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LOW-TEMPERATURE SPECTRAL PROPERTIES OF THE RESPIRATORY CHAIN CYTOCHROMES OF MITOCHONDRIA FROM *CRITHIDIA FASCICULATA*

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SUMMARY

Highly purified mitochondrial preparations from the trypanosomatid hemoflagellate, *Crithidia fasciculata* (A.T.C.C. No.11745), were examined by low-temperature difference spectroscopy. The cytochrome $a+a_3$ α maximum of hypotonically-treated mitochondria reduced with succinate, was shifted from 605 nm at room temperature to 601 nm at 77 °K. The Soret maximum, found at 445 nm at 23 °C, was split at 77 °K into two approximately equally absorbing species with maxima at 438 and 444 nm. A prominent shoulder observed at 590 nm with hypotonically-treated mitochondria was not present in spectra of isotonic controls.

The cytochrome *b* maxima observed in the presence of succinate *plus* antimycin A were shifted from the 431 and 561 nm positions observed at 23 °C to 427 and 557 nm at 77 °K. Multiple *b* cytochromes were not apparent.

Unlike other soluble *c*-type cytochromes, the α maximum of cytochrome c_{555} was not shifted at 77 °K although it was split to give a 551 nm shoulder adjacent to the 555 nm maximum. This lack of a low-temperature blue shift was true for partially purified hemoprotein preparations as well as *in situ* in the mitochondrial membrane.

Using cytochrome c_{555}-depleted mitochondria, a cytochrome c_1 pigment was observed with a maximum at 420 nm and multiple maxima at 551, 556, and 560 nm. After extraction of non-covalently bound heme, the pyridine hemochromogen difference spectrum of cytochrome c_{555}-depleted preparations exhibited an α maximum at 553 nm at room temperature.

The reduced rate of succinate oxidation by cytochrome c_{555}-depleted mitochondria and the ferricyanide requirement for the reoxidation of cytochrome c_1, even in the presence of antimycin, indicated that cytochrome c_{555}-mediated electron transfer between cytochromes c_1 and $a+a_3$ in a manner analagous to that of cytochrome *c* in mammalian mitochondria.

INTRODUCTION

Previous studies have demonstrated the presence of *a*-, *b*-, and *c*-type cytochromes in both intact cells and subcellular preparations of the trypanosomatid *Crithidia*

Abbreviations: TMPD, *N,N'*-tetramethylphenylenediamine; MOPS, morpholinopropane sulfonic acid.

fasciculata[1–4] and in the culture forms of several related species of *Trypanosoma*[5–8]. The extent to which the respiratory chain carriers in this protozoan resemble or differ from those in mitochondria from higher animals and higher plants, as well as those in other unicellular organisms, is a point of considerable biological interest, especially regarding the evolution of mitochondria. In addition, such comparative studies may assist in locating those components common, and hence necessary, to energy-conserving respiratory pathways in all aerobic eukaryotes.

C. fasciculata contain a hemoprotein analagous to cytochrome *c* in mammalian mitochondria, with an absorbance peak at 555 nm in the reduced form. This soluble cytochrome c_{555} has been highly purified and extensively characterized[9–11]. Relatively little is known concerning the respiratory chain carriers not readily extractable from the mitochondrial membrane in this organism. Mitochondrial preparations used in previous studies were composed predominantly of kinetoplasts and mitochondrial fragments[1–4]. They oxidized succinate about as rapidly as did mitochondria from mammalian sources[1–3], but were lacking in respiratory control[12] with this substrate and phosphorylated ADP with ADP/O ratios of only 0.5–1.0[4,13]. Recently we[14], and Toner and Weber[15] have succeeded in obtaining mitochondrial preparations from *C. fasciculata* which show respiratory control ratios of 2–3 with succinate as substrate and ADP as phosphate acceptor and with ADP/O ratios of 0.8–1.2. This work demonstrated that the relatively low ADP:O ratio observed with these mitochondria was due to two sites of energy conservation operating at low efficiency of oxidative phosphorylation, rather than due to one site operating at high efficiency. Further, it was shown that these sites function at the same location and with the same substrates as do Sites II and III in mammalian mitochondria. This mitochondrial preparation is therefore suitable for characterizing the respiratory chain carriers of *C. fasciculata* and their involvement in the energy conservation process. In this paper, we report the results of such a characterization obtained by means of low temperature spectrophotometry[16,17].

