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CO-BINDING PIGMENTS AND THE FUNCTIONAL TERMINAL OXIDASE OF THE TRYPANOSOMATID HEMOFLAGELLATE *CRITHIDIA FASCICULATA*

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SUMMARY

CO-difference absorbance spectra of both intact cells and of mitochondrial preparations isolated from *Crithidia fasciculata* were obtained after anaerobiosis was attained either with substrates or with dithionite. Under both sets of conditions, the CO-difference spectrum of cytochrome a_3, with difference absorbance maxima at 430 and 589 nm and minima at 443 and 612 nm, was readily identified in both the intact cells and in the mitochondria. In addition to the difference absorbance bands of cytochrome a_3–CO, three difference absorbance maxima at 417, 538 and 570 nm and a minimum at 556 nm were observed. The magnitude of the maximum at 570 nm relative to the maximum of cytochrome a_3–CO at 589 nm was less for mitochondria rendered anaerobic with substrate than for mitochondria rendered anaerobic with dithionite. This difference was taken to define operationally two groups of mitochondrial CO-binding pigments: Group I is that group observed on anaerobiosis with substrate: Group II is the additional group observed on anaerobiosis with dithionite. The Group I CO-binding pigments were virtually absent from submitochondrial particles derived by sonication, but the Group II pigments remained.

Photochemical action spectra were obtained with isolated mitochondria and intact cells to ascertain if cytochrome *o* was present. These action spectra, obtained in CO *plus* O_2 atmospheres, had maxima only at 432, 550 and 588 nm, attributable to the photodissociation of cytochrome a_3–CO. Even after suppression of cytochrome a_3 activity to 10% of the normal value, no contribution of cytochrome *o* activity to the photochemical action spectrum was observed. Cytochrome a_3 is therefore the only functional terminal oxidase present in the mitochondria of *Crithidia fasciculata*.

INTRODUCTION

The presence of at least three CO-binding hemoproteins in cells of the *Trypanosomatidae* has been reported[1–5]. These are cytochrome a_3, cytochrome P-450, and a pigment having absorbance maxima at 419, 540, and 570 nm in its CO-difference spectrum. Based on the similarity of this latter spectrum to that of one of the hemo-

Abbreviation: MOPS, morpholinopropane sulfonic acid.

protein terminal oxidases found in prokaryotes, namely cytochrome *o*[6–12], it has been suggested that this pigment observed in *Crithidia fasciculata*[4] and in *Crithidia oncopelti*[5] is cytochrome *o*. Proof of this tentative assignment would be of evolutionary significance, since cytochrome *o* has not yet been convincingly demonstrated to be a functional oxidase in any unicellular eukaryote. Nor do multi-cellular eukaryotes possess cytochrome *o*; the CO-binding pigment present in plant mitochondrial preparations originally thought to be cytochrome *o* was shown to be a peroxidase[13]. Another pigment present in mitochondrial preparations from intestinal parasites has a similar CO-difference spectrum. However, this hemoprotein seems to function as a fumarate reductase[14,15] and has no oxidase activity in photochemical action spectra[16], in marked contrast to prokaryotic cytochrome *o*[6,7].

The tentative identification of cytochrome *o* in *C. fasciculata* and in *C. oncopelti* is uncertain for the following reasons: (1) Kusel[1], Kusel and Weber[2] and deBoiso and Stoppani[3] all observed an almost identical CO-binding pigment in the microsomal fractions derived from homogenates of *C. fasciculata* and *Trypanosoma cruzi*, respectively. The microsomal P-450 in the hands of the former authors was unstable to the addition of dithionite, and was rapidly converted to a cytochrome P-420 with CO-difference absorbance maxima at 421, 540, and 572 nm. Thus, microsomal contamination of the mitochondrial preparations of Hill and White[4] could have contributed to the CO-binding pigment which was observed. (2) *C. fasiculata* has an absolute growth requirement for either hemin or protophorphyrin IX[17], and hemin was employed in the studies of Hill and White[4]. These authors were careful to note the possible formation of artificial heme–protein complexes[18–21] which might give rise to CO-difference spectra similar to cytochrome *o*; but this formation was not excluded in their reports. (3) While *C. oncopelti* does not require the addition of hemin for growth[17], it contains a Feulgen-positive body which appears to be a bacterial endosymbiont[22–26]. In studies with intact cells of *C. oncopelti* such as have been reported[5], the possibility that the observed CO-binding pigment might be of bacterial origin cannot be excluded. (4) There are several naturally-occurring non-cytochrome hemoproteins which have CO-difference spectra of the cytochrome *o* type, such as ferroperoxidase[27], hemoglobin[27], myoglobin[27], and sulfite reductase[28]. These are widely distributed in nature and have been identified in yeasts, molds, and protozoa[29–32] in addition to their better-known occurrence in animals[27], parasites[33,34] and plants[13]. Purified yeast hemoglobin[35] forms a CO-compound with absorbance maxima at 420, 540, and 570 nm which ist very similar to the absorbance spectra of the pigment reportedly present in *Crithidia* sp. For these reasons, it seems premature to conclude on the basis of spectral evidence alone that the CO-binding pigment described by Hill and White[4] and by Srivastava[5] is cytochrome *o*.

