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PROPERTIES OF *ASCARIS* MUSCLE MITOCHONDRIA

1. CYTOCHROMES

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SUMMARY

1. The cytochrome system in *Ascaris* muscle mitochondria was further characterized using purer preparations.

2. Difference spectra (at 22 °C and −196 °C) of the mitochondrial preparations using succinate and ascorbate plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine show that *Ascaris* muscle mitochondria contain cytochromes c_1, c and aa_3, and also at least three *b*-type cytochromes. The *b*-type cytochrome is the predominant component.

3. Cytochrome *c* and *Ascaris* cytochrome *b*-560 can be extracted from the mitochondrial preparations with 150 mM KCl, leaving the membrane-bound cytochromes c_1, *b* and aa_3 in the KCl residue.

INTRODUCTION

The presence of functional cytochromes in several large intestinal [1–5] and other parasites [6] living in an environment of low oxygen tension is well established. However, not all the mitochondrial cytochromes in the muscle of *Ascaris lumbricoides*, the intestinal roundworm of pigs, have been characterized because of the difficulty in satisfactorily obtaining spectra both at room (22 °C) and at liquid nitrogen (−196 °C) temperature. For example, neither cytochrome c_1 nor the reduced α-peak of cytochrome aa_3 have been observed in all the mitochondrial preparations so far reported [4, 5, 7–9].

This paper describes further studies of the cytochromes in *Ascaris* muscle mitochondria and also shows that these mitochondria have cytochromes aa_3, c_1, c and a complex *b*-type cytochrome.

MATERIALS AND METHODS

Antimycin A (Type III), sodium succinate and rotenone were purchased from Sigma Chemical Corp.; sodium salt of L-ascorbate, EDTA, TMPD and CO from the British Drug Houses. All other reagents were of analytical grade. Crystalline *Bacillus*

Abbreviation: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

subtilis proteinase was obtained from Teikoku Chemical Co., Japan. *Ascaris* muscle mitochondria were isolated using *B. subtilis* proteinase [10].

The difference spectra (at 22 °C and at −196 °C) were recorded with the Aminco-Chance Dual-wavelength/split-beam spectrophotometer using 10 and 2 mm light-path cells, respectively. The mitochondria were suspended in the reaction medium (pH 7.2) containing 1.0 mM EDTA, 30.0 mM KCl, 6.0 mM $MgCl_2$, 75.0 mM sucrose and 20.0 mM KH_2PO_4.

The kinetic reductions of the cytochromes were measured using the following wavelength pairs: 561-575 nm for cytochrome *b*, 550-540 nm for cytochromes $c+c_1$ and 445-465 nm for cytochrome $a_3(a)$. The percentage reduction of the aerobic steady state of the cytochromes was estimated by assuming a 100 % reduction with dithionite.

Cytochrome c_1 and other membrane-bound cytochromes were detected in the $20\,000 \times g$ mitochondrial residue after the mitochondria had been extracted with 150 mM KCl by rapid freezing of the mitochondrial suspension in liquid nitrogen followed by thawing of the frozen samples in warm water.

The approximate concentrations of the cytochromes were calculated from difference spectra recorded at 22 °C and −196 °C. The following wavelength pairs and difference millimolar extinction coefficients were employed: cytochrome aa_3