MATERIALS AND METHODS

Reagent grade succinic and ascorbic acids were employed as molar solutions after adjusting the pH to 7.5 with KOH or $KHCO_3$, respectively. *N,N'*-Tetramethylphenylenediamine (TMPD) was employed as a 0.1 M aqueous solution after crystallization as the dihydrochloride from ethanol. $K_3Fe(CN)_6$ was dissolved in water to 0.2 M after crystallization from water. Antimycin A, obtained from Sigma Chemical Co., St. Louis, Mo., was dissolved to 2.0 mg/ml in dimethylformamide.

Cytochrome c_{555} was extracted from *C. fasciculata* and purified through the ammonium sulfate precipitation step as previously described[9]. After dialysis to remove excess salt, it was oxidized with ferricyanide and recovered by gel filtration[9].

Mitochondria were prepared as described previously[14]. Hypotonic swelling was accomplished by diluting freshly prepared mitochondrial suspensions in 0.3 M mannitol with 30 vol, of ice-cold 10 mM morpholinopropane sulfonic acid (MOPS) buffer, adjusted to pH 7.5 with KOH. The suspension was stirred gently at 0–4 °C for 40–60 min. Mitochondria were recovered by centrifuging for 15 min at 16 000 × *g*. One half of the pellet of hypotonically-swollen mitochondria was resuspended in in 20 vol. of 10 mM MOPS buffer, pH 7.5, containing 0.15 M KCl in order to extract

the soluble cytochrome c_{555} (ref. 18). The other half, the hypotonic control, was resuspended in 10 mM MOPS buffer. After 18 h at 0–4 °C, both preparations were centrifuged for 15 min at 16 000 × *g*. The cytochrome *c*-depleted mitochondria were washed three times by resuspension in KCl buffer followed by centrifugation as above, then washed once with 10 mM MOPS buffer, and once with 0.3 M mannitol buffered with 10 mM MOPS at pH 7.5. The hypotonic control preparation was subjected to the identical procedures of resuspension and centrifugation except that 10 mM MOPS buffer was used in place of KCl-containing buffer. Both preparations were finally resuspended in 0.3 M mannitol buffered with 10 mM MOPS, pH 7.5, to a final protein concentration of 5–15 mg/ml. Isotonic control mitochondrial were similarly diluted in mannitol–MOPS medium just prior to use but after storage for about 24 h at 0–4 °C in the wash medium used in preparing the mitochondria[14].

Difference spectra were obtained at 77 °K with a Johnson Foundation split-beam spectrophotometer as described by Estabrook[16] with the modifications introduced by Bonner[17]. The samples were frozen in lucite cuvettes with a 0.2 cm light path. The slits of the spectrophotometer were adjusted to produce incident light with a spectral half-band width of 3 nm. Spectra were scanned over the interval 400–650 nm. A low-pressure Hg arc was used to calibrate the wavelength indicator dial of the spectrophotometer. Wavelengths of absorption maxima were determined with a reproducibility of ± 1 nm.

Protein concentrations were determined by the method of Miller[19] using crystalline bovine serum albumin (Fraction V, Nutritional Biochemical Corp.) as the standard.