Respiration in the closely related organisms *Trypanosoma brucei*[36,37], *Trypanosoma mega*[38,39], and *Trypanosoma rhodesiense*[40,41] has been reported to be only partially sensitive to inhibition by cyanide or antimycin A. Ray and Cross[38,39] have attributed the cyanide insensitive but CO and salicylhydroxamic acid sensitive respiration in *T. mega* to cytochrome *o*, interacting with the respiratory chain in the region of ubiquinone. Their evidence for this assignment was based on CO-difference spectra of the type described above for *C. fasciculata* and *C. oncopelti*.

A direct method for demonstrating the existence and functionality of CO-binding hemoprotein terminal oxidases, first devised by Warburg and Negelein[42], is

the determination of photochemical action spectra for CO dissociation. All known hemoproteins which bind or react with O_2 in the ferrous form also bind CO in this form and are photodissociable. The activity of a terminal oxidase exposed to a mixed atmosphere of CO *plus* O_2 is markedly inhibited in the dark; during illumination with light of the proper wavelengths, the CO inhibition is relieved and the resulting O_2 uptake can be measured. An instrument for determining the photochemical action spectrum of CO-binding pigments in this manner was developed by Castor and Chance[6], and was further refined by Hyde[43]. The latter instrument was used, in collaboration with Dr Paul Kronick of these laboratories, to determine the CO-binding hemoprotein terminal oxidases present in whole cells and in mitochondrial preparations of *C. fasciculata*. Preliminary results indicated that cytochrome *o* might be present[44]. In this paper, we report a detailed examination of the CO-binding hemoproteins of *C. fasciculata* using both spectrophotometric and photochemical action spectrum techniques. The results rule out the existence of a functional cytochrome *o* in this protozoan.

MATERIALS AND METHODS

Growth medium

Crithidia fasciculata (A.T.C.C. No. 11745) was cultivated in the undefined medium of Kidder and Dutta[45] prepared as previously described[1] (Method No. 2) but with the following modifications:

(1) Glycerol (10 g/l) replaced glucose as added carbohydrate source.

(2) Crystalline triethanolamine·HCl (*N,N',N''*-nitrilotriethanol hydrochloride) (Sigma) replaced Eastman Kodak liquid triethanolamine, and was used at a concentration of 15 g/l (0.08 M).

(3) Tween 80 (polysorbitan monooleate) was omitted.

(4) After adjusting the pH to 8.0 with KOH, 950 ml aliquots of hemin-free but otherwise complete medium were transferred to 2-liter erlenmeyer flasks and were steam sterilized for 25 min at 121 °C.

(5) Hemin (Nutritional Biochemical Co.) was dissolved to 5.0 mg/ml in 0.1 M KOH, and 1.0 M triethanolamine·HCl was added to pH 8.5. The hemin solution was steam sterilized for 15 min at 121 °C and was stored at −20 °C in the dark. Hemin was added aseptically to the growth medium immediately prior to cell transfer to provide 5.0 mg/l (7.5 μM).

Cell culture and isolation of mitochondria

Cultures grown at 28–30 °C in New Brunswick gyratory shakers operated at 250 cycles/min exhibited a doubling time of 4.5 h in the logarithmic growth phase, which persisted until culture densities of $2.0 \cdot 10^8$–$2.5 \cdot 10^8$ organisms/cm^3 were obtained. Cells were collected in the logarithmic to late logarithmic phases of growth with a Sharpless centrifuge. They were washed by resuspension in 10 vol. of buffer containing 0.3 M mannitol and 5 mM morpholinopropane sulfonic acid (MOPS) buffer, adjusted with KOH to pH 7.5; this was the standard buffer used throughout this study unless otherwise noted. Mitochondria were prepared as previously described[46], but the concentration of MOPS buffer in the isolation media was reduced from 10 mM to 5 mM, permitting better separation of the "fluffy" layer from mitochondrial pellets. Mitochondrial protein was determined as described by Miller[47].

Nitrite treatment

Mitochondrial suspensions (50–70 mg protein/ml) were diluted with 15–20 vol. of buffer containing 1.0% (w/v) of KNO_2. After stirring for 30–90 min at 0–4 °C, mitochondria were recovered by centrifuging for 10 min at 16000 × *g*. They were subsequently washed 3 times and suspended in buffer for use.

Preparation of submitochondrial particles

Mitochondrial suspensions in isolation medium[46] containing 0.3 M mannitol were diluted with 20 vol. of 10 mM MOPS buffer, pH 7.5. After standing for 10–20 min at 0–4 °C, the hypotonically-swollen mitochondria were recovered by centrifuging for 10 min at 16000 × *g*. The sediment was resuspended in 10 mM MOPS buffer to contain about 10 mg protein/ml. Mitochondria were sonically disrupted using two 15-s treatments with the microtip attachment of a Heat Systems, Inc. Model W185 sonifier operated at near-maximum output. Unbroken mitochondria were removed by centrifugation as above. The supernatant fluid was centrifuged for 90 min at 105000 × *g*. The sedimented submitochondrial particles were washed once with MOPS buffer, and were resuspended in standard buffer.

