *A. castellanii* 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 the attainment of anaerobiosis in these suspensions. Suspensions of mitochondria or submitochondrial fractions were similarly treated, except that succinate (10 mM) or NADH (1 mM) were the reductants; CO was then bubbled through the suspensions after spraying the surface of the cuvette contents with a trace of anti-foam (Anti-foam A; Dow Corning, Midland, MI, U.S.A.). A further 2 min incubation at room temperature preceded immersion in a cooling bath containing ethanol and solid CO₂ at -20°C. After equilibration in the dark the cuvette was transferred to an ethanol/solid CO₂ bath at

Fig. 1. Room-temperature CO difference spectrum of whole cells of *A. castellanii*

A suspension of whole cells (10⁹ cells/ml) suspended in 50 mM-MgCl₂, pH 7.4, was allowed to become anaerobic by endogenous respiration for 5 min at room temperature. A spectrum was recorded in a dual-wavelength scanning spectrophotometer and stored in the digital memory. This spectrum was automatically subtracted from all subsequent scans. Spectrum (a) (----) is the reduced-reduced baseline and (b) the spectrum obtained after sparging the cuvette with CO for 15 s (—). The reference wavelength was 500 nm.

−80°C. Spectrophotometric analysis was performed in a dual-wavelength scanning spectrophotometer (Chance *et al.*, 1972); baseline corrections (spectra of CO-ligated anaerobic 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 (Chance *et al.*, 1975)] before photolysis (several flashes of a 20 J xenon flash lamp).

Results

CO-binding and photodissociable haemoproteins detectable in intact cells

A suspension of intact cells, in 50 mM-MgCl₂, was allowed to become anaerobic for 10 min at room

temperature and then a spectrum was scanned and stored in the digital memory of a dual-wavelength scanning spectrophotometer; this spectrum was then subtracted from all subsequent scans. Fig. 1(a) shows the baseline (reduced—reduced) and the spectrum obtained (Fig. 1b) after sparging the contents of the cuvette with CO for 15 s (CO-reduced—reduced). Absorption maxima were evident at 424 and 565–572 nm, with minima at 444, 562 and 585 nm. After bubbling with CO for 2 min, a similar spectrum was obtained, except that the maximum at 424 nm was shifted to 421 nm (spectrum not shown).

In another experiment, a sample of intact cells in 50 mM-MgCl₂ and 30% ethylene glycol was allowed to become anaerobic for 10 min at room temperature and then bubbled with CO for 45 s. The base-

Fig. 2. Low-temperature photodissociation spectra of intact cells of *A. castellanii*

A suspension of intact cells in 50 mM-MgCl₂, pH 7.4, containing 30% ethylene glycol (10⁹ cells/ml) was allowed to respire to anaerobiosis with endogenous substrates. The suspension was then bubbled with CO for 45 s, cooled to −126°C, as described in the Experimental section, and a spectrum was recorded and stored in the memory of the dual-wavelength scanning spectrophotometer. (a) is the baseline, CO-reduced—CO-reduced, and (b) the spectrum obtained after flashing the sample four times. (c) and (d): A sample of whole cells was treated exactly as above except that the time of bubbling with CO was 2 min. (c) CO-reduced—CO-reduced baseline and spectra obtained after one flash (d) and four flashes (e). Spectrum (f) was recorded 19.5 min after the fourth flash.

line in Fig. 2(a) (broken line) is CO-reduced—CO-reduced difference spectrum of this suspension at -126°C . After photodissociation by four flashes, the spectrum in Fig. 2(b) was obtained with absorption maxima (due to the photodissociation of CO-reduced to reduced components) evident at 447, 553, 562, 564, 571 and 614 nm and with minima at 429, 542, 546, 588 and 632 nm. A sample of in-

tact cells was treated exactly as above except that the bubbling time with CO was increased to 2 min. Fig. 2(c) shows the baseline (CO-reduced—CO-reduced) and the photodissociated spectrum after one flash (Fig. 2d) with maxima at 445 and 556–572 nm, and the minima at 429, 546 and 588 nm. Repetitive scans, after a further three flashes, which did not result in further photodissociation, revealed

