The role of succinate in the respiratory chain of *Trypanosoma brucei* procyclic trypomastigotes

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Trypanosoma brucei procyclic trypomastigotes were made permeable by using digitonin (0–70 µg/mg of protein). This procedure allowed exposure of coupled mitochondria to different substrates. Only succinate and glycerol phosphate (but not NADH-dependent substrates) were capable of stimulating oxygen consumption. Fluorescence studies on intact cells indicated that addition of succinate stimulates NAD(P)H oxidation, contrary to what happens in mammalian mitochondria. Addition of malonate, an inhibitor of succinate dehydrogenase, stimulated NAD(P)H reduction. Malonate also inhibited intact-cell respiration and motility, both of which were restored by further addition of succinate. Experiments carried out with isolated mitochondrial membranes showed that, although the electron transfer from succinate to cytochrome *c* was inhibitable by antimycin, NADH–cytochrome *c* reductase was antimycin-insensitive. We postulate that the NADH–ubiquinone segment of the respiratory chain is replaced by NADH–fumarate reductase, which reoxidizes the mitochondrial NADH and in turn generates succinate for the respiratory chain. This hypothesis is further supported by the inhibitory effect on cell growth and respiration of 3-methoxyphenylacetic acid, an inhibitor of the NADH–fumarate reductase of *T. brucei*.

INTRODUCTION

Energy metabolism in the genus *Trypanosoma* differs from that of mammalian cells in several respects, including the compartmentalization of glycolytic enzymes in a unique organelle (glycosome), a rotenone-insensitive respiratory chain, and the presence of an alternative KCN-insensitive respiration (for reviews see Hill, 1976; Fairlamb & Opperdoes, 1986). The proportion of KCN-resistant respiration varies from species to species and also in the same species when compared among different stages during the life cycle of the trypanosome. For example, in *T. brucei* bloodstream trypomastigotes (which do not have cytochromes), 100% of the respiration is KCN-resistant, whereas in *T. brucei* procyclic trypomastigotes (where cytochromes are present) only 5–10% of the total respiration is insensitive to KCN. Another interesting difference is that glycolysis in *T. brucei* glycosomes leads to the formation of malate from phosphoenolpyruvate, since the enzyme pyruvate kinase is missing from these organisms (Hart *et al.*, 1984; Fairlamb & Opperdoes, 1986). Malate in turn may enter the mitochondria, being converted into either oxaloacetate (producing NADH) or into fumarate, through the enzyme fumarate hydratase (Fairlamb & Opperdoes, 1986). Thus, malate may be utilized either to reduce NAD⁺ to NADH or to oxidize indirectly through the NADH–fumarate reductase.

Previous studies indicated that NADH-dependent oxygen consumption in mitochondrial membranes isolated from *T. brucei* procyclic trypomastigotes was due to the generation of H₂O₂, rather than to the complete reduction of oxygen at the cytochrome *c* oxidase level (Turrens, 1987). Because it is likely that isolation of subcellular membranes may have altered the integrity of the respiratory chain, we decided to evaluate the capability of the respiratory chain to oxidize NADH through

different experiments in which the mitochondrial integrity was preserved. Our results suggest that the NADH–ubiquinone segment in the respiratory chain of *T. brucei* procyclic trypomastigotes is not active, and it is instead replaced by the direct generation of succinate from fumarate reduction, which in turn enters the respiratory chain, regenerating fumarate.

MATERIALS AND METHODS

T. brucei procyclic trypomastigotes were cultured in a semi-defined medium, as described by Cunningham (1977). The cells were collected in a medium containing 0.23 M-mannitol, 0.07 M-sucrose, 0.1 mM-EDTA, 0.2% bovine serum albumin and 5 mM-Tris/HCl (pH 7.4).

For the experiments using digitonin-permeabilized cells, the cells were incubated at 30 °C for 10 min with several concentrations of digitonin. After that period of time, the cells were diluted 20-fold and immediately used for monitoring oxygen consumption. Mitochondrial fragments were isolated by freezing and thawing cells, followed by a single passage through a 27-gauge needle and centrifugation in a table-top Eppendorf microfuge. The membranes were then resuspended in the same buffer.