RESULTS

Reduced *minus* oxidized difference spectra are shown in Figs 1A and 1B for *C. fasciculata* mitochondria treated with isotonic medium and in Figs 1C and 1D for mitochondria treated with hypotonic medium. With succinate as substrate, both preparations had a maximum at 600 nm and a double maximum at 438–440 and 444 nm attributable to cytochromes $a+a_3$ (refs 20, 21). Maxima at 422–424 nm, 512–521 nm, and 556 nm were attributable to combined contributions from the *b* and *c* cytochromes. A flavoprotein trough was also evident at 456–458 nm. The mitochondria treated with hypotonic medium had an additional band near 590 nm as a shoulder on the 601 nm maximum of cytochromes $a+a_3$. Succinate failed to reduce all of the cytochromes present in the isotonic mitochondrial preparations. The difference spectrum shown in Fig. 1B, obtained with $Na_2S_2O_4$ in the experimental cuvette and succinate in the reference, had absorbance maxima attributable to cytochromes *b* and/or *c* with only slight contributions from cytochromes $a+a_3$. As shown in Fig. 1D, hypotonically treated mitochondria did not exhibit these absorption bands under the same conditions, indicating that these pigments may be in the outer membrane or intermembrane space which would be removed by the hypotonic washing procedure. To avoid interference from these pigments, hypotonically treated mitochondria were used to obtain the rest of the spectra presented in this paper.

The spectra of Fig. 1 indicate the difficulties inherent in the spectrophotometric resolution of the *b* and *c* cytochromes of *C. fasciculata*. The maxima of the α-bands were

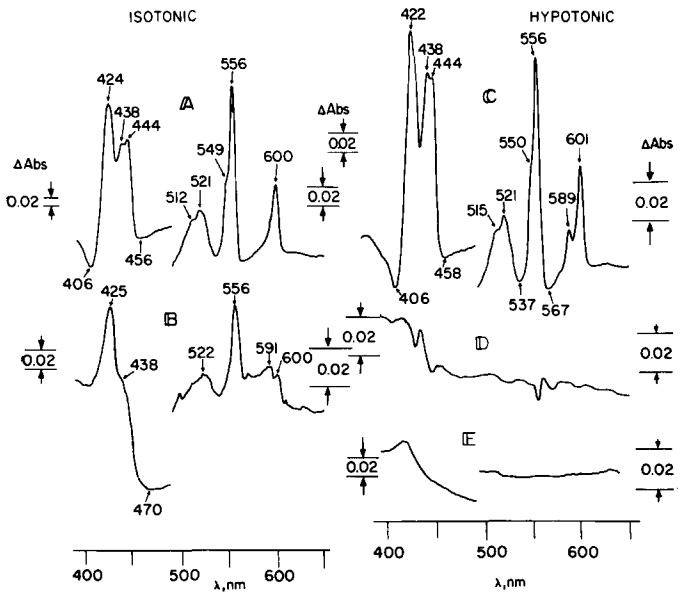


Fig. 1. Difference spectra obtained at 77 °K with mitochondrial preparations before and after exposure to hypotonic conditions. (A) Isotonic mitochondrial suspension (Preparation 1; 11.7 mg protein/ml) reduced with 10 mM succinate *minus* oxygenated reference. (B) As in A but $Na_2S_2O_4$-reduced *minus* reference reduced with 10 mM succinate. (C) Hypotonically treated mitochondrial suspension (Preparation 2; 6.6 mg protein/ml) reduced with 10 mM succinate *minus* oxygenated reference. (D) Hypotonically treated mitochondrial suspension (Preparation 1; 11.7 mg protein/ml) reduced with $Na_2S_2O_4$ *minus* reference reduced with 10 mM succinate. (E) Oxidized *minus* oxidized base line of hypotonically treated mitochondria (Preparation 2; 6.6 mg protein/ml), obtained with the same instrument gain settings as were used to record the difference spectra in Figs 1, 2, 4, 5 and 6.

very close together and were not resolved, even at 77 °K. The cytochrome *b* component was resolved with the aid of antimycin A, as shown in Fig. 2A, and cytochrome *c* components were resolved as shown in Fig. 2B. The difference spectrum of Fig. 2A was obtained with hypotonically treated mitochondria with succinate in the presence of antimycin A; both experimental and reference samples were oxygenated immediately before freezing. In Fig. 2A, prominent maxima at 427, 527, and 557 nm were observed. Essentially the same spectrum was obtained with cytochrome *c*-depleted mitochondria under similar conditions (Fig. 2C). The cytochrome difference spectrum of Fig. 2A can therefore be attributed to cytochrome *b* alone. In both cases, the antimycin inhibition was not complete as can be seen by the 444 nm shoulder and small 600 nm peak of cytochromes $a+a_3$. The incompletenes of the antimycin block permitted the recording of difference spectra of cytochromes situated on the electron transport pathway beyond the site of antimycin inhibition. This was done by allowing the experimental and reference samples to become anaerobic in the presence of both succinate and antimycin, followed by oxygenation of the reference sample just prior to freezing. The resulting spectrum obtained with hypotonically treated mitochondria may be seen in Fig. 2B. Absorption maxima at 439, 444, and 601 nm were ascribed to cytochromes $a+a_3$, while those at 421, 522 and 555 nm were attributed to *c*-type cytochromes.

