Oxidative activities in mitochondria-like particles from *Setaria digitata*, a filarial parasite

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The oxidative metabolic potential of *Setaria digitata*, a filarial parasite found in the intraperitoneal cavity of cattle, was investigated. These worms showed active wriggling movements which were not affected by respiratory poisons such as cyanide, rotenone and malonate. They also possessed cyanide-insensitive and glucose-independent oxygen consumption pathways. By differential centrifugation of sucrose homogenates, a fraction containing mitochondria-like particles was obtained in which the activity of the marker enzyme, succinate dehydrogenase, was recovered. This fraction catalysed succinate- and NADH-dependent reduction of both cytochrome *c* and dyes. Oxygen uptake found with succinate, NADH and ascorbate as substrates was not sensitive to cyanide. Cytochromes could not be detected in either this fraction or homogenates of the worms. H_2O_2 generation with a number of substrates and lipid peroxidation by measuring malondialdehyde formed as well as by accompanying oxygen uptake were demonstrated in the mitochondria-like particles. A lipid quinone, possibly with a short side chain and related to ubiquinone, was detected in the worms. The results suggested the existence of two cyanide-insensitive oxygen-consuming reactions in *Setaria*: one respiratory substrate-independent lipid peroxidation, and a second substrate-dependent reaction that requires an auto-oxidizable quinone but not a cytochrome system.

INTRODUCTION

Complete oxidation of substrates to CO_2 and water does not occur in parasitic helminths, which indicates little or no terminal oxidation (Barrett, 1976). They accumulate organic end products. At the same time oxygen uptake is known in some parasite systems (Saz, 1981). Nematodes such as *Ascaris lumbricoides* and cestodes such as *Hymenolepis diminuta*, *H. nana* and *Taenia crassiceps* require no oxygen in their energy-yielding pathways and appear to survive equally well either anaerobically or aerobically. Some helminths are obligate aerobes, e.g. the adult stages of the filarial worm *Litomosoides carinii* which resides in the pleural cavity of some rodents and the nematode of the rat intestine *Nippostrongylus brasiliensis* (see Cheah, 1976, for a review). Some parasites such as microfilariae of *Litomosoides carinii*, *Brugia pahangi* and *Dipetalonema vitae* at larval stages require oxygen for motility, but can survive under anaerobic conditions (Wang & Saz, 1974). Most adult filarial parasites studied such as *B. pahangi*, *D. vitae* and *Dirofilaria uniformis* appear to be homolactate fermenters and depend on energy derived from the oxidative breakdown of pyruvate to acetate and CO_2 . Pyruvate is decarboxylated and only the carboxyl carbon gives rise to respiratory CO_2 in these systems. From this evidence, it was speculated that oxygen may be required only for the single oxidation of pyruvate to acetate and CO_2 and that the single oxidation might provide additional energy for survival for the parasite (see Barrett, 1976, and Saz, 1981, for full discussion).

The requirement of oxygen for survival suggested a role for the mitochondrial systems of these parasites despite their production of essentially anaerobic metabolic end-products. The cytochrome system, for instance,

had been reported to be different from the classical mammalian type. Muscle and reproductive tissues of *Ascaris* and *L. carinii* had no measurable cytochrome *c* or cytochrome oxidase (Bueding & Charms, 1952). *Ascaris* was later reinvestigated using sensitive techniques and shown to contain the multiple terminal oxidases cytochrome a_3 , cytochrome *o* and cytochrome a_1 (Cheah, 1976). It was also shown to contain systems of cyanide-sensitive and cyanide-insensitive oxygen uptake (Cheah, 1976).

Wuchereria bancrofti and *B. malayi* are two parasites responsible for filariasis in many parts of the world. *Setaria digitata*, located in the peritoneal cavity of cattle, is a filarial parasite reported to be similar to *W. bancrofti* and *B. malayi*, especially in the response to the drug diethylcarbamazine citrate. Our studies have shown that *Setaria* can tolerate high concentrations of cyanide. This prompted a detailed study on the nature of the mitochondrial system of *S. digitata*. The work presented here forms part of a study undertaken to elucidate the oxidative metabolic potential of the mitochondrial system of this filarial parasite.