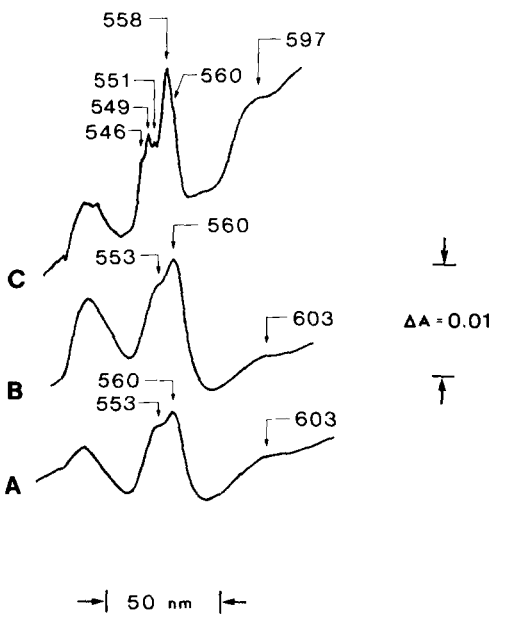

Fig. 1. Difference spectra showing the presence of cytochromes in *Ascaris* muscle mitochondria. Trace A: Difference spectrum [(CN^- + rotenone + succinate + ascorbate + TMPD) minus (oxidized)] recorded at 22 °C. Trace B: Difference spectrum (dithionite minus oxidized) recorded at 22 °C. Trace C: Same as Trace A but recorded at −196 °C. Final concn: CN^-, 3.0 mM; rotenone, 1.0 μM; succinate, 10.0 mM; ascorbate, 4.0 mM; TMPD, 0.4 mM; dithionite, 1.0 mg. The reaction medium (pH 7.4) contained 1.0 mM EDTA, 30.0 mM KCl, 6.0 mM $MgCl_2$, 75.0 mM sucrose and 20.0 mM KH_2PO_4. Both the sample and reference cuvettes contained 2.7 ml mitochondrial suspension for spectra recorded at 22 °C and 1.0 ml for spectra recorded at −196 °C. Protein concn: 2.44 mg per ml.

(603-630 nm), 13.1 [11]; cytochrome *b* (560-575 nm), 22.0 [12]; cytochrome *c* (549-538 nm from difference spectra at −196 °C), 18.9 [13]; cytochrome c_1 (551-538 nm from difference spectra at −196 °C), 17.1 [14]; cytochrome *o* (417-432 nm), 80.0 [15]. The latter was estimated as the cytochrome *o* · CO complex from the CO difference spectrum at 22 °C.

Protein was determined with the Folin phenol reagent [16] using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Cytochromes in Ascaris muscle mitochondria

Substrates, in the presence of CN^-, reduced a high percentage of the *b*, *c* and *a*-type cytochromes in *Ascaris* muscle mitochondria. The difference spectrum (Fig. 1) recorded at 22 °C of the substrate (Trace A) and of the dithionite (Trace B) reducible cytochromes shows that approximately 73, 83 and 100 % of the *b*, *c* and *a*-type cytochromes were reduced by the combined substrates, succinate and ascorbate plus TMPD. The data (Trace C) suggest the presence of cytochrome c_1 (551 nm), cytochrome *c* (549 and 546 nm), possibly two *b*-type cytochromes (560 and 558 nm), and cytochrome aa_3 (603 nm in Traces A and B; 597 nm in Trace C). The reduced α-peak of cytochrome aa_3 had not been observed in previous reports [4, 5, 7–9] even with difference spectra recorded at −196 °C. Cytochrome c_1 was also not detected in previous mitochondrial preparations [4, 5, 7–9] and was suggested to be missing in *Ascaris* muscle mitochondria [4, 5].

Ascorbate plus TMPD, in the presence of CN^-, could reduce cytochrome

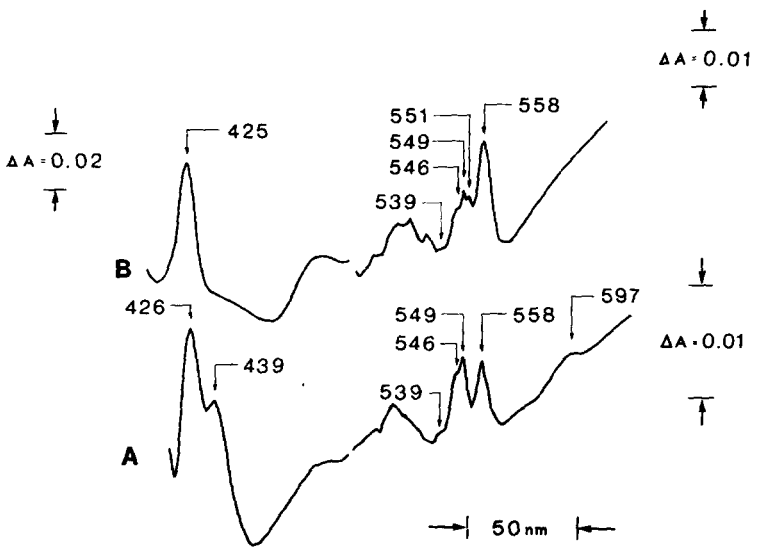

Fig. 2. Difference spectra (−196 °C) showing the ascorbate plus TMPD-reducible and non-reducible cytochromes in *Ascaris* muscle mitochondria. Trace A: Difference spectrum [(CN^- + ascorbate + TMPD) minus (oxidized)] recorded 4 min after reduction. Trace B: Difference spectrum [(CN^- + succinate) minus (CN^- + ascorbate + TMPD)] recorded 4 min after reduction. Other experimental details are given in the legend to Fig. 1. Protein concn: 5.85 mg per ml.