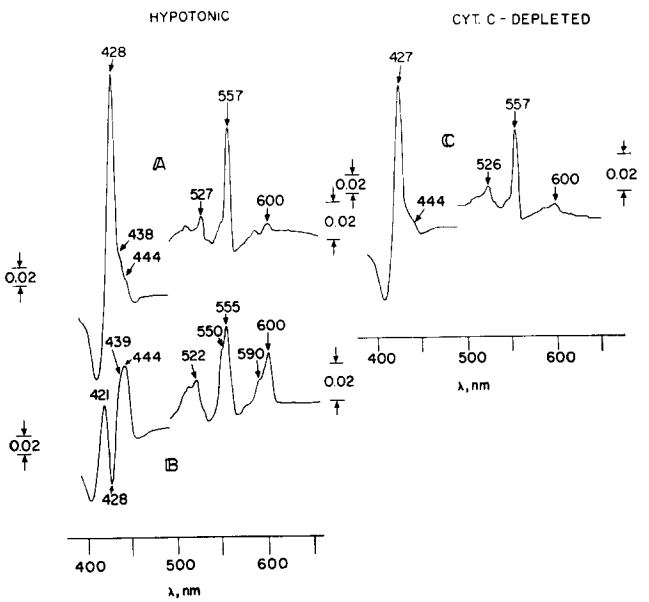


Fig. 2. Difference spectra obtained at 77 °K in the presence of antimycin A. (A) Hypotonically treated mitochondrial suspension (Preparation 2; 6.6 mg protein/ml) reduced with 10 mM succinate *plus* 10 μg/ml antimycin *minus* oxygenated reference. (B) As in A, but both cuvettes contained 10 mM succinate *plus* 10 μg/ml antimycin; the experimental cuvette was permitted to become anaerobic while the reference cuvette was oxygenated immediately before freezing. (C) Cytochrome *c*-depleted mitochondria (Preparation 2; 7.2 mg protein/ml) reduced with 10 mM succinate *plus* 10 μg/ml antimycin *minus* oxygenated reference.

Identification of the latter was confirmed by obtaining the reduced *minus* oxidized difference spectrum of purified cytochrome c_{555} at 77 °K. As may be seen in Fig. 3, essentially the same major peak positions were observed in both the mitochondrial preparation and the sample of partially purified hemoprotein. The α maximum of *Crithidia* cytochrome c_{555} was split at low temperature to give prominent α_1 and α_2 maxima as was the case with horse, pigeon and moth cytochromes c[22]. Unlike the latter, however, the α_1 maximum of the *Crithidia* hemoprotein was not shifted to shorter wavelengths at low temperature; it remained at the same position observed at room temperature[2–4]. In contrast, the cytochrome *b* absorption maxima were shifted from 431 and 561 nm observed at room temperature[1–3] to 427 and 557 nm respectively at 77 °K. This explains why cytochromes *b* and c_{555} were not spectrally resolved at low temperatures except for the α_2 absorbance of cytochrome c_{555} seen in Figs 1A and 1C as a shoulder at 549–550 nm.