MATERIALS AND METHODS

Handling of parasites

The filarial parasite, *S. digitata*, used for the study was collected from local slaughterhouses. The worms, located in the peritoneal cavity of the cattle, were collected, immediately after opening the abdomen, in modified Tyrode solution (NaCl, 0.8%; KCl, 0.02%; $CaCl_2$, 0.02%; $MgCl_2$, 0.01%; $NaHCO_3$, 0.015%; Na_2HPO_4 , 0.05%; glucose, 0.1%; pH adjusted to 7.4), brought to the laboratory and washed free of extraneous materials

Abbreviations used: MCP, mitochondria-like particles; TMPD, *NNN'*-tetramethyl-*p*-phenylenediamine.

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using the same salt solution. Worms of average length of about 5 cm and width of about 1 mm were selected in these experiments.

Studies with whole worms

For this study three worms, suspended in 100 ml of Tyrode solution, were used in each experiment and observations were made every hour for a period of 6 h. The testing was always done in amber-coloured bottles held under laboratory fluorescent light. Lids were removed for observing wriggling movements which occur as a reaction of the parasites to light. Peristaltic-type wriggling movements of the parasite of curvature from one side to the other of the median and back was scored as 1 and counts were made for 1 min on each organism at the periods indicated.

Oxygen uptake studies using whole worms

The worms for this purpose were removed before blotting and maintained at 37 °C in the medium. The same medium was also used in the Warburg flasks. Two worms were suspended in a total of 5 ml of medium for oxygen uptake studies.

Cell fractionation of *Setaria*

The weighed live worms were suspended in 0.25 M-sucrose containing 0.1% bovine serum albumin and 5 mM-EDTA (10 ml of solution per g wet weight of the worm) and the mixture was homogenized in a Potter-Elvehjem tissue homogenizer. The homogenate was centrifuged in a refrigerated Sorvall centrifuge. The pellet collected at 800 g was discarded and those collected at 8000 g, 12000 g and 16000 g were used in the first set of experiments to determine the distribution of oxidative activities in these particles. The pellet collected between 800 g and 12000 g was used in subsequent studies.

Assays

The activity of succinate dehydrogenase was estimated by the method of reduction of dichlorophenolindophenol measured by decrease in absorbance at 600 nm, in a Shimadzu spectrophotometer (Arrigoni & Singer, 1962). Protein estimation was carried out using Folin's reagent (Lowry *et al.*, 1951). Malondialdehyde formed was measured as thiobarbituric acid reacting material and the product measured at 535 nm for the assay of lipid peroxidation (Buege & Aust, 1978).

Oxygen uptake studies were carried out using the Warburg manometer (Umbreit *et al.*, 1972). Activities involving oxygen uptake were estimated using a Gilson K-ICT-C oxygraph with Clark-type oxygen electrode having a cell capacity of 1.4 ml. Protein used per experiment was about 0.5 mg with relevant substrates for the assay systems. Catalase activity was estimated as described by Lück (1963). Cytochrome *c* oxidoreductase activities using succinate and NADH were estimated by the change in absorbance at 550 nm (Tisdale, 1967). The decrease in fluorescence of scopoletin at 360 nm (excitation) and 465 nm (emission) was measured in a spectrofluorimeter for the estimation of H₂O₂ generation in the presence of horseradish peroxidase (Swaroop & Ramasarma, 1981). Mitochondrial preparations were scanned in a spectrophotometer from 650 to 500 nm for the detection of cytochromes (Rieske, 1967). Extraction and identification of the lipid quinones were carried out using the methods of saponification, fractionation of

unsaponifiable lipids on deactivated alumina columns (Ramasarma & Jayaraman, 1971) and examining the 5% diethyl ether/light petroleum fractions for spectra in the u.v. region before and after reduction with borohydride, and also by reverse phase paper chromatography (Lester & Ramasarma, 1959) followed by testing for quinone by Leucomethylene Blue spray (Linn *et al.*, 1959).