aa_3 (597 and 439 nm), cytochrome *b* (558 nm) and cytochrome *c* (549, 546 and 539 nm) but appeared not to reduce cytochrome c_1 (551-552 nm). Furthermore, only a partial reduction of both the cytochromes *b* and *c* was obtained with ascorbate plus TMPD. The difference spectrum (Fig. 2, Trace B) recorded between ascorbate plus TMPD and succinate (sample cuvette) and ascorbate plus TMPD (reference cuvette) shows that a further reduction was obtained with both the *b*- (558 nm) and the *c*- (551 nm for cytochrome c_1, and 549, 546 and 539 nm for cytochrome *c*) type cytochromes, but not with cytochrome aa_3 by succinate.

The spectral evidence (Fig. 1, Trace C and Fig. 2, Trace B) suggests that *Ascaris* muscle mitochondria contained cytochrome c_1, and that this component could be reduced by succinate (Fig. 2, Trace B). The presence of cytochrome c_1 is conclusively demonstrated in Fig. 3. The difference spectrum of the KCl residue (Trace A) shows the membrane-bound cytochrome c_1 (552 nm), cytochrome *b* (559 nm) and cytochrome aa_3 (603 nm). The KCl extract (Trace B), on the other hand, contained cytochrome *c* (549 and 546 nm) and the novel *Ascaris* cytochrome *b*-560 [17], the latter component being represented by the peaks at 552 and 559 nm. Both of these cytochromes had been separated by chromatography with carboxyl methylcellulose (CM-52) [17]. No CO-reactive pigment was observed in the KC-extract.

The CO difference spectrum (Fig. 4) shows the presence of two CO-reactive cytochromes in *Ascaris* muscle mitochondria. Cytochrome $a_3 \cdot$ CO complex is repre-

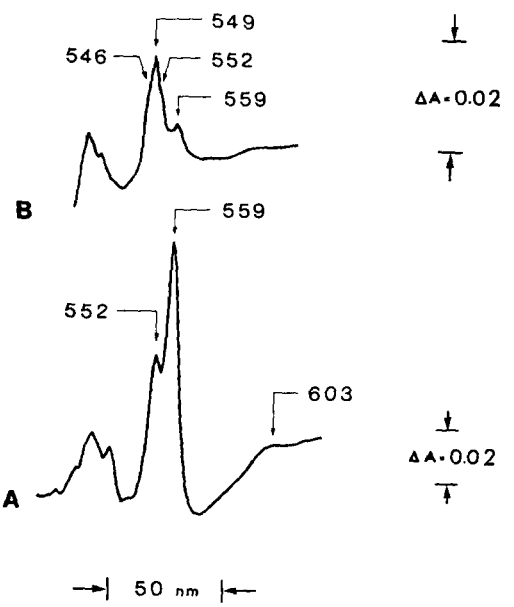

Fig. 3. Difference spectra (−196 °C) showing the soluble and the membrane-bound cytochromes of *Ascaris* muscle mitochondria. Trace A: Difference spectrum (dithionite-reduced minus ferricyanide-oxidized) of the mitochondrial residue after KCl extraction. Protein concn: 11.3 mg per ml. Trace B: Difference spectrum (dithionite-reduced minus ferricyanide-oxidized) of the concentrated KCl extract of *Ascaris* muscle mitochondria. The KCl extract was concentrated by ultra-filtration at 0 °C with the Amicon cell (10 ml capacity) with a PM-10 membrane. Protein concn: 9.1 mg per ml.