All the spectrophotometric determinations were carried out in a Perkin–Elmer Lambda 3A spectrophotometer. NADH–fumarate reductase activity was assayed as previously described (Klein *et al.*, 1975), by determining the fumarate-dependent rate of NADH oxidation at 340 nm. For these experiments, approx. 0.1 mg of protein and 0.1 mM-NADH were added to both sample and reference cuvettes. The reaction was started by addition of 2 mM-fumarate to the sample cuvette. Since the incubation of membranes with NADH in the absence of fumarate leads to the formation of H₂O₂ (Turrens, 1987), both cuvettes were also

supplemented with 200 units of catalase (Boehringer Mannheim). Succinate- and NADH-cytochrome *c* reductase activities were monitored at 550 nm by using 20 μ M-cytochrome *c* and either 5 mM-succinate or 0.2 mM-NADH in 50 mM-phosphate buffer (pH 7.4).

The fluorescence experiments were performed in a Perkin-Elmer model LS-5 fluorescence spectrophotometer, in the same buffer used for isolating cells. Oxygen consumption was monitored with a Clark electrode, at 30 °C, in the isolation buffer supplemented with 5 mM-phosphate.

The NADH-fumarate reductase inhibitor 3-methoxyphenylacetic acid (3-MPA) was given by Merck, Sharp and Dohme Laboratories (Rahway, NJ, U.S.A.). All other substrates were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

RESULTS

Isolation of coupled mitochondria from *T. brucei* procyclic trypomastigotes is complicated by the presence of microtubules in the cell membrane, which require stronger forces for homogenizing the cells than those necessary for disrupting the mitochondrial membrane. Cannata & Cazzulo (1984) demonstrated that *T. cruzi* epimastigotes could be selectively permeabilized by using digitonin at different concentrations. This method was adapted to *T. brucei* procyclic trypomastigotes, in order to obtain coupled mitochondria in which to study better the electron-transport chain in these cells.

After the cells were treated with digitonin, different mitochondrial substrates were added in order to determine optimal conditions for measuring oxygen consumption. Several NADH-dependent substrates (malate, β -hydroxybutyrate, glutamate, α -oxoglutarate and alanine) were added to permeabilized cells to stimulate respiration, but none of these substrates stimulated oxygen consumption. However, both succinate and glycerol phosphate (both substrates of flavoproteins that donate electrons to ubiquinone) were

Table 1. Effect of several substrates on respiration of starved *T. brucei* procyclic trypomastigotes

For these experiments the cells were starved by incubating them at room temperature for 20 min, and then supplemented with 4 mM of either succinate, glucose or proline. All the determinations were carried out with a Clark electrode as described in the Materials and methods section.

Substrate	Oxygen consumption (nmol/min per mg of protein)
None	8 $\pm$ 3
Succinate	27*
Glucose	40 $\pm$ 7
Proline	43 $\pm$ 4

* This value is the average of two different cell preparations.

capable of stimulating respiration. Cells permeabilized by 40–60 μ g of digitonin/mg of protein, and incubated with succinate, showed a respiratory control ratio close to 1.6 on addition of ADP, indicating that the mitochondria remained intact (although poorly coupled) during the experiment (Fig. 1).

The failure of NADH-dependent substrates to increase oxygen consumption by permeabilized cells suggested that the NADH dehydrogenase-ubiquinone segment (Complex 1) is not very active in *T. brucei* procyclic trypomastigotes. If that were the case, one would expect that succinate would have a predominant role as an electron donor for the respiratory chain of these trypanosomes.

Experiments using 'starved' intact cells indicated that glucose, succinate and proline (Evans & Brown, 1972a,b) were suitable substrates for stimulating oxygen consumption (Table 1). Both glucose and proline may generate succinate during their metabolism. Proline generates α -oxoglutarate, which in turn enters the tricarboxylic acid cycle to produce succinate, ultimately donating electrons to the enzyme succinate dehydrogenase. Glucose metabolism in the glycosome of trypanosomatids produces malate (Hart *et al.*, 1984; Fairlamb & Opperdoes, 1986), which in turn could either generate cytoplasmic pyruvate or enter the mitochondrial matrix, generating intramitochondrial fumarate or NADH. In the second case, both products may form succinate by the reaction catalysed by NADH-fumarate reductase.

