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ATP generation in *Leishmania donovani* amastigote form

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Abstract

Leishmania is the causative agent of various forms of leishmaniasis, a significant cause of morbidity and mortality. The clinical manifestations of the disease range from self-healing cutaneous and mucocutaneous skin ulcers to a fatal visceral form named visceral leishmaniasis or kala-azar. The differentiation of *Leishmania* parasites from the insect stage, the promastigote, towards the pathogenic mammalian stage, the amastigote, is triggered primarily by the rise in ambient temperature encountered during the insect to mammal transmission. The survival of amastigote stage is dependent on that of the host. Regarding energy metabolism, which is an essential factor for the survival, parasites adapt to the environment under low oxygen tension in the host using metabolic systems which are very different from that of the host mammals. The amastigote form of *L. donovani* is independent on oxidative phosphorylation for ATP production. Indeed, its cell growth was not inhibited by 20-fold excess oligomycin and dicyclohexylcarbodiimide, which are the most specific inhibitors of the mitochondrial F_oF_1-ATP synthase. In contrast, mitochondrial complex I inhibitor rotenone and complex III inhibitor antimycin A inhibited amastigote cell growth, suggesting the role of complex I and complex III in cell survival. Complex II appeared to have no role in cell survival. To further investigate the site of ATP production, we studied the substrate level phosphorylation, which was involved in the synthesis of ATP. Succinate-pyruvate couple showed the highest substrate level phosphorylation, whereas NADH-fumarate and NADH-pyruvate couples failed to produce ATP. In contrast, NADPH-fumarate showed the highest rate of ATP formation in promastigotes. We conclude that substrate level phosphorylation is essential for the growth of *L. donovani* amastigotes.

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ATP, *Leishmania*, amastigote, substrate level phosphorylation, oligomycin.

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INTRODUCTION

Leishmania donovani, a digenic trypanosomatid protozoan infects and resides within macrophages during its mammalian cycle of existence resulting in visceral leishmaniasis (VL). VL might be fatal if not treated properly and was reported to cause mortality in several parts of the developing world [1]. *Leishmania* infection was also detected as

coinfection in HIV patients [1]. After successful entry into macrophages, promastigote form of the parasite survives and proliferate within the mature phagolysosome compartment as an amastigote, where it multiplies and finally bursts the compartment to infect neighbouring macrophages [2]. During its stay within parasitophorous vacuoles (PV) of macrophages, parasite scavenges nutrients from the host cell at pH around 5.5 [3], prevents host cell apoptosis, and alters host cell gene expression [4].

Studies during the last decade indicated that shifting promastigotes to an intralysosomal-like environment (37°C and pH 5.5 at 5% CO_2 environment), induced differentiation into amastigotes in host free culture [5,6]. Acidic pH and heat induced growth, direct the heat-adapted promastigotes to differentiate into amastigotes [7]. Such an experimental system has already been used by several laboratories to investigate various state regulated functions in *L. donovani* [8,9] and has also been developed in our laboratory to investigate stage-regulated drug susceptibility and metabolic capabilities in wild, resistant field isolate and *in vitro* generated drug resistant *L. donovani*. Characterization of axenic amastigotes of the *Leishmania* species has demonstrated that they resemble animal derived amastigotes [6].

Amastogotes adapt to life in the intracellular acidic phagolysosome. This adaptation is reflected through a metabolic profile distinct from promastigotes. In this context, axenic amastigotes closely resemble intracellular amastigotes which exhibit raised levels of acid ribonuclease, protinase, reduced activity of adenine deaminase, peroxidase, increased incorporation of thymidine, uridine, proline, and altered metabolism of glucose and linolenic acid when compared with promastigotes [10,11]. The major products of glucose catabolism were demonstraterd for amastigotes and promastigotes under aerobic and anaerobic conditions. Under aerobic conditions the major products for both forms are carbon dioxide, succinate, malate, acetate and alanine. Under anaerobic conditions, promastigotes produce glycerol as the dominant metabolite, along with lesser amounts of succinate, acetate and alanine, whereas acetate and alanine are the major metabolites in amastigotes. Amastigotes are relatively less affected by lack of oxygen and produced carbon dioxide at a rate comparable to promastigotes under anaerobic conditions [12]. Succinate and valine are found in higher quantities in intracellular *L. donovani* amastigotes and axenic amastigotes than in promastigotes. Acetoacetate is present only in intracellular and axenic amastigotes [13].