Fig. 3. Room-temperature CO difference spectra of succinate-reduced mitochondria

A suspension of mitochondria was allowed to become anaerobic in the presence of 10 mM-succinate in both the sample and reference cuvettes. A baseline spectrum was recorded and subtracted from all scans. (a) Spectrum recorded 1 min after bubbling the contents of the sample cuvette with CO for 10 s; (b) spectrum recorded 18 min later, without any further CO additions, and (c) contents of the sample cuvette bubbled with CO for a total of 25 s and recorded 1 min later. (d) Shows a scan of the α -region at higher sensitivity. The protein content was 2.8 mg/ml.

a gradual change in the profile of this spectrum, especially in the α -region (Fig. 2e), until, by 19.5 min after photodissociation, maxima were evident at 543, 563 and 574 nm and minima at 551 and 577 nm (Fig. 2f). This change in spectral characteristics may be

partially explained by selective recombination of CO to different component(s).

Room-temperature CO difference spectra of isolated mitochondria

A suspension of mitochondria was allowed to become anaerobic in the presence of 10 mM-succinate for 10 min at room temperature. The reference and sample cuvettes both contained this suspension,

Fig. 4. Room-temperature CO difference spectra of NADH-reduced mitochondria

A suspension of mitochondria was allowed to become anaerobic in the presence of 1 mM-NADH in both the sample and reference cuvettes. A reduced-reduced baseline spectrum was recorded and subtracted from all subsequent scans. (a) Spectrum recorded 3 min after bubbling the sample cuvette with CO for 10 s, (b) spectrum obtained 5 min later, and (c) spectrum recorded 8 min after (b), both without any further CO addition. The sample cuvette was then bubbled with CO for a further 15 s and (d) was recorded 6 min later, and (e) 44 min later. The protein content was 3.7 mg/ml.

Fig. 5. Room-temperature CO difference spectra of NADH-reduced mitochondria recorded at high sensitivity

Spectra were obtained under conditions identical with those used in Fig. 4. Spectra were recorded (a) 6 min, (b) 8 min, and (c) 10 min after bubbling the contents of the sample cuvette with CO for 25 s. The protein content was 3.7 mg/ml.

and a baseline was recorded; this baseline was subtracted from all spectra shown in Fig. 3. The spectrum in Fig. 3(a) was recorded 1 min after bubbling the sample cuvette with CO for 10 s and shows maxima at 433 and 592 nm and minima at 417 and 447 nm. The contents of both cuvettes were then allowed to stand, with no further additions for another 18 min, and the spectrum in Fig. 3(b) was recorded, with absorption maxima at 428, 432, 542, 580 and 592 nm and minima at 416, 446, 553, 563 and 605 nm. The contents of the sample cuvette were then bubbled with CO for a further 15 s, and the spectrum shown in Fig. 3(c) was recorded 1 min after the bubbling; maxima were evident at 434, 542 and 593 nm and minima at 420, 446, 554, 563 and 606 nm. Scanning of the α -region at higher sensitivity (Fig. 3d) showed maxima at 542, 582 and 592 nm and minima at 553, 562, 606 and 614 nm.

Fig. 4 shows a similar experiment but with 1 mM-NADH used to reduce the mitochondrial suspension. The spectrum shown in Fig. 4(a) was scanned 3 min after bubbling the sample cuvette with CO for 10 s, and shows maxima at 416, 422, 540 and 575 nm, with minima at 446 and 554 nm. The cuvettes were then left for a further 5 min and the spectrum in Fig. 4(b) was recorded; it showed

maxima at 414, 422, 432, 463 and 542 nm, and minima at 447, 562 and 610 nm. Fig. 4(c) was obtained 8 min later (maxima at 410, 434, 463, 540, 562, 576 and 592 nm, minima at 446, 524, 554 and 605 nm). A spectrum scanned 6 min after bubbling with CO for a further 15 s (Fig. 4d) was similar to Fig. 4(c), except in the Soret region (minimum at 432 and maximum at 437 nm), whereas 44 min later the spectrum in Fig. 4(e) was obtained, which showed less complexity in the α -region.