Spectrophotometry

CO-difference spectra were obtained at room temperature using the split-beam spectrophotometer described by Chance[48]. Slits were adjusted to provide incident light with a spectral band width at half peak height of 3 or 6 nm as indicated in the figure legends. Suspensions of cells or mitochondria were placed in cuvettes of 1.0-cm light path. Base-line spectra were recorded after anaerobiosis was attained either by endogenous respiration, or by addition of substrates or dithionite ($Na_2S_2O_4$). The sample cuvette was then bubbled gently with CO (Matheson) for 1 min, and spectral differences between CO-treated samples and untreated controls were recorded.

Action spectra

Photochemical action spectra were obtained over the interval 400–700 nm by the method of Hyde[43]. A Bausch and Lomb monochromator of 6.4 nm/mm dispersion and 500-nm blaze was employed. Slits were adjusted to provide incident light with spectral band width at half peak height of 2 or 4 nm as indicated in legends to the figures. The wavelength indicator of the monochromator was calibrated with a low pressure Hg source. Light from a Zeiss xenon source was filtered through 3 cm of water in a clear, flat-surfaced vessel. Light energy was uncompensated: no correction was made for the variation of intensity and quantum energy content with wavelength. Atmospheres of CO *plus* O_2 were used in ratios (v/v) varying from 95:5 to 50:50; the most frequently used mixture was 80:20. Respiring samples were suspended as a droplet in an annulus of 1/8-inch diameter in a 1/32-inch-thick lucite disc mounted in the center of a cylindrical chamber which was flushed with CO *plus* O_2 mixtures. A teflon-covered Ag–Pt oxygen microelectrode was inserted into the sample droplet. The electronic circuitry, described by Hyde[43], converted the electrode signal into a time derivative to yield dO_2/dt directly. This was recorded with a strip chart recorder as a deflection from a constant base line. Intermittent illumination of the sample was performed using increments of 1 nm in regions of maximal changes in activity with wavelength.

RESULTS

CO-difference spectra obtained with suspensions of intact, late-logarithmic phase cells are shown in Fig. 1. The trace in Fig. 1A was obtained by bubbling CO through samples which had attained anaerobiosis by respiring endogenous substrate. The CO-difference of cytochrome a_3 is readily identified by the maxima at 430 and 589 nm and the minima at 443 and 613 nm. The existence of another pigment or group of pigments binding CO is evident from the maxima at 417, 538 and 570 nm and the minimum at 556 nm. Upon addition of dithionite (Fig. 1B), there is a marked increase

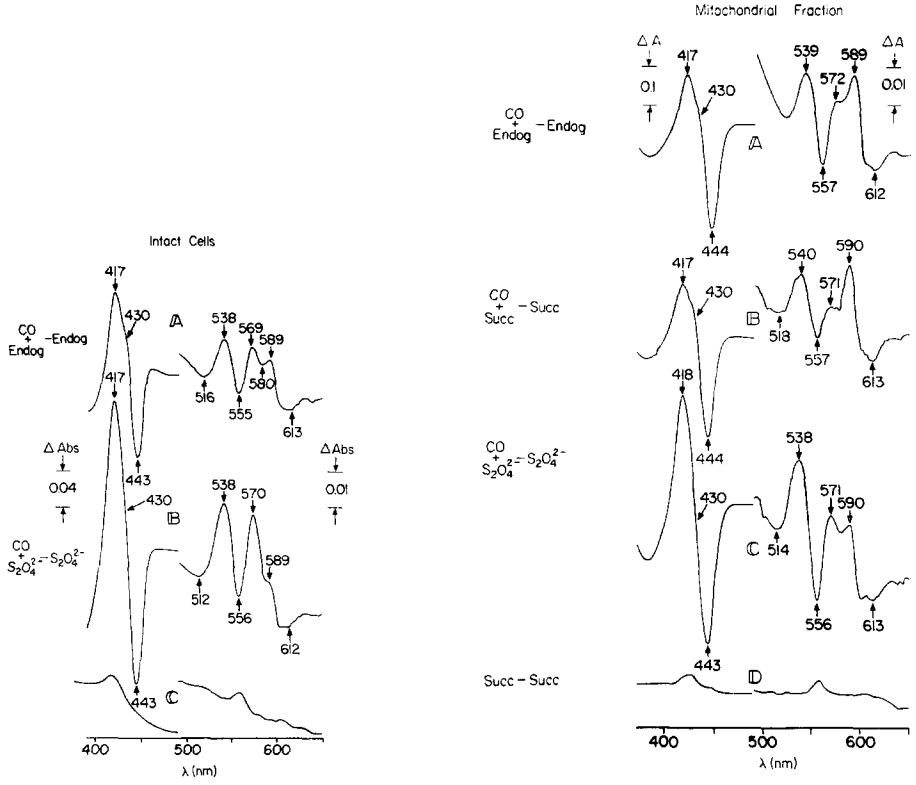

Fig. 1. CO-difference spectra of late-logarithmic phase cells. Cells were collected at a culture density of $1.8 \cdot 10^8$ organisms/cm^3. After washing in standard buffer, they were resuspended to 20% (w/v) in the same buffer to contain approximately $3 \cdot 10^9$ organisms/cm^3. Room-temperature difference spectra were obtained using incident light of 6-nm spectral band width. (A) CO was added to the sample cuvette (10-mm light path) after endogenous respiration had rendered the suspension anaerobic. (B) As in A, but dithionite was added to both cuvettes. (C) As in A, but before the addition of CO (base line).