RESULTS

Effect of inhibitors on wriggling movements of *Setaria*

The *Setaria* worms have active wriggling movements in the salt medium used. The wriggling score was maintained constant at 10 for 6 h. The uncoupler, 2,4-dinitrophenol, the inhibitor of Na⁺ + K⁺-ATPase, vanadate (Cantley, 1977) and the inhibitor of H₂O₂ generation, *p*-coumarate (Swaroop & Ramasarma, 1981) all showed negligible effects. Of the compounds tested, only azide showed progressive damage, and by 6 h most worms became virtually inactive (Table 1). These results indicated that energy production by this parasitic organism must be obtained through cyanide-insensitive reactions.

Oxygen uptake by *Setaria*

The worms placed in the salt medium were able to consume oxygen as measured in shaken Warburg manometers. There was an initial lag in the rate of oxygen consumption. After 40 min constant rates were obtained (Fig. 1). Rates of O₂ consumed were 32 and 20 nmol/min per mg of protein in the control and glucose-supplemented flasks, respectively. That cyanide (30 mM) gave only 20% inhibition correlates with the observation of cyanide insensitivity of wriggling movements.

Preparation of mitochondria-like particles from *Setaria*

Adult live worms were washed well, blotted dry and homogenized in a medium (10 ml/g wet wt.) containing 0.25 M-sucrose, 0.1% bovine serum albumin and 5 mM-EDTA with pH adjusted to 7.0 prior to fractionation. The pellets obtained at 8000 g, 12000 g and 16000 g were

Table 1. Effect of inhibitors on wriggling movements of *Setaria*

Three active worms were placed in flasks containing 100 ml of a salt/glucose medium adjusted to pH 7.0, as described in the Materials and methods section. The average rate per min of wriggling scored by each movement across a median and back was recorded. The inhibitors were added at concentrations indicated and the medium was kept at 37 °C.

Addition	Concn. (mM)	Wriggling score at:		
		2 h	4 h	6 h
None	—	10	10	10
KCN	30	10	10	9
Rotenone	0.3	10	9	7
Malonate	25	10	10	7
Dinitrophenol	0.3	10	10	9
Vanadate	10	10	10	9
<i>p</i> -Coumarate	25	10	9	8

Fig. 1. Oxygen uptake by whole worms

The details of the medium and incubation are given in the Materials and methods section. Where mentioned, concentrations of glucose and KCN were 0.1% and 30 mM, respectively. Oxygen uptake was measured in a Warburg apparatus.

washed once with 0.25 M-sucrose and suspended in the same medium before analysis for protein and succinate dehydrogenase as the mitochondrial marker enzyme. Typical data from one of four experiments are given in Table 2. The specific activity of the enzyme varied in these fractions with the highest in the 12000 g fraction. Maximum amount of activity and protein were recovered in 8000 g fraction. In the subsequent experiments the fractions collected between 800 g and 12000 g were used for further characterization of oxidative activities. The yields of protein were about 5 mg/g wet wt. of worms and nearly 90% of succinate dehydrogenase activity was recovered in this 'mitochondria-like particles' (MLP) fraction (Table 2).

Oxidative activities in MLP

The MLP fractions also showed the activities of NADH-dependent reduction of ferricyanide, and succinate- and NADH-dependent reduction of cytochrome *c*. The relative rates of these activities are given in Table 3. With succinate the K_m value was found to be about 0.6 mM. Addition of antimycin A (10 µg/ml) inhibited by 60% the reduction of cytochrome *c* both by succinate and NADH. Addition of rotenone (10 µg/ml) inhibited by 75% the reduction of cytochrome *c* by NADH, but not by succinate.