Changes occurring in the range 520–610 nm (with NADH as reductant) studied at higher sensitivity are shown in Fig. 5. The minimum at 554 nm, which was detected 6 min after bubbling with CO for 25 s (Fig. 5a), gradually diminished by 10 min (Fig. 5c).

Photodissociation of CO-bound complexes in mitochondria

A suspension of intact mitochondria was allowed to become anaerobic in the presence of 10 mM-succinate for 10 min at room temperature, and then bubbled with CO for 2 min. A spectrum was then scanned at -100°C and stored in the digital memory and subtracted from all subsequent scans. The baseline (broken line) in Fig. 6 is a CO-

Fig. 6. Low-temperature photodissociation of CO-bound haemoproteins of mitochondria

A sample of mitochondria, in disruption buffer, containing 30% (v/v) ethylene glycol was allowed to become anaerobic for 5 min in the presence of 10 mM-succinate before bubbling with CO for 2 min at room temperature. The cuvette was cooled to -100°C and a spectrum was recorded, stored in the digital memory of the dual-wavelength scanning spectrophotometer, and subtracted from all subsequent scans. The baseline (----) is a reduced—reduced difference spectrum, and spectra 1 → 8 were recorded at 90 s intervals after four flashes of the xenon lamp.

reduced—CO-reduced difference spectrum. The sample was then flashed four times and eight spectra were recorded at 90 s intervals. The initial scan revealed maxima at 447, 450 and 614 nm and minima at 432 and 592 nm, and repetitive scans indicated recombination of CO with reduced cytochromes (indicated by the progressive loss of absorption maxima and minima) in the Soret region, with the emergence of a distinct maximum at 445.5 nm. In the α -region, changes were more complex; the second and subsequent scans revealed maxima at 546, 562, 599.5 and 614 nm and minima at 553.5, 566 and 606 nm.

Low-temperature difference spectra

In order to elucidate further the nature of the CO-reacting components present in *A. castellanii*, submitochondrial particles were prepared to determine whether any components were easily removed by treatment with ultrasound. Fig. 7(a) shows the difference spectrum, at 77 K, of the high-speed-supernatant fraction, showing maxima at 430, 521, 549 and 562 nm. Difference spectra of mitochondria and submitochondrial particles were essentially similar, except that the ratio cytochrome c_{550} /cytochrome a_{604} was lower in submitochondrial particles (Figs. 7b and 7c).

Fig. 7. Low-temperature difference spectra

Reduced—oxidized difference spectra, at 77 K were recorded as follows: (a) supernatant fraction (protein concn. 8.5 mg/ml) reduced by dithionite, oxidized by 0.1% (v/v) H_2O_2 ; (b) mitochondria (protein concn. 41 mg/ml) reduced by dithionite, oxidized by 0.1% (v/v) H_2O_2 ; (c) submitochondrial particles (protein concn. 13.5 mg/ml), reduced by dithionite, oxidized by vigorous aeration.

Fig. 8. Room-temperature CO difference spectra of NADH-reduced submitochondrial particles

A suspension of submitochondrial particles (5.3 mg of protein/ml) was allowed to become anaerobic for 5 min in the presence of 1 mM-NADH in both the sample and reference cuvettes. A reduced—reduced spectrum was recorded and subtracted from all subsequent scans. (a) Spectrum recorded 1 min after bubbling the contents of the sample cuvette with CO for 10 s, and (b) spectrum recorded 8 min later without any further CO addition. The sample cuvette was bubbled with CO for a further 10 s and spectra recorded (c) 1 min, and (d) 9 min after bubbling.