The use of ascorbate *plus* TMPD as reductant interacting with the mitochondrial respiratory chain at the level of cytochrome c[23,24] yielded a reduced *minus* oxidized difference spectrum (Fig. 4A) which was quite similar to that obtained in Fig. 2B. However, two observations suggested that ascorbate *plus* TMPD also partly reduced cytochrome *b*: (1) the ratio of the difference absorbance $\Delta A_{555-565\,nm}$ to the difference absorbance $\Delta A_{600-630\,nm}$ was greater in Fig. 4A than in Fig. 2B, and (2) the α_2 (550 nm) shoulder of cytochrome c_{555} was displaced vertically from the α_1 (555 nm) maximum to a greater extent in Fig. 4A than in Fig. 2B. With

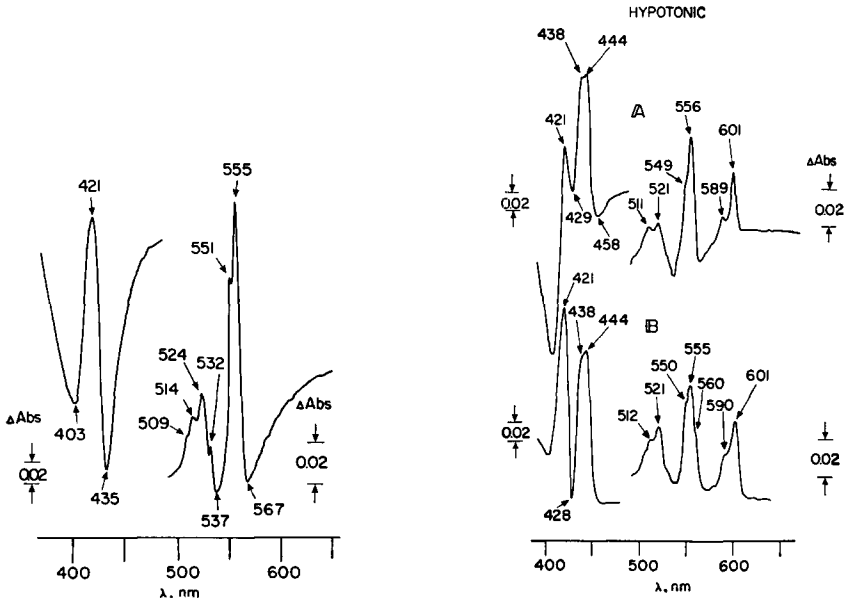


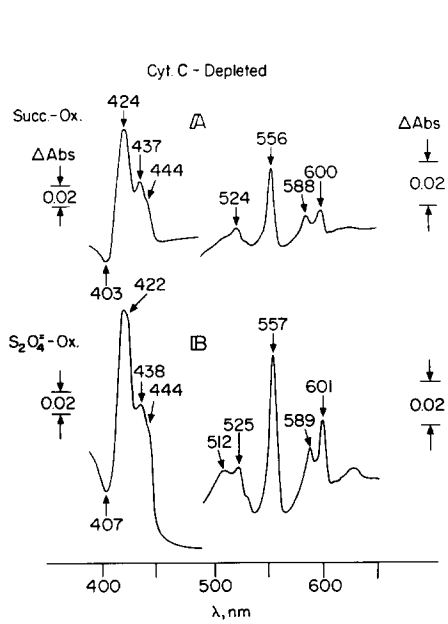
Fig. 3. Difference spectrum obtained at 77 °K with partially purified cytochrome c_{555}. The hemoprotein was dissolved in 0.3 M mannitol buffered with 10 mM MOPS, pH 7.5, to a concentration of 1.1 μM. Reduced with $Na_2S_2O_4$ *minus* oxidized.

Fig. 4. Difference spectra obtained at 77 °K with hypotonically treated mitochondria reduced with ascorbate and TMPD in the presence and absence of antimycin A. (A) Mitochondrial suspension (Preparation 2; 6.6 mg protein/ml) reduced with 5 mM ascorbate *plus* 0.5 mM TMPD *minus* oxygenated reference. (B) As in A, but both samples contained 10 mM succinate *plus* 10 μg/ml antimycin. Ascorbate and TMPD were added to reduce the experimental sample; the reference sample was oxygenated before freezing.

succinate and antimycin A added at room temperature to the mitochondria to reduce cytochrome *b*, and ascorbate *plus* TMPD added to the experimental sample and O_2 to the reference, the difference spectrum seen in Fig. 4B was obtained. The difference absorbance $\Delta A_{555-570\,nm}$ was lower relative to $\Delta A_{600-630\,nm}$ than in the absence of succinate plus antimycin (Fig. 2A), and the α_1 and α_2 cytochrome c_{555} absorbance maxima were again closer together as in Fig. 2B. A small shoulder at 560 nm was observed in both Figs 2B and 4B, but not in 4A and was thus correlated with the presence of succinate and antimycin A in both the experimental and reference samples. This experimental condition ruled out a red shift due to antimycin A[25] as the source of this band.