When tested for oxygen uptake in an oxygraph, substrate-dependent activity was observed with succinate, NADH and ascorbate (+TPMD) (Table 3). These activities were found to be insensitive to addition of cyanide (2 mM). This is illustrated in Fig. 2 with ascorbate as the substrate. The oxygen uptake by MLP with ascorbate increased on addition of TPMD but addition of cyanide (2–6 mM) did not affect the rate. These results indicated that cytochrome oxidase was not involved. Oxidation of added reduced cytochrome *c* was obtained with MLP but this also was cyanide-insensitive (results not shown).

Table 2. Distribution of succinate dehydrogenase in subcellular fractions

The worms were homogenized in 0.25 M-sucrose containing 0.1% bovine serum albumin and 5 mM-EDTA, subcellular fractions were obtained by differential centrifugation, and the activity of succinate dehydrogenase was measured in each fraction, suspended in 0.25 M-sucrose.

Sub-cellular fraction	Protein (mg/g wet wt.)	Succinate dehydrogenase activity		
		(nmol/min per mg of protein)	(nmol/min per g wet wt.)	(%)
8000 g	3.7	45	166	58
12000 g	0.9	99	89	30
16000 g	0.6	60	36	12

Table 3. Some oxidative activities in MLP of *Setaria*

The measurements of the dehydrogenase and the reductase activities are described in the Materials and methods section. Oxygen uptake with the substrates mentioned was measured in an oxygraph, and these are insensitive to 2 mM cyanide. The rates indicated are dependent on addition of the respective substrates.

Activity tested	Test method	Activity (nmol/min per mg of protein)
Succinate dehydrogenase	Dichlorophenol-indophenol reduction	52 ± 8
NADH dehydrogenase	Ferricyanide reduction	85 ± 5
Succinate-cytochrome <i>c</i> reductase	Cytochrome <i>c</i> reduction	23 ± 2
NADH-cytochrome <i>c</i> reductase	Cytochrome <i>c</i> reduction	17 ± 2
Succinate oxidation	Oxygen uptake	18 ± 5
NADH oxidation	Oxygen uptake	8 ± 3
Ascorbate + TMPD oxidation	Oxygen uptake	33 ± 5

Cytochrome not detected in MLP

The above observations indicated that *a*-type cytochromes are not detectable. This was confirmed by spectroscopic examination. Both MLP fractions and homogenates of *Setaria* (1.5–5.5 mg of protein) were tested for the presence of cytochromes by the pyridine hemochromogen method. All the samples tested did not show any absorption or characteristic spectrum in the range 500–650 nm (results not shown). This organism and the MLP fraction obtained therefrom seem to have no detectable cytochrome system.

H₂O₂ generation by MLP

Production of H₂O₂, measured by decrease of scopoletin fluorescence in the presence of horseradish peroxidase, was detected in MLP fractions oxidizing

Fig. 2. Cyanide insensitivity of ascorbate (+ TMPD) oxidation by MLP

Oxygen uptake was measured in an oxygraph in a medium (1.4 ml) containing 0.05 M-Tris buffer (pH 7.4), with additions of MLP (0.3 mg of protein), ascorbate (1 mM), TMPD (0.1 mM) and KCN (2 mM each addition, increasing to 6 mM final) in sequence.

Table 4. H_2O_2 generation in MLP fractions

The assay method for H_2O_2 is described in the Materials and methods section and essentially consists of measuring the decrease in scopoletin fluorescence on oxidation with H_2O_2 in the presence of horseradish peroxidase. Several MLP fractions were used for these experiments and one set of representative data with substrate concentration giving maximum rates is given.