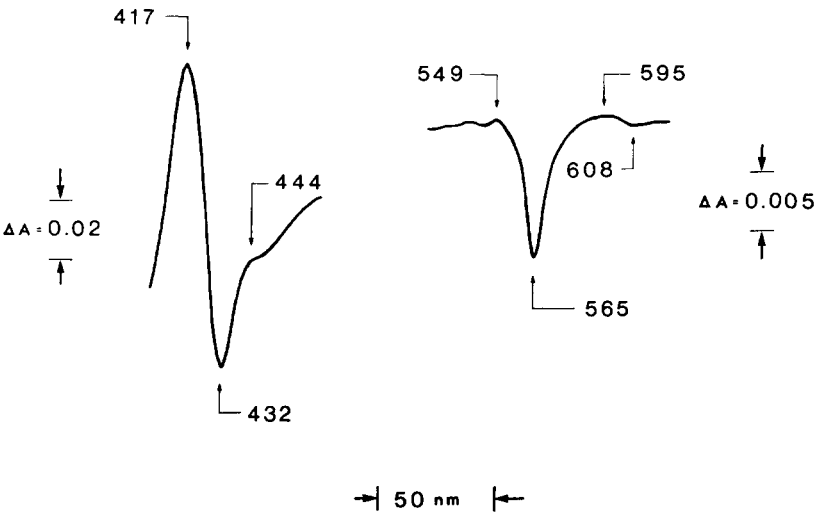

Fig. 4. CO difference spectrum (dithionite+CO minus dithionite) of *Ascaris* muscle mitochondria. Nitrite (1 mg) was added to the mitochondrial suspension in both cuvettes to eliminate the possible contribution of haemoglobin, if any, to the difference spectrum. Protein concn: 4 mg per ml.

sented by the broad absorption maximum (partly due to the absorption of cytochrome *o* · CO complex) at about 595 nm (sample cuvette) and the trough at about 608 nm was contributed by reduced cytochrome a_3 (reference cuvette). Cytochrome a_3 was also previously demonstrated to be present by the photochemical action spectrum of the CO-inhibited respiration of *Ascaris* muscle mitochondria [5]. The absorption maximum at about 444 nm was probably due to cytochrome *a*. Cytochrome *o* · CO complex is represented by the absorptions peaks at 417 and 549 nm (sample cuvette) and the troughs at 432 and 565 nm, the reduced form of cytochrome *o* (reference cuvette). The addition of nitrite to the mitochondrial suspensions was to eliminate the possible contribution of haemoglobin, if any, to the CO difference spectrum.

Table I illustrates the approximate concentrations of some of the cytochromes in *Ascaris* muscle mitochondria. The contents of all the cytochromes were much higher than those values previously reported [5] because of the purer mitochondrial

TABLE I

CONCENTRATION OF CYTOCHROMES IN *ASCARIS* MUSCLE MITOCHONDRIA

The concentration of cytochrome $a+a_3$ and cytochrome b_{560} was estimated from difference spectrum recorded at 22 °C, and that of cytochrome *c* and cytochrome c_1 from difference spectrum recorded at −196 °C. The values were calculated from data obtained from two different mitochondrial preparations. The CO-reactive cytochrome *b* was calculated from the CO difference spectrum recorded at 22 °C (Fig. 4). The soluble *Ascaris* cytochrome *b*-560 [17] was not estimated.

Cytochromes	Concn (nmol per mg protein)
$a+a_3$	0.037
b_{560}	0.163
b (CO-reactive)	0.120
c_1	0.120
c	0.078

preparations used. The data show that the *b*-type cytochromes were the major component, and cytochrome aa_3 the minor component in *Ascaris* muscle mitochondria. *Ascaris* cytochrome *b*-560 [17], a mitochondrial haemoprotein (Cheah, K. S., in preparation), was difficult to estimate accurately in the mitochondria by difference spectroscopy.

Kinetic studies of substrate-reducible cytochromes

CO inhibited *Ascaris* mitochondrial respiration [5], and was thus expected to have some effect on the aerobic steady state reduction of the *b* and *b*-type cytochromes in these mitochondria. The effect of CO on the aerobic steady state reduction of cytochromes *b*, *c* and c_1 (Table II) shows that CO increased the reduction of these cytochromes. The reduction of the *b*-type cytochromes was least affected when compared with the reduction of cytochromes $c+c_1$. This was due to the presence of cytochromes *o* (Fig. 4) causing a decrease in the absorbance in the wavelength pair (561-575 nm) selected for cytochrome *b*.

TABLE II

EFFECT OF CO ON THE AEROBIC STEADY STATE REDUCTION OF CYTOCHROMES IN *ASCARIS* MUSCLE MITOCHONDRIA

The effect of CO on the aerobic steady state reduction was assayed using the following wavelength pairs: *b*-type cytochrome, 561-575 nm; cytochromes $c+c_1$, 550-540 nm. The reaction medium (see Materials and Methods) containing approximately 4 mg protein from *Ascaris* muscle mitochondria was saturated with CO to give a final concentration of about 1 mM. Reaction was initiated by adding succinate (10 mM) to the mitochondrial suspension after the addition of rotenone (1 μM). The control experiments were carried out exactly as the test samples except that the reaction medium contained no CO. The percentage reduction was estimated by assuming that dithionite gave a 100 % reduction with all the cytochromes in *Ascaris* muscle mitochondria. Total volume, 2.80 ml; temperature, 22 °C. –, not tested.