The *in vivo* efficiencies of drugs have been reported to be under the control of different parameters such as pharmacokinetic parameters. One of the most important parameters is the drug's direct activity against a parasite at the mammalian stage. Axenically grown amastigotes may thus become a powerful tool in the isolation of new compounds and characterization of biochemical parameters for new drug targets. Extracellular amastigotes clearly resemble intracellular amastigotes in their ultra-structural, biological, biochemical and immunological properties [6]. Moreover, characterized amatigotes such as intracellular ones, differ from promastigotes in having a variety of biochemical characteristics, including proteinase, ribonuclease, adenine deaminase, peroxidase, dehydrogenase activities and glucose catabolism, nucleic acid synthesis and nitric oxide activity [14-17].

The present study compares the results of energy metabolisms in wild AG83 promastigotes and amastigotes. To our knowledge this is the first report regarding the differences in energy metabolisms between promastigotes and amastigotes. Energy metabolism of *L. donovani* amastigotes differs significantly from that of their hosts. In light of this present report, we may expect that this type of bioenergetic analysis provides essential mechanisms

of amastigote bioenergetics leading to the identification of tractable targets for therapeutic intervention.

MATERIALS AND METHODS

Materials. Antimycin A, rotenone, thenoyltrifluoroacetone (TTFA), oligomycin, dicyclohexylcarbodiimide (DCCD), medium M-199 and fetal calf serum (FCS) were purchased from Sigma-Aldrich (St. Louis, MO). Other analytical chemicals were purchased from local source.

Parasites and culture conditions. Promastigotes of *Leishmania donovani* clones, AG83 (MHOM/IN/83/AG83) was VL isolates obtained as a gift from Indian Institute of Chemical Biology, Council of Scientific and Industrial Research, Kolkata, India. Strain AG83 has been used in India as reference standard strain of *L. donovani*. Parasites were routinely grown as promastigotes in medium M-199 with 10% heat–inactivated fetal calf serum (FCS) at 24°C [6,18].

Generation of axenic amastigotes. *Leishamnia donovani* amastigote forms were grown and maintained as described by Debrabant *et al.* [6]. Axenically grown amastigotes of *L. donovani* were maintained at 37°C in 5% CO_2 /air by weekly sub-passages in MMA/20 at pH 5.5 in petri dishes [19]. Under these conditions, promastigotes differentiated to amastigotes within 120 hours. Cultures were maintained by 1:3 dilutions once in a week.

Axenic amastigote inhibitor susceptibility assay. Axenic amastigote inhibitor susceptibility assays were carried out by determining the 50% inhibitory concentration (IC_{50}) by cell counting method using a hemocytometer. To measure the IC_{50} values for different inhibitors, the parasites were seeded into 96-well plates at a density of $2x10^5$ amastigotes/ well in 200µl culture medium containing 10µl of different inhibitors. Parasite multiplication was compared to that of untreated control (100% growth). After 72 hours of incubation, cell count was taken microscopically. The results were expressed as the percentage of reduction in parasite number compared to that of untreated control wells, and the 50% inhibitory concentration (IC_{50}) was calculated by linear regression analysis (MINITAB V.13.1, PA) or linear interpolation [20]. All experiments were repeated three times, unless otherwise indicated.