Malonate, a competitive inhibitor of the enzyme succinate dehydrogenase, was added to glucose-supplemented cells to determine its effect on the intracellular redox state of NAD(P)H (Fig. 2a) and on respiration of intact cells (Fig. 2b). Malonate inhibited the rate of oxygen consumption by intact cells by almost 50%, and the inhibition was reversible on addition of excess succinate (Fig. 2b). The fluorimetric determination of NAD(P)H indicated that, upon incubation with malonate, *T. brucei* procyclic trypomastigotes accumulate reducing equivalents (Fig. 2a), rather than oxidizing NADH as in mammalian mitochondria (Fig. 3), suggesting that the inhibition of respiration was not due to a lack of reducing equivalents for the respiratory chain.

Fig. 1. Effect of digitonin concentration on the respiratory control of *T. brucei* procyclic trypomastigotes

After digitonin treatment the cells decreased their rate of O₂ consumption (dilution of endogenous substrates). O₂ consumption was restored by 5 mM-succinate and the respiratory control was determined upon addition of 0.1 mM-ADP.

Fig. 2. Effect of malonate on fluorescence at 366–450 nm (a) and on the rate of oxygen consumption (b) by intact *T. brucei* procyclic trypomastigotes

The numbers by the traces are rates of oxygen consumption, in nmol/min per mg of protein.

The same experiment was repeated in permeabilized cells (using digitonin), in order to eliminate the contribution of cytoplasmic NAD(P)H to the changes in fluorescence observed in intact cells. The changes seen immediately after addition of digitonin to the cells are likely to be the consequence of decreased light scattering, rather than real changes in fluorescence (Fig. 4a). These initial changes are accompanied by inhibition of the rate of oxygen consumption owing to the dilution of endogenous substrates (Fig. 4b). Addition of malonate to permeabilized cells produced the same changes in the steady-state concentration of NAD(P)H, indicating that the changes in fluorescence shown in Fig. 2 represented, at least in part, changes in the mitochondrial NAD(P)H concentration. Malonate also inhibited both proline- or succinate-supported respiration by 40–50% (results not shown).

When examined under the microscope, the motility of intact cells was greatly decreased upon incubation with 0.5 mM-malonate. Further addition of 6 mM-succinate restored motility, suggesting that malonate substantially compromised the intracellular generation of energy. Excess fumarate instead of succinate did not reverse the inhibition caused by malonate (results not shown). This result is not surprising, since fumarate does not permeate to the mitochondrial membrane, at least in mammalian mitochondria (Wainio, 1970).

Both succinate- and NADH-cytochrome *c* reductase activities were determined in isolated membranes from *T. brucei* procyclic trypomastigotes, to test the effect of mitochondrial inhibitors in these activities. Succinate-cytochrome *c* reductase activity was completely inhibitable by antimycin (Table 2). However, although NADH was capable of reducing cytochrome *c*, this activity was insensitive to antimycin, and partially inhibitable by either superoxide dismutase or fumarate (Table 2), as previously described (Turrens, 1987). Table 2 also shows the specific activity of NADH-fumarate

Fig. 3. Changes in fluorescence of rat liver mitochondria (RLM) (366–450 nm) with different substrates

Addition of succinate increases the reduced steady-state concentration of NAD(P)H by competing with NADH for the respiratory chain. Malonate inhibits the enzyme succinate dehydrogenase, stimulating NADH oxidation.

Fig. 4. Changes in fluorescence at 366–450 nm (a) and in respiration rate (b) on addition of malonate to permeated *T. brucei* procyclic trypomastigotes (0.6 mg of protein)

The experiments were run in two different pieces of equipment, the same preparation being used for both traces. The numbers of the traces indicate rates of oxygen consumption, as in Fig. 2.