Additions	Aerobic steady state reduction (%) of cytochromes		
	b	$c+c_1$	a_3
None	25	40	10
CO	33	52	–

CN^-, like CO, also affected the extent of reduction of the aerobic steady state of the cytochromes in *Ascaris* muscle mitochondria. Addition of CN^- (3 mM) increased the aerobic steady state reduction of cytochrome $a_3(a)$ by about 1-fold (Fig. 5). The marked enhanced reduction following the subsequent addition of dithionite was due to the predominant contributions of the reduced Soret bands of the *b*- and *c*-type cytochromes. This was supported by the fast reduction observed with dithionite, since the cyanide-bound cytochrome a_3 would be reduced only very slowly under these conditions. Thus, the increase from 11 to 22 % (Fig. 5), assuming dithionite giving a 100 % reduction, did not represent the true increase in the percentage reduction of the aerobic steady state of cytochrome $a_3(a)$ by CN^-. Similar concentration of CN^- also increased the extent of the aerobic steady state reduction of cytochrome *b* and cytochromes $c+c_1$ by 10 and 21 %, respectively.

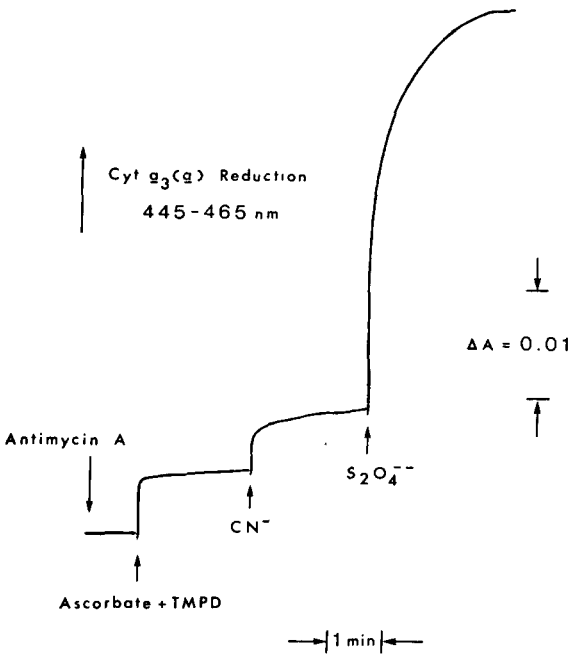

Fig. 5. Effect of CN^- on the aerobic state reduction steady of cytochrome $a_3(a)$ of *Ascaris* muscle mitochondria. The reaction medium (pH 7.2) contained 1.0 mM EDTA, 30.0 mM KCl, 6.0 mM $MgCl_2$, 75.0 mM sucrose and 20 mM KH_2PO_4. Final concn: Antimycin A, 0.5 μg per mg protein; ascorbate, 4.0 mM; TMPD, 0.4 mM; CN^-, 3.0 mM; dithionite, 1.0 mg. Protein concn: 5.9 mg per ml.

The present data of *Ascaris* muscle mitochondria are complementary to earlier reports [4, 5, 7–9] and further elaborates by showing the existence of cytochrome c_1, cytochrome *a* and the complex *b*-type cytochrome. *Ascaris* muscle mitochondria, like mitochondria from other parasites [1–3, 6] living in an environment of low oxygen tension, thus have a branched electron transport chain system with two CO-reactive cytochromes, i.e. cytochrome a_3 and cytochrome *o*. With all these parasites, cytochrome a_3 is the minor CO-reactive component [1–3, 6]. The *b*-type cytochrome is the major component in the respiratory chain system of *Ascaris* muscle mitochondria. The *b*-type cytochrome includes the CO-reactive component and the soluble *Ascaris* cytochrome *b*-560 [17], both of which have not been observed in mammalian mitochondria.

After the submission of this paper, Hayashi et al. [18] reported that *Ascaris* muscle mitochondria contained at least four cytochromes: a double-peak *b*-type cytochrome, a c_1-like cytochrome, cytochrome *c* and a small amount of cytochrome aa_3.

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