CO difference and photodissociation spectra of submitochondrial particles

A suspension of submitochondrial particles was allowed to become anaerobic in the presence of 1 mM-NADH in both the sample and reference cuvettes. The resulting baseline was recorded and

subtracted from all subsequent scans. Fig. 8(a) shows the CO difference spectrum obtained 1 min after bubbling the sample cuvette with CO for 10 s; the only major feature of this spectrum was a trough at 413 nm. The suspension was left for a further 8 min and a spectrum revealed maxima at 437 and 541 nm, with minima at 420, 445, 552 and 607 nm

Fig. 9. Low-temperature photodissociation of CO-bound haemoproteins detectable in submitochondrial particles
A suspension of submitochondrial particles (protein concn. 15 mg/ml) in disruption buffer plus 30% (v/v) ethylene glycol was allowed to become anaerobic for 5 min in the presence of 1 mM-NADH before bubbling with CO for 2 min. The cuvette was cooled to -80°C and then a spectrum recorded and stored in the memory of the dual-wavelength scanning spectrophotometer; this spectrum was subtracted from all subsequent scans. The broken line is the reduced—reduced baseline and the solid line is the spectrum obtained after flashing the cuvette five times.

Fig. 10. Room-temperature CO difference spectra of the supernatant fraction
The high-speed supernatant fraction, obtained after ultrasound treatment of mitochondria, was allowed to become anaerobic in the presence of 1 mM-NADH in both the sample and reference cuvettes. A reduced—reduced baseline spectrum was recorded and subtracted from all subsequent scans. The contents of the sample cuvette were then bubbled with CO for 10 s and spectra were recorded (a) 1 min, and (b) 15 min after this. The sample cuvette was then bubbled with CO for a further 20 s and spectra were recorded (c) 1 min and (d) 13 min later. The protein content was 8.5 mg/ml.

(Fig. 8b). The sample cuvette was then bubbled with CO for a further 10 s, and 1 min later the spectrum shown in Fig. 8(c) was recorded, which revealed maxima at 435, 540, 562 and 592 nm. After 8 min a spectrum showed that the minima at 553 and 607 nm had become less prominent (Fig. 8d).

Photodissociation spectra at -80°C of NADH-reduced submitochondrial particles, after bubbling with CO for 2 min, show maxima at 421, 554, 566 and 598 nm, with minima at 411, 429, 446, 546, and 592 nm (Fig. 9).

Room-temperature CO difference spectra of the supernatant fraction

Difference spectra (Fig. 7) suggested that the high-speed supernatant contained mainly a *c*-type cytochrome (maximum at 549 nm) and some contribution from a component with an absorption maximum at 562 nm. CO difference spectra revealed some minor variations in the positions of absorption maxima and minima after different exposure times to CO (Fig. 10) with maxima at 418, 542 and 574 nm, and minima at 430 and 560 nm.

Discussion

Whereas cytochrome a_3 readily reacts with CO to give a spectrally distinct complex, apart from in the case of some bacterial cytochromes, cytochromes *b* and *c* do not react with CO unless previously modified or denatured (Lemberg & Barrett, 1973). The CO-reacting cytochromes *b* and *c* in *Acanthamoeba castellanii* may have been subjected to adverse conditions during the procedures used to prepare mitochondria and submitochondrial particles, but the observation that these components are identifiable in CO-difference and photodissociation spectra of intact cells strongly suggests that at least this is not entirely the case; similar experiments performed with the yeast *Schizosaccharomyces pombe* revealed only cytochrome a_3 (Poole *et al.*, 1979). However, particularly for cytochrome *b*, the slow CO binding suggests that denaturation may be occurring and may be necessary for the reaction with CO.