Substrate	Concn. (mM)	Rate (pmol of H_2O_2 generated/min per mg of protein)
Succinate	5	650
Glutamate	10	340
α -Glycerophosphate	1	170
Pyruvate	10	180
Malate	10	90

succinate, glutamate, α -glycerophosphate, pyruvate or malate (Table 4). Succinate gave the highest rates and the apparent K_m value was found to be about 0.66 mM; that this was similar to that obtained with succinate dehydrogenase is taken to indicate that the same dehydrogenase was participating in both systems. This is in contrast to low substrate saturation for H_2O_2 generation obtained in animal tissue mitochondria (Swaroop & Ramasarma, 1981). The relative rates of H_2O_2 generation compared to dye reduction (dehydrogenase assay) were low, e.g., for the *Setaria* MLP fractions these were about 1:80. It was obvious from these results that, although the same substrates are used for these two activities, the H_2O_2 generation rates are too low to account for the high substrate-dependent oxygen uptake observed. A high activity of catalase (about 3.6 μ mol of H_2O_2 degraded/min per mg of protein) was detected in MLP fractions. Therefore, part of the H_2O_2 formed may have been degraded, although horseradish peroxidase could

measure H_2O_2 in presence of catalase in view of its high affinity for H_2O_2 .

Lipid peroxidation in MLP

Lipid peroxidation supported by NADPH and iron chelates occurring in microsomes of animal tissues produces malondialdehyde (Buege & Aust, 1978) and is accompanied by several-fold higher rates of oxygen uptake. Such a reaction was found to occur in mitochondria (Szabados *et al.*, 1987), and addition of Fe(III) gluconate alone was found to induce peroxidation of mitochondrial lipids (Masini *et al.*, 1985). We investigated whether *Setaria* MLP fraction possessed a capacity for lipid peroxidation, and also whether this was dependent on succinate. Prolonged incubation of MLP fractions for 90 min under conditions of oxygen uptake led to formation of malondialdehyde which was unaffected by adding cyanide or catalase, and increased nearly 2-fold in the presence of Fe^{2+} -ADP. In all cases, succinate was inhibitory (Table 5). Addition of Fe^{2+} -ADP (15 μ M each) by itself to the MLP fraction resulted in a rate of malondialdehyde production of 380 pmol/min per mg of protein and considerable oxygen uptake.

This effect of Fe^{2+} on oxygen uptake was observed in presence of succinate (Fig. 3) and the ratio of succinate-dependent oxygen uptake remained the same after the transient burst occurring in the presence of Fe^{2+} . The ratio of Fe^{2+} /O is about 2 for this oxygen burst. It therefore appears that this represents the oxidation of Fe^{2+} to Fe^{3+} . Succinate-dependent oxygen uptake also is not related to this Fe^{2+} effect, as it is unaffected on addition of EDTA (Fig. 3).

Identification of a lipid quinone

Ubiquinone is the link between dehydrogenases and cytochromes in animal tissue mitochondria (Crane, 1977) and also is a site of reduction to H_2O_2 (Boveris *et al.*, 1976). Therefore it was of interest to find out whether this lipid quinone is present in *Setaria*. Large quantities of worms were pooled and processed for

Table 5. Lipid peroxidation in MLP fractions

The reaction mixture consisted of 0.05 M-Tris buffer, pH 7.4, 0.9% NaCl and 2.5 mg of MLP protein in a total volume of 3 ml. After incubation at 37 °C for 90 min, 1 ml of 20% (w/v) trichloroacetic acid was added and an aliquot of the supernatant was tested for thiobarbituric acid-reacting material. The values represent the total product formed per mg of protein.

Additions	Peroxidation (pmol of malondialdehyde per mg of protein)	
	No succinate	+ Succinate (5 mM)
None	2900	2380
Cyanide (2 mM)	2380	2000
Catalase (10 µg/ml)	2570	1720
Fe ²⁺ -ADP (15 µM, 15 µM)	5350	3630

Fig. 3. Oxygen uptake dependent on succinate and Fe²⁺

Oxygen uptake was measured in an oxygraph in a medium (1.4 ml) containing 0.05 M-Tris buffer (pH 7.4) with additions of MLP (0.86 mg of protein), succinate (5 mM), FeSO₄ (100 µM) and EDTA (1 mM) in sequence.