Table 2. Enzymic activities in the membrane-rich fraction of *T. brucei* procyclic trypomastigotes

The enzymic activities were determined as described in the Materials and methods section. The activities are expressed as means $\pm$ S.E.M. of three or four experiments. Numbers without S.E.M. are means of two different experiments.

Enzyme	Specific activity (munits/mg of protein)
Succinate–cytochrome <i>c</i> reductase	38 $\pm$ 4
+ 1 μ M-Antimycin	0
NADH–cytochrome <i>c</i> reductase	74 $\pm$ 7
+ 1 μ M-Antimycin	71
+ 200 units of superoxide dismutase	38
+ 4 mM-Fumarate	17
NADH–fumarate reductase	52 $\pm$ 3
+ 5 mM-Malonate	43

reductase, in order to compare these values with the rate of NADH–cytochrome *c* reductase. Addition of 5 mM-malonate inhibited this enzyme by 20% (Table 2).

The role of fumarate reductase in cell respiration was further evaluated by using enzyme inhibitors. The effect of the drug 3-MPA (developed by Merck, Sharp and Dohme Laboratories to inhibit the fumarate reductase of *Haemonchus contortus*) was analysed in *T. brucei* procyclic trypomastigotes respiration and growth in culture. 3-MPA inhibited the NADH–fumarate reductase

Fig. 5. Effect of 3-MPA on NADH–fumarate reductase activity (Δ), cell respiration ($\bullet$) and growth in culture ($\blacksquare$)

Experiments were performed as described in the Materials and methods section.

activity of *T. brucei* procyclic trypomastigotes, with an ID₅₀ of 3 µg/ml (Fig. 5). The ID₅₀ for both intact-cell respiration and cell growth in culture was 5-fold higher (Fig. 5).

DISCUSSION

The results shown in this paper suggest that the rotenone-insensitive mitochondrial respiratory chain of *T. brucei* procyclic trypomastigotes is quite different from that in mammalian mitochondria. We propose that the main mechanism by which these cells reoxidize NADH is through the enzyme NADH–fumarate reductase, generating succinate, which in turn is oxidized by the mitochondrial respiratory chain. The relatively high NADH–fumarate reductase activity of *T. brucei* procyclic trypomastigotes (Table 2) may, under physiological conditions, produce enough succinate to support the electron flow into the respiratory chain.

When incubated with malonate, mammalian mitochondria tend to oxidize NADH, owing to the inhibition of succinate oxidation. However, *T. brucei* procyclic trypomastigotes tend to reduce even more NADH, as if malonate were inhibiting NADH oxidation (Figs. 2 and 4). The accumulation of NADH on addition of malonate suggests a major difference between the mammalian mitochondrion and that of *T. brucei* procyclic trypomastigotes. It is unlikely that malonate is directly inhibiting the respiratory chain. Several factors may contribute to the inhibition of the reaction catalysed by the NADH–fumarate reductase. First, malonate is a competitive inhibitor of the mitochondrial succinate dehydrogenase, and, to a limited degree, inhibits directly the NADH–fumarate reductase (Table 2, and Boveris *et al.*, 1986). Malonate will also compete for the mitochondrial dicarboxylic acid carrier, inhibiting the incorporation of malate into the mitochondrial matrix, thus decreasing extramitochondrial sources of fumarate. The inhibition of succinate dehydrogenase will decrease the intramitochondrial production of fumarate. These three processes (inhibition of malate transport, succinate dehydrogenase and fumarate dehydrogenase) may lead to the accumulation of intramitochondrial NADH through both depletion of substrate (intramitochondrial

fumarate) and accumulation of product (succinate). When the inhibition of succinate dehydrogenase is reversed by addition of excess succinate, this produces intramitochondrial fumarate, reoxidizing NADH.

It is worth noting that, on addition of malonate, glucose-supported respiration is only inhibited by about 50%, indicating that the effect of malonate is not complete. This may be due to the oxidation of other substrates (i.e. glycerol phosphate), and eventually to some remnant NADH oxidase activity which could not be detected in either permeabilized cells or isolated membranes.