Fig. 2. CO-difference spectra of the mitochondrial fraction. Cells were collected at a culture density of $1.6 \cdot 10^8$ organisms/cm^3. Mitochondria were prepared and were diluted in standard buffer to contain 6.8 mg mitochondrial protein/ml. Room temperature difference spectra were obtained using incident light of 6-nm spectral band width. (A) CO was added to the sample cuvette after endogenous respiration had rendered the suspension anaerobic. (B) As in A, but with the addition of 5 mM succinate to both cuvettes. (C) As in B, but with the addition of dithionite to both cuvettes. (D) As in B, but before the addition of CO (base line).

in the latter difference absorbance bands relative to those of cytochrome a_3–CO. The CO-difference spectra obtained with suspensions of isolated mitochondria, rendered anaerobic with endogenous substrate, succinate, and dithionite are shown in Figs 2A, 2B and 2C, respectively. The difference absorbance bands due to cytochrome a_3–CO are dominant in the spectra of Figs 2A and 2B. With dithionite there is an increase in the difference absorbance bands at 417, 538 and 570 nm, such that the latter band is about equal in intensity to the 590-nm band of cytochrome a_3–CO. The magnitude of the difference absorbance band at 570 nm relative to that of cytochrome a_3–CO at 589 nm in the spectra of Figs 1 and 2 makes it possible to define operationally four groups of CO-binding pigments which all have essentially the same CO-difference spectrum. Group I is mitochondrial and is observed on anaerobiosis with endogenous substrate or succinate (Figs 2A and 2B). Group II is also mitochondrial and is observed on anaerobiosis with dithionite (Fig. 2C). Group III is cytoplasmic and is observed in whole cells on anaerobiosis with endogenous substrate (compare Figs 1A and 2A). Group IV is also cytoplasmic and is observed in whole cells on anaerobiosis with dithionite (compare Figs 1B and 2C). The work in this paper deals exclusively with the mitochondrial pigments, namely Groups I and II.

The dependence of the observed absorbance changes of the mitochondrial CO-binding pigments upon reducing conditions is quantitated in Table I. The magnitudes of the difference-absorbance changes calculated for the wavelength pairs 590–612 nm, corresponding to cytochrome a_3–CO, and the wavelength pair 572–556 nm, corresponding to Group I and Group II CO-binding pigments, have been rendered in units of absolute absorbance per milligram of mitochondrial protein. The effects of reducing conditions on the observed absorbance changes were quite reproducible

TABLE I

QUANTITATION OF MITOCHONDRIAL CO-BINDING PIGMENTS

The following numbers give the concentration of mitochondrial protein for each preparation and the culture density at time of harvest of cells from which mitochondrial fractions were prepared: Preparation No. 1: 4.8 mg/ml, $2.0 \cdot 10^8$ cells/cm^3; Preparation No. 2: 7.5 mg/ml, $1.6 \cdot 10^8$ cells/cm^3; Preparation No. 3: 6.8 mg/ml, $1.6 \cdot 10^8$ cells/cm^3; Preparation No. 4: 8.0 mg/ml, $1.4 \cdot 10^8$ cells/cm^3

Mitochondrial preparation	$10^3 \times \Delta A$ *(589–612 nm)/mg protein**			$10^3 \times \Delta A$ *(572–556 nm)/mg protein**		
	Endogenous	*Reductant:*		*Endogenous*	*Reductant:*	
		5 mM succinate	*Dithionite*		*5 mM succinate*	*Dithionite*
No 1	2.27 (104)	2.19 (100)	2.02 (92)	0.93 (184)	0.51 (100)	1.26 (250)
No. 2	2.53 (100)	2.53 (100)	2.27 (90)	1.73 (108)	1.60 (100)	6.84 (433)
No. 3	3.56 (96)	3.71 (100)	2.97 (80)	2.37 (200)	1.18 (100)	3.26 (275)
No. 4	—	3.12 (100)	2.50 (80)	—	1.50 (100)	3.00 (200)
$10^3 \times \Delta A$ (average)	2.78	2.89	2.44	1.67	1.20	3.59
% succinate value	100	100	84	139	100	289
% endogenous value	—	—	—	100	72	215

* Data in parentheses represent % succinate value.

for cytochrome a_3–CO, but were rather more variable for the other CO-binding pigments. Endogenous substrate, succinate and dithionite elicited about the same absorbance change at 590–612 nm. Addition of succinate resulted in a slight apparent decrease in the 572 − 556-nm difference absorbance compared with endogenous substrate, but there was no change in the absorbance at 417 nm. Addition of dithionite resulted in an average increase in the 572 − 556-nm difference absorbance to 215% relative to endogenous substrate and to 289% relative to succinate; analogous increments were seen at the 417-nm absorbance maximum. These dithionite-induced absorbance increments define the Group II pigments.