isolation of ubiquinone by saponification, extraction of unsaponifiable lipids and fractionation of these on deactivated alumina column. The 5% diethyl ether fraction was dried and dissolved in ethanol. The spectrum of this fraction showed a broad absorption in the range 260–280 nm and a decrease in absorbance over this range of wavelengths on addition of borohydride. The fraction on reverse phase paper chromatography showed a positive quinone spot of Leucomethylene Blue spray at R_F about 0.9. These properties of extractability into unsaponifiable lipids, recovery in 5% diethyl ether on fractionation on deactivated alumina, spectral characteristics with and without addition of borohydride and the quinone-positive spot on reverse-phase paper chromatography point to the presence of ubiquinone. The high R_F value of 0.9 relative to 0.25 for Q_{10} indicates that it has a short chain.

DISCUSSION

The vigorous wriggling movements of this parasite require a high rate of energy transduction. Particles

containing succinate dehydrogenase corresponding to mitochondria have been isolated by differential centrifugation; other tricarboxylic acid cycle enzymes also occur in these particles (our unpublished results), but cytochromes could not be detected. For this reason we tentatively labelled these as mitochondria-like particles (MLP). It thus appears these MLP are deficient in some respiratory chain components.

It is of interest to explain the presence of substrate-dependent cyanide-insensitive oxygen uptake. Electron transport from the flavoprotein dehydrogenases occurs to a common electron transfer component equivalent to ubiquinone represented by the lipid quinone isolated from *Setaria*. We suspect that the high mobility of this quinone on reverse phase paper chromatography, greater than even Q_6 , with six isoprene units in its side chain, is likely to have a very short side chain. Such lower homologues with three to five isoprene units are now known in some fungi (Olszak, 1973). Quinones without or with small side chains are auto-oxidizable more readily than natural lipid quinones (Ramasarma & Lester, 1960). This type of electron transfer to oxygen explains lack of sensitivity to cyanide and independence of a terminal cytochrome oxidase. Stimulation of cyanide-insensitive respiration by menadione in rat liver mitochondria may be an analogous process (Meera Rau & Ramasarma, 1979).

Such a reaction would be expected to yield H_2O_2 as the product of reduction of oxygen, and the presence of catalase ensures detoxification of H_2O_2 and prevention of formation of H_2O_2 -derived toxic free radicals (Halliwell & Gutteridge, 1986). That azide is more toxic to this organism than other respiratory poisons (e.g. cyanide), suggests that azide inhibits catalase and thus allows H_2O_2 concentration to increase to toxic levels. Although we could demonstrate H_2O_2 generation with all substrates tested, the rates were small presumably because of the abundance of catalase in MLP.

The other possibility for high rates of oxygen uptake is through peroxidation of lipids. In *Trichinella* larvae, lipid content decreased in presence of oxygen, possibly due to its oxidation (von Brand *et al.*, 1952). Lipid peroxidation, measured by malondialdehyde formation, occurred in MLP of *Setaria* and this activity was insensitive to both cyanide and catalase. Rapid oxygen uptake accompanied lipid peroxidation when Fe^{2+} , but not Fe^{3+} , was added to rat liver mitochondria in the absence of any substrate (our unpublished results). A similar activity was also found in *Setaria* MLP and its involvement in lipid peroxidation and possible relationship to the wriggling movements merits further studies.

There are thus at least two major cyanide-insensitive oxygen-consuming reactions occurring in *Setaria* MLP. The consumption of oxygen by the worms, represented in Fig. 1, which occurs in the absence of glucose is most probably due to peroxidation of lipids. Since this activity occurs in the absence of respiratory substrates, the oxygen uptake that is obtained in presence of succinate and other substrates uses another pathway. Our results favour the view that an auto-oxidizable electron transfer component will act as the electron donor to oxygen and the lipid quinone detected in *Setaria* is likely to serve this function. It will be of interest to study how these reactions are integrated into the metabolic potential of this parasite for its survival.

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