Keilin and Hartree[26] were able to differentiate cytochrome c_1 from cytochrome *c* in spectra obtained at liquid air temperatures. This differentiation is difficult, however, and usually the absorbance bands of the two cytochromes in the reduced form have not been resolved in low temperature difference spectra obtained with mitochondria from mammalian[27] and from plant[28] tissues. The spectrophotometric characterization of cytochrome c_1 is best done by extraction of cytochrome *c*. *C. fasciculata* mitochondria depleted of cytochrome c_{555} respired with succinate at a rate one-tenth that of unextracted mitochondria. The succinate reduced *minus*

oxidized difference spectrum of such a preparation is shown in Fig. 5A. The efficacy of *c* extraction can be assessed by the observation that the cytochrome $a+a_3$ complement was only partially reduced by succinate after incubation at room temperature for 15 min, which was ample time to attain anaerobiosis. In hypotonically treated but unextracted mitochondria all of the cytochrome $a+a_3$ was reduced in less than 1 min by succinate. The size of the α-band of cytochrome $a+a_3$ at 601 nm was comparable to the band at 590 nm. There was one at 556 nm which included the cytochrome *b* and part of cytochrome c_1. In the reduced *minus* oxidized spectrum shown in Fig. 5B with dithionite as reductant, there was a larger band at 557 nm comprised of *b* and c_1 cytochromes. The cytochromes $a+a_3$, were also reduced under these conditions. With ascorbate *plus* TMPD alone as substrate, some cytochrome *b* was also reduced in the cytochrome *c*-depleted mitochondria as shown by an absorbance peak at 426 nm seen in the spectrum of Fig. 6A. This cytochrome *b* contribution could be eliminated by prior reduction of both samples with succinate *plus* antimycin A. The experimental sample was then rendered anaerobic with ascorbate *plus* TMPD while the reference sample was oxygenated, and the difference spectrum seen in Fig. 6B was obtained. The α-bands of cytochromes $a+a_3$ were readily apparent,



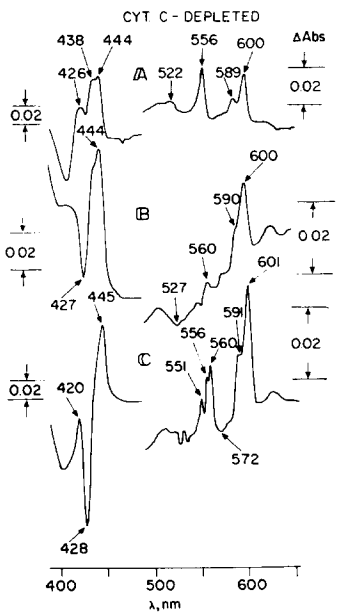


Fig. 5. Difference spectra obtained at 77 °K with cytochrome *c*-depleted mitochondrial preparations. (A) Mitochondrial suspension (Preparation 2; 7.2 mg protein/ml) reduced for 15 min with 10 mM succinate *minus* oxygenated reference. (B) As in A but $Na_2S_2O_4$ replaced succinate.