The Group I CO-binding pigment or pigments, observed in anaerobiosis with endogenous substrate or succinate could be of two types: either a ferrous hemoprotein such as hemoglobin or myoglobin whose oxidation–reduction state was independent of electron flux through the respiratory chain, or an oxidation-reduction component in intimate communication with the respiratory carriers, which became reduced on anaerobiosis with endogenous or added substrates. In order to distinguish between these two possibilities, mitochondrial preparations were exposed to nitrite which oxidizes ferrohemoproteins to the ferric oxidation state[49,50]. CO-difference spectra were then obtained with nitrite-treated preparations and suitable controls using succinate and dithionite as reductants. As may be seen in Figs 3A and 3B, control preparations contained the same ratios of the two CO-binding pigments as previously observed after addition of succinate or dithionite. In contrast, the nitrite-treated preparation reduced with succinate (Fig. 3D) exhibited only a trace of the Group I CO-binding pigments in comparison to cytochrome a_3–CO (compare Figs 3A and 3D). Addition of dithionite to nitrite-treated preparations (Fig. 3E) resulted in formation of Group I and II CO-binding pigments, but no increase in cytochrome a_3–CO. Nitrite treatment apparently decreased the absorbance changes of all the CO-binding pigments by about 50% relative to control preparations (compare Figs 3B and 3E). However, the final ratio of the Group I and II CO-binding pigments to cytochrome a_3–CO observed after dithionite reduction was about the same with both nitrite-treated mitochondria and untreated controls. This indicated that the Group I CO-binding pigments were oxidized by exposure to nitrite. Subsequent to nitrite oxidation, these were not readily reducible by succinate, but were reducible by dithionite.

The CO-difference spectrum of submitochondrial particles reduced with succinate (Fig. 4A) also contained very little of the CO-binding pigments relative to cytochrome a_3–CO. This indicated that the fraction of the Group I pigments seen in intact mitochondria after substrate-induced anaerobiosis was either not membrane-bound or was readily solubilized by sonification. A CO-binding pigment with difference absorbance maxima at 419, 540 and 570 nm was observed in the soluble supernatant obtained after sonic description of mitochondria; this pigment was not further characterized. The dithionite-reducible Group II CO-binding pigments remained associated with submitochondrial particles as indicated by the difference spectrum seen in Fig. 4B.

In order to determine whether one of the CO-binding pigments represented a functional terminal oxidase such as cytochrome *o*, photochemical action spectra were obtained. As may be seen in Fig. 5, intact cells obtained from either mid-logarithmic or early stationary phase cultures as well as isolated mitochondria gave

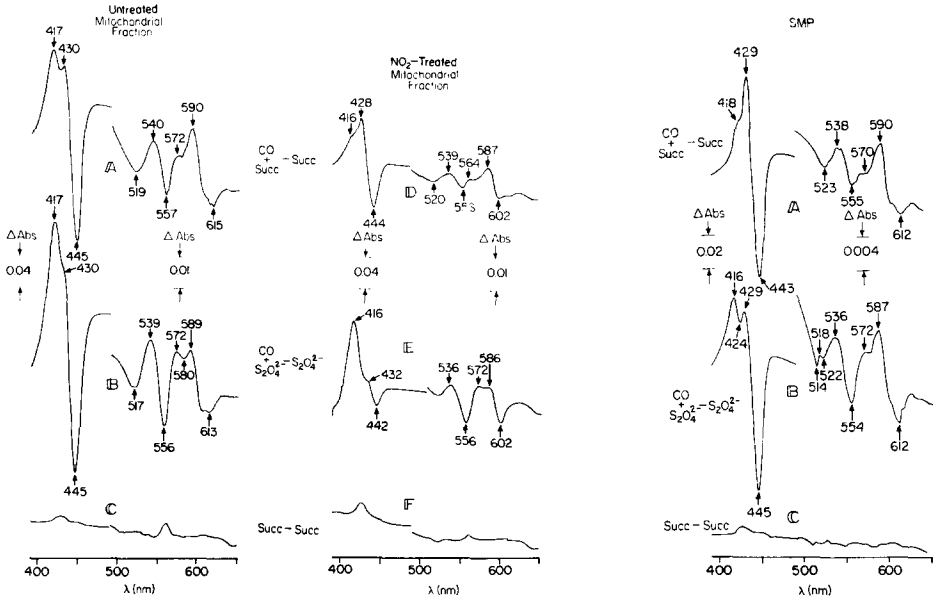

Fig. 3. CO-difference spectra of mitochondrial fraction before and after exposure to KNO_2. Mitochondria were prepared from cells collected at a culture density of $1.4 \cdot 10^8$ organisms/cm^3. Room temperature difference spectra were obtained using incident light of 6-nm spectral band width. (A) CO was added to a suspension of untreated mitochondria (8.0 mg protein/ml) in standard buffer rendered anaerobic with 5 mM succinate. (B) As in A, but with dithionite added to both cuvettes. (C) As in A, but before the addition of CO (base line). (D) CO was added to a KNO_2-treated mitochondrial suspension (7.1 mg protein/ml) in standard buffer rendered anaerobic with succinate. (E) As in D, but with dithionite added to both cuvettes. (F) As in D, but before the addition of CO (base line).