The possibility that the fumarate reductase system is by-passing Complex I in the respiratory chain could also explain why several enzymes of the tricarboxylic acid cycle are missing from these cells, including citrate synthase and isocitrate dehydrogenase (Fairlamb & Opperdoes, 1986), which are required for starting the cycle, and to produce intramitochondrial reduced nicotinamide nucleotides. NADH may instead be generated in other reactions and may be transported into the mitochondrial compartment by the malate shuttle. Malate in turn may produce both fumarate and intramitochondrial NADH, depending on the intracellular demands.

The transcription product of a gene that may code for the enzyme NADH dehydrogenase has been isolated from maxi-circles from different forms of the parasite (Jasmer *et al.*, 1985; Feagin *et al.*, 1986), indicating that the enzyme NADH dehydrogenase should be present in the mitochondria of these parasites. The results reported in the present paper suggest that, although an NADH dehydrogenase activity exists in mitochondria from *T. brucei* procyclic trypomastigotes, it may act by transferring electrons to fumarate in an alternative pathway to that present in mammalian mitochondria. Previous reports (Cheah, 1967) had demonstrated that in *Moniezia expansa* the respiratory chain is branched, and the electrons from NADH oxidation may be transferred to either the cytochrome *c* oxidase or the fumarate reductase. In *T. brucei* procyclic trypomastigotes only those electrons coming from succinate can reduce cytochrome *c* through the fragment of the respiratory chain that resembles the mammalian electron-transport chain (Table 2). When incubated with NADH, membranes isolated from *T. brucei* can only transfer electrons to cytochrome *c* in a pathway that may or may not involve superoxide production (Table 2), but certainly does not involve the ubiquinone–cytochrome *b* segment, since it is not inhibitable by antimycin. The mitochondrial NADH–cytochrome *c* reductase activity is apparently catalysed by a direct electron transfer from the NADH–fumarate reductase to cytochrome *c* (Turrens, 1987); it is only detected in mitochondrial membranes, and is inhibited by fumarate. In intact mitochondria the active site of fumarate reductase for NADH is facing the mitochondrial matrix, whereas cytochrome *c* is located in the intermembrane space, thus making impossible a direct electron transfer from the nucleotide, even in the absence of fumarate.

Several species of trypanosomes are known to excrete succinate into the medium on reaching the stationary phase. Our results indicate that succinate itself can be incorporated as a source of electrons for the respiratory chain when the cells have been starved at room temperature. If our hypothesis is correct, and the

NADH–fumarate reductase is the main mitochondrial system for reoxidizing NADH, this also would explain why several trypanosomatids secrete succinate into the culture medium (Fairlamb & Opperdoes, 1986), since it should be a final secretion product from NADH oxidation, even when the energy level is relatively high.

Proline, which also generates intracellular succinate, is actually the preferred substrate for growing *T. brucei* procyclic trypomastigotes (Evans & Brown, 1972a). Proline metabolism has the advantage that it avoids going through glycolysis, generating malate and then succinate from fumarate. Nevertheless, these cells can obtain energy from any source, including glucose (Table 1, and Evans & Brown, 1972a).

Finally, a significant role for the NADH–fumarate reductase in cell metabolism is also suggested by the effect of 3-MPA, an inhibitor of this reductase which not only blocks its activity *in vitro* but also inhibits respiration *in vivo* and cell growth. There are, however, large differences between the concentrations that block the enzyme *in vitro* and those that inhibit both respiration and cell growth (Fig. 5), which may represent low permeability for the drug, or secondary effects of the drug in the respiratory metabolism.

Although the proposed mechanism for the electron-transport chain in *T. brucei* procyclic trypomastigotes has not been completely elucidated, we may conclude that it is definitely different from that present in mammalian cells. If these differences are also present in other trypanosomatids, these findings open a new door for the potential treatment of different forms of human trypanosomiasis (i.e. *T. cruzi*, and eventually other members of the genus *Leishmania*), in which the parasite contains mitochondria when in the host. Specific inhibitors of the enzyme NADH–fumarate reductase may directly affect the survival of the parasite without inhibiting the normal metabolism of the mammalian cell, thus becoming specific anti-trypanosomal agents.

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