Fig. 6. Difference spectra at 77 °K with cytochrome *c*-depleted mitochondria reduced with ascorbate and TMPD in the presence and absence of antimycin A. (A) Mitochondrial suspension (Preparation 2; 7.2 mg protein/ml) reduced with 5 mM ascorbate *plus* 0.5 mM TMPD *minus* oxygenated reference. (B) As in A, but both samples contained 10 mM succinate *plus* 10 μg/ml antimycin. Ascorbate and TMPD were added to reduce the experimental sample; the reference sample was oxygenated before freezing. (C) As in B, but 0.1 mM $K_3Fe(CN)_6$ was added to the reference immediately before freezing.

but there was no significant absorption in the 550–560 nm region. If ferricyanide was added to the reference sample just before freezing, the spectrum of Fig. 6C was obtained. The Soret band was at 420 nm, and there were three maxima in the α region at 551, 556 and 560 nm. These spectra were obtained only when samples were frozen as soon as possible following addition of ferricyanide. When freezing was delayed by 1 min or more, the spectrum reverted to the type shown in Fig. 6B with no significant absorption in the 550–560 nm regions. This indicated that the pigment oxidized by ferricyanide was an active oxidation–reduction component since it could be reduced again by succinate through the antimycin A "leak" once the added ferricyanide had been completely reduced. This finding eliminated the possibility that the observed pigment might be a reduced hemoprotein present in the preparation but not intimately linked to the succinate respiratory chain. It also demonstrated that extraction of cytochrome c_{555} prevented the reoxidation of this cytochrome by cytochrome *a*, in accord with the results obtained by Wohlrab[29] with rat liver mitochondria depleted of cytochrome *c*. In addition, pyridine hemochromogen difference spectra ($Na_2S_2O_4$-reduced *minus* oxidized) of the cytochrome *c*-depleted mitochondria, after extraction of noncovalently-bound heme by the acid–acetone extraction method of Falk[30], exhibited an absorbance peak at 552–553 nm, consistent with the presence of a *c*-type cytochrome with a covalently bound heme group[30]. From these results, it seemed reasonable to conclude that the 551, 556–557 nm and 560 nm absorbance maxima seen in Fig. 6C were due to *C. fasciculata* cytochrome c_1.

DISCUSSION

Cytochrome $a+a_3$

Reduced *minus* oxidized difference spectra of isotonically and hypotonically treated mitochondria at 77 °K showed that cytochrome $a+a_3$ had two Soret maxima at 438 and 444 nm. Split Soret maxima with identical positions have been reported previously for both rat liver[20,21] and plant cytochrome $a+a_3$ (refs 17, 18). Whereas the 600 nm band of *Crithidia* cytochrome oxidase was usually found as a symmetrical peak in isotonically treated mitochondria, hypotonically treated preparations usually exhibited an additional 590 nm shoulder adjacent to the 600 nm peak. Such shoulders have also been reported in low temperature difference spectra of plant[31] and rat liver mitochondria[20,21], but only in the presence of KCN or CO. Neither of these substances were present in our preparations. The possibility that mitochondrial suspensions might have absorbed traces of CO was ruled out by bubbling pure O_2 for 5 min through mitochondrial suspensions illuminated with bright light from a tungsten source; there was no effect on the presence or relative size of the 590 nm shoulder. Since isotonically treated mitochondria lacked this absorption band while hypotonically treated mitochondria showed it, the possibilities exist that:

(1) the 590 nm shoulder was produced by some alteration of native cytochrome $a+a_3$ by exposure to hypotonic conditions; or

(2) that it was unmasked by removal of other pigments during hypotonic treatment.

The first possibility was placed in doubt by the lack of change in the Soret bands of cytochrome $a+a_3$ in the hypotonically treated mitochondria, and by the greater prominence of the 590 nm band in the reduced *minus* oxidized difference

spectra of cytochrome *c*-depleted mitochondria reduced with succinate compared to dithionite (Fig. 5). The second possibility, namely that the shoulder was rendered visible upon removal of interfering pigments during hypotonic shock, would be a reasonable explanation for its appearance, provided that one assumes that the difference spectrum of Fig. 5A shows the true α region spectrum of a_3 alone, which then would have two α bands at 590 nm and 601 nm, while the difference spectrum of Fig. 5B shows cytochrome $a+a_3$. Under the conditions of cytochrome *c* depletion, cytochrome a_3 of more positive midpoint potential[32] would be expected to become reduced in preference to cytochrome *a*. We tentatively suggest that the 590 nm band may be attributable to one of two α bands of cytochrome a_3 in these mitochondria.