Fig. 4. CO-difference spectra of submitochondrial particles (SMP). Submitochondrial particles were prepared from mitochondria isolated from cells which were collected at a culture density of $1.6 \cdot 10^8$ organisms/cm^3. Room-temperature difference spectra were obtained using incident light of 3-nm spectral band width. (A) CO was added to suspensions containing 11.8 mg protein/ml of submitochondrial particles after anaerobiosis was achieved with 5 mM succinate. (B) As in A, but after the addition of dithionite. (C) As in A, but before the addition of CO (base line).

identical action spectra with maxima at 432, 550 and 588 nm, corresponding to that for photodissociation of cytochrome a_3–CO[6,42]. The same result was obtained with early logarithmic phase cells. Except for a slight asymmetry in the region 408–412 nm, there was no evidence for a functional cytochrome *o*. The small peak seen here at 465 nm was also found in photochemical action spectra obtained with *Saccharomyces carlsbergensis* under the same conditions. This artifact was caused by the rather sharp maximum in the emission spectrum of the xenon arc at this wavelength, because no compensation was made for the variation of light intensity with wavelength.

The possibility that the asymmetry seen in photochemical action spectra in the 408–412-nm region was caused by a very small amount of cytochrome *o* was tested by obtaining photochemical action spectra of cells treated with respiratory inhibitors or rendered deficient in cytochrome a_3. The effect on the action spectrum of adding 1 mM azide to intact cells is shown in Fig. 6. The relative rate of light-stimulated O_2