Mitochondria isolated from *Acanthamoeba castellanii* contain at least four spectrally identifiable components that react with CO. In CO difference spectra these have the absorption characteristics shown in Table 1, although precise assignment of wavelength positions of maxima and minima is difficult, especially in regions where these overlap (e.g. the absorption maximum of the β -band of the cytochrome a_3 -CO complex at 548 nm and the trough at about 550 nm due to the reaction of cytochrome *c* with CO). The identification of different components is crucially dependent on both the time of bubbling with CO and the time of subsequent exposure to the gas in solution. Thus the CO-reacting

Table 1. Absorption characteristics of CO-binding haemoproteins in *A. castellanii*

The values presented are those obtained from room-temperature CO difference spectra of mitochondrial preparations, except in the case of cytochrome a_{614} , which has been designated on the basis of its appearance in low-temperature photodissociation spectra.

Cytochrome	Absorption characteristics in CO difference spectra	
	Maxima	Minima
a_{614}	—	614
a_3	592, 546–548, 434–432	605–607, 446
<i>b</i> -Type	574, 542, 418	558, 430
<i>c</i> -Type	412–409	551–554, 420

cytochrome *c* is identified after a much shorter exposure time than is the *b*-type cytochrome. The nature of the reductant is also important; this cytochrome *c* is more easily distinguished when the reductant is NADH.

Whereas treatment of mitochondria with ultrasound releases cytochrome *c* plus a small amount of a *b*-type cytochrome, as seen in reduced–oxidized difference spectra, only the latter appears to react with CO. The cytochrome *c* retained in the submitochondrial particles, however, does react with CO. This clearly suggests that mitochondria of *A. castellanii* contain at least two *c*-type cytochromes, with an absorption maximum, in reduced–oxidized difference spectra, at 549–550 nm; one of these is firmly attached to the mitochondrial membrane and reacts with CO, whereas the other is easily removed and has little or no reaction with CO. The CO-reacting *b*-type cytochrome is also easily removed from mitochondria. Similarly, a cytochrome *c* and a CO-reacting cytochrome *b* are released from mitochondria of *Tetrahymena pyriformis* after treatment with ultrasound (Lloyd & Chance, 1972; Kilpatrick & Erecińska, 1977b).

Although previously unequivocal identification of cytochrome a_3 in CO-difference spectra of mitochondria of *A. castellanii* was not possible (Edwards *et al.*, 1977), inhibitor studies and measurements of oxygen affinities (Lloyd *et al.*, 1979), together with the spectral evidence presented here, confirm the presence of this cytochrome in this organism. The identity of the second *a*-type cytochrome reacting with CO is more obscure. Whereas the occurrence of cytochromes a_1 and a_2 is common in many bacteria (Lemberg & Barrett, 1973; Degn *et al.*, 1978), the only other eukaryotic micro-organism with unusual *a*-type cytochromes is *T. pyriformis* (Lloyd & Chance, 1972; Kilpatrick & Erecińska, 1977a). However, the spectral characteristics of their complexes with CO suggest that the unusual *a*-type cyto-

chromes in *A. castellanii* and *T. pyriformis* are different. Further work is necessary to characterize this *a*-type cytochrome in *A. castellanii* and establish a possible physiological function. Although no mitochondrial CO-reacting cytochrome *c* has been described, such a cytochrome (*c'* or *cc'*) has been reported in several photosynthetic bacteria, but whether these function as oxidases is uncertain (Lemberg & Barrett, 1973). On the other hand, CO-reacting *b*-type cytochromes have been found in a variety of eukaryotes, such as *T. pyriformis* (Yamanaka *et al.*, 1978), *Crithidia fasciculata* (Edwards & Lloyd, 1973; Kusel & Storey, 1973), *Polytoma uvella* and *Astasia longa* (Webster & Hackett, 1965), *Euglena gracilis* (Raison & Smillie, 1969) and many trypanosomes (Hill, 1976); only in these latter organisms has there been any evidence for the existence of a functional cytochrome *o*. Further work is necessary to establish whether any CO-binding haemoprotein in *A. castellanii* has a role as a functional oxidase alternative to cytochrome *a₃*.

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