Cytochrome b

There seemed to be but one spectrophotometrically detectable cytochrome *b* in mitochondria from *C. fasciculata*, as seen in the spectra of Figs 2A and 2C. A single, sharp maximum at 557 nm was observed, corresponding to a 4 nm shift to shorter wavelengths from the maximum observed at room temperature[1]. With succinate *plus* antimycin, no shoulder was seen at shorter wavelengths comparable to the one seen under similar conditions with yeast[33], plant[34], mammalian[35] and avian[36] mitochondria. Such a shoulder is diagnostic of reduced cytochrome b_T in vertebrate mitochondria which with cytochrome b_{561} or b_K comprises the dual *b* cytochrome components of these mitochondria. Based on the spectral evidence, therefore, mitochondria from *C. fasciculata* would seem to have but a single cytochrome *b*. Potentiometric measurements are required to confirm or deny this supposition and these are being undertaken. For the present, this cytochrome has been designated cytochrome b_{561}, the subscript corresponding to the α maximum observed at room temperature.

Cytochromes c and c_1

The most striking spectral property of cytochrome c_{555} found in this study was the failure of the α_1 maximum to shift to shorter wavelengths, while the cytochrome *b* α maximum shifted 4 nm closer to the cytochrome *c* maximum. Previous investigation indicated that *Crithidia* cytochrome c_{555} has an unusual α maximum at 553 nm for the reduced pyridine hemochromogen[9,10]. Recently, Pettigrew and Meyer[11] have obtained evidence that this may be due to the presence of but a single thioester linkage of the heme to the protein moiety. This alone, however, seems insufficient to account for the lack of a spectral shift at 77 °K since *Euglena* cytochrome c_{558}, which also has a 553 nm maximum for the pyridine hemochromogen and was also suggested to contain a single thioester heme–protein linkage, is shifted to 555.6 nm at low temperatures[37]. It was noted that at low temperatures the wavelength maxima of the complex β-band substructure of the *Euglena* and *Crithidia* hemoproteins were virtually identical. (In this regard, it should be noted that euglenids were suggested to have been progenitors of the *Trypanosomatidae*[38].) Since the lack of a shift was also observed in the purified solubilized cytochrome, the membrane environment cannot be held responsible for this spectral property. The reasons for the stability of this maximum remain obscure at present.

The reduced *minus* oxidized difference spectrum of cytochrome c_1 seen in Fig. 6C was complex, but no more so than the spectrum of cytochrome c_1 purified

from beef heart by Yu *et al.*[39]. The oxidized cytochrome from beef heart was relatively featureless in the spectrum obtained at 77 °K, but the reduced cytochrome had three maxima at 549, 550, and 552.5 nm so identified by Yu *et al.*[39] and a shoulder on the long wavelength side not identified as to wavelength. This makes more plausible the idea that the three maxima at 551, 556 and 560 nm are all attributable to cytochrome c_1 in *C. fasciculata*. The α_1 maximum of this component is observed at 560 nm at 77 °K, a 7.5 nm shift to the red compared to the α_1 maximum of mammalian cytochrome c_1. A similar shift of 7 nm is observed for the α_1 maximum of 555 nm at 77 °K for the cytochrome c_{555} of this organism compared to that mammalian cytochrome *c* at 548 nm[22].

The results of this study indicate that mitochondria of *C. fasciculata* have a respiratory chain remarkably similar in its cytochrome components to the respiratory chain of the higher animals. The sequence:

$$\text{Succinate} \rightarrow\rightarrow b_{561} \rightarrow c_1 \rightarrow c_{555} \rightarrow a \rightarrow a_3 \rightarrow O_2$$

was deduced from these results as well as previous work with respiratory inhibitors[1–4]. In particular, the role of the readily solubilized cytochrome c_{555} in equilibrating the redox state of cytochromes c_1 and *a*, as shown for mammalian cytochrome *c* by Wohlrab, was evident from the experiment in Fig. 5A. The previous identification of two coupling sites in this region[14] was in accord with the results obtained with animal mitochondria, and indicates that mitochondria from this protozoan strongly resemble those from metazoa.

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