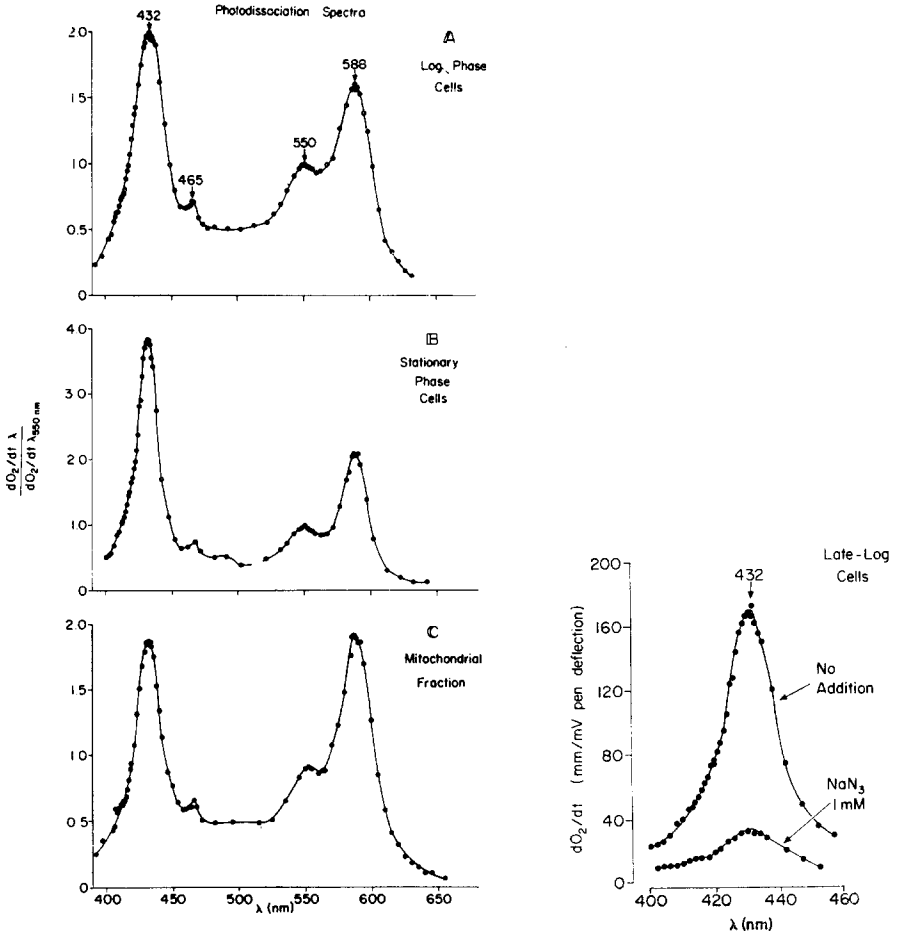

Fig. 5. Photochemical action spectra of intact cells and mitochondrial fractions. (A) Cells were collected at a culture density of $1.6 \cdot 10^8$ organisms/cm^3. They were washed and resuspended in 50 mM MOPS buffer, pH 7.5, to 20% (w/v). A gas mixture of CO–O_2 (80:20, v/v) was employed. Incident light had a spectral band width of 4 nm. (B) Cells were collected at a culture density of $2.4 \cdot 10^8$ organisms/cm^3. They were washed and resuspended as in A. A gas mixture of CO–O_2 (80:20, v/v) was employed. Incident light had a spectral band width of 2 nm. (C) Mitochondrial fractions were prepared from cells collected at a culture density of $1.5 \cdot 10^8$ organisms/cm^3. Mitochondrial suspensions in standard buffer contained 26.7 mg/ml protein, 6 mM $MgCl_2$ and 25 mM DL-α-glycerophosphate. A gas mixture of CO–O_2 (90:10, v/v) was employed. Incident light had a spectral band width of 4 nm.

Fig. 6. Photochemical action spectra of intact cells in the presence and absence of azide. Spectra were obtained with the preparation and conditions given in the legend to Fig. 5B, with the addition of 1 mM NaN_3 as indicated.

consumption at the 432-nm maximum was decreased in the presence of azide to about 15% of the control preparation without azide. However, no increase in relative activity in the 410–420-nm region was seen as might have been expected if an azide-insensitive cytochrome *o* were present even in very low concentrations relative to cyto-

chrome a_3. A similar experiment was performed using antimycin A as the respiratory inhibitor. Mid-logarithmic phase cells were titrated with antimycin until the rate of oxygen uptake was reduced by 80%. After washing, the cells were used to obtain the action spectrum seen in Fig. 7A. Even though the absolute rates of O_2 consumption were lower compared to controls, the action spectrum of the antimycin-inhibited cells was virtually identical with those seen in Figs 5A and 5B obtained with uninhibited cell suspensions. Again, no significant enhancement of the activity in the 410–420-nm region was observed. Growth of *C. fasciculata* in the presence of acriflavin (a mixture of proflavin and euflavin) has been shown to inhibit formation of cytochrome a_3 oxidase activity[51–53]. Cells grown in the presence of 10 μM euflavin (2,8-diamino-10-methylacridinium hydrochloride) and 19 μM hemin[51] for 4.5 cell doublings contained less than 10% of the normal amount of spectrophotometrically-detectable cytochromes $a+a_3$. The photochemical action spectrum obtained with these cells is shown in Fig. 7B. Although the absolute rates of O_2 consumption were 5 to 10% those of normal cells, the relative positions of the maxima in the spectrum indicated the presence of but a single functional oxidase, namely cytochrome a_3, with no enhancement of relative activity in the 410–420-nm region of the spectrum. Attempts to induce the formation of cytochrome *o* by growing cells under reduced O_2 tension,

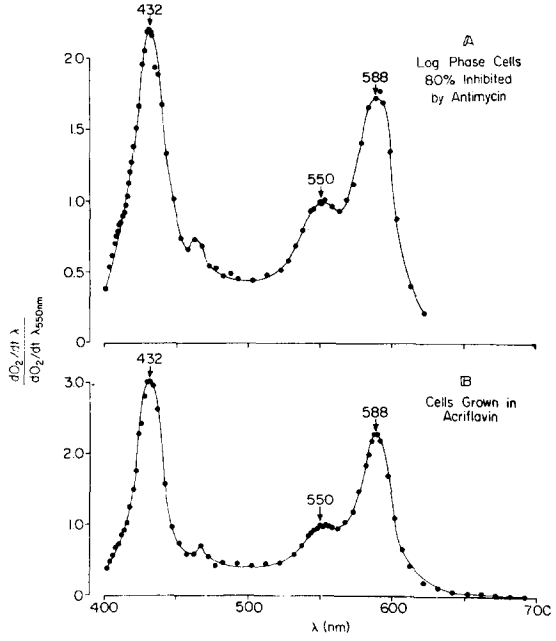

Fig. 7. Photochemical action spectra of intact cells in the presence of antimycin, or after growth in the presence of acriflavin. (**A**) Cells were grown in 0.01% glycerol to a density of $9 \cdot 10^7$ organisms/cm^3. They were titrated with antimycin as described in the text, and were resuspended to 20% (w/v) in 50 mM MOPS buffer, pH 7.5. A gas mixture of CO–O_2 (70:30, v/v) was employed. Incident light had a spectral band width of 4 nm. (B) Cells were grown in the presence of 10 μM euflavin and 19 μM hemin for 4.5 cell doublings to a culture density of $1.1 \cdot 10^8$ organisms/cm^3. They were washed and resuspended with 50 mM MOPS buffer, pH 7.5, to a concentration of 20% (w/v). A gas mixture of CO–O_2 (80:20, v/v) was employed. Incident light had a spectral band width of 2 nm.

using diffusion-limited, unshaken cultures with either glycerol or glucose as carbohydrate sources, were completely unsuccessful; cytochrome a_3 oxidase was the only functional oxidase found in the photochemical action spectra obtained with such cells.

Succinoxidase activity of mitochondria isolated from *C. fasciculata* was cyanide sensitive, with 50% inhibition observed at 5 μM KCN and 99% inhibition at 50 μM KCN. Antimycin A at 2 μg/mg mitochondrial protein consistently inhibited respiration by at least 98% with all substrates tested, including succinate, α-glycerophosphate, α-ketoglutarate, and L-proline. These results are consistent with the presence of cytochrome a_3 as the sole terminal oxidase, rather than with the presence of a branched respiratory chain terminating in cytochromes a_3 and *o*.

DISCUSSION

In addition to cytochrome a_3, the presence of two groups of CO-binding hemoproteins with spectra similar to each other, but different from cytochrome a_3–CO was demonstrated operationally in these mitochondria. Group I was seen after addition of CO to cells or mitochondria rendered anaerobic by endogenous respiration or by addition of succinate, and Group II was observed only after addition of dithionite. The Group I pigments have absorbance bands similar to those of the slowly autooxidizable CO-reactive cytochrome b_{560} of *Tetrahymena* described by Perlish and Eichel[54], by Lloyd and Chance[55], and by Yamanaka *et al.*[56], who reported solubilization of the *Tetrahymena* hemoprotein by treatment with ultrasound. The latter observation would be consistent with the decreased content of Group I pigments observed in this study with sonically prepared submitochondrial particles. However, the observation that the Group I pigments treated with nitrite were not readily reduced after addition of succinate implies that this group consists of components which are not redox members of the respiratory chain, but exist normally in the ferrous oxidation state. They would be oxidized to the ferric oxidation state by nitrite from which they could be reduced to the ferrous state only with dithionite. Such components would not contribute to the reduced-*minus*-oxidized difference spectra of these mitochondria[1,2,4,57], and should not be classified as cytochromes. They might be hemoglobin- or myoglobin-like compounds, and/or proteins complexed with ferrous heme similar to those reported by Drabkin[18], by Tohjo and Shibata[19], and by Yoshida *et al.*[20], and which may function in the transport[58] or storage[59] of hemin required for formation of functional hemoproteins in the heme-requiring *Trypanosomatidae*.

At least two possibilities exist for the nature of the Group II CO-binding pigments reducible only with dithionite: (a) a heme–protein "storage" complex of the kind discussed above, but with the heme group in the oxidized state, and (b) a peroxidase-like hemoprotein, which functions in the ferric state, but which is reducible by dithionite. The two possibilities are not mutually exclusive. The latter possibility finds analogy in the presence of cytochrome *c* peroxidase in yeast mitochondria[60]; the existence of this type of peroxidase in *C. fasciculata* mitochondria has been demonstrated in this laboratory (Kusel, J. P., Boveris, A. and Storey, B. T., unpublished results).

Photodissociation action spectra obtained with both intact cells and mitochondrial preparations demonstrated the presence of a single functional terminal oxidase, identified as cytochrome a_3, with maxima at 432, 550, and 588–590 nm.

Except for a slight asymmetry on the ascending limb of the Soret maximum at about 410 nm, there was no evidence for a functional cytochrome *o*. All attempts to increase the relative contribution to this region of the action spectrum by a putative cytochrome *o* were unsuccessful. Even after inhibition of cellular cytochrome a_3 activity to one-fifth the original activity using respiratory inhibitors, or after inhibition of cytochrome a_3 formation by euflavin to less than one-tenth that of normal cells, no activity attributable to cytochrome *o* could be found. From these findings, we conclude that cytochrome *o* is absent from *C. fasciculata*.

These results apply to *C. fasciculata* grown as described. The unsuccessful attempts to induce cytochrome *o* activity by altering growth conditions, do not absolutely preclude the possibility of induction of such an oxidase under appropriate conditions but render it most unlikely. The nature of the cyanide-insensitive respiration reported in *Trypanosoma brucei*[36,37], *Trypanosoma mega*[38,39], and *Trypanosoma rhodensiense*[40,41] remains unresolved. This type of respiration in *Euglena*[61,62], *Neurospora*[63–66], yeast[67], and in higher plants[68–71] does not involve hemoproteins of the respiratory chain, however. There is some evidence that the alternate terminal oxidase for cyanide- and antimycin-insensitive respiration in plant mitochondria involves an iron–sulfur protein[70] linked to flavoprotein[69,70], but the lack of specific absorbance changes correlating with the redox state of this oxidase has made it most difficult to characterize. The benzhydrozamic acids were found in this laboratory to be specific inhibitors of this oxidase in plant mitochondria[70]; they also inhibit this oxidase in *Neurospora*[63] and in certain yeast strains[67] which have antimycin-insensitive respiration. Salicylhydroxamic acid, a member of this class of inhibitors, was shown by Ray and Cross[38,39] to inhibit the cyanide-insensitive pathway in *T. mega*. It seems rather more probable that cyanide-insensitive respiration in trypanosomes proceeds through this type of oxidase rather than through cytochrome *o*. While the results of our work do not preclude the existence of functional cytochrome *o* in this class of eukaryotes, they render it less probable and support the hypothesis that cytochrome *o* is strictly a prokaryotic terminal oxidase.

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