Assay of ATP formation activity. ATP formation activities in digitonin permeabilized *L. donovani* cells in presence of electron donor and acceptor couple was studied by phosphate estimation [21], where phosphate was eliminated exclusively in presence of ADP. Phosphate elimination assay in presence of ADP was carried out in the assay buffer (KCl-50 mM, sucrose-300 mM, Tris-HCl-50 mM, EGTA-2 mM at pH 7.0). Digitonin permeabilized *Leishmania donovani* cells were added at a conc. of 120 µg/ml. ADP and Mg-acetate were added at a conc. of 2 mM and 4 mM, respectively. NADPH (1 mM), NADH (1 mM), K-lactate (10 mM), dihydroorotate (10 mM), succinate (10 mM) were the electron donors and K-fumarate (10 mM)) and K-pyruvate ((10 mM) were the electron acceptors in the assay of ATP formation by digitonin permeabilized cells. Cells were incubated for 20 mins for assay of ATP formation and terminated by perchloric acid. Perchloric extract was neutralized by KOH and was centrifuged at 20,000x g for 15 mins at 4°C. Supernatant was preserved for phosphate estimation leading to ATP formation.

Preparation of digitonin permeabilized *Leishmania* cells. *Leishmania donovani* promastigote and/or amastigote cells were collected, washed once by buffer A (140 mM NaCl, 20 mM KCl, 20 mM Tris, 1 mM EDTA, pH 7.5), and resuspended in isolation buffer (20 mM MOPS-NaOH, 0.3% BSA, 350 mM sucrose, 20 mM potassium acetate, 5 mM magnesium acetate, 1 mM EGTA, pH 7.0). Cells were permeabilized in a separate tube with digitonin (100 µg/mg protein) and incubated on ice for 10 mins. After incubation, the cells were centrifuged at 6000x g for 7 mins. Pellets were resuspended in assay buffer.

Protein estimation. Total cell protein was determined by the biuret method in the presence of 0.2% deoxycholate [22,23]. One milligram of protein corresponds to $1.75x10^8$ promastigote cells and $1.14x10^8$ amastigote cells.

Statistical analysis. All experiments were performed in triplicate, with similar results obtained in at least three separate experiments. Statistical significance was determined by Student's t-test. Significance was considered as $P < 0.05$.

RESULTS AND DISCUSSION

The findings that heat transformed, acidic pH stabilized *L. donovani* cells down-regulate plasma membrane and mitochondrial electron transport as well as oxygen uptake [18], insisted us to explore the nature of enzymes involved in metabolism of *L. donovani* promastigotes and amastigotes. Many lower eukaryotes can survive in hypoxic or anaerobic condition via a fermentative pathway that involves the use of the reduction of endogenously produced fumarate as an electron sink. They are highly adapted for prolonged survival or even continuous functioning in the absence of oxygen, whereas many of them are adapted to alternating periods in the presence and absence of oxygen. Some anaerobically functioning eukaryotes, such as yeast and certain fishes, can survive without mitochondrial energy metabolism via cytosolic fermentations in which NADH/NADPH produced during glycolysis is consumed during the reduction of pyruvate to lactate or fumarate to succinate, which are subsequently excreted as end products.

It appears from Table 1 that mitochondrial complex II electron transport inhibitor TTFA and F_oF_1-ATP synthase inhibitor oligomycin and DCCD are refractory to growth inhibition of AG83 amastigotes, whereas mitochondrial complex I inhibitor rotenone and complex III inhibitor antimycin A are sensitive to growth inhibition. Growth inhibition studies on AG83 amastigotes reveal that complex II of amastigote mitochondria is not essential for the maintenance of amastigote cell growth. In contrast, involvement of complex II is essential for the growth of normal mammalian cells. Inhibition of amastigote cells, growth by rotenone and antimycin A suggests that amastigote cell growth is dependent on the functioning of complex I and complex III. Table 1 also shows that mitochondrial F_oF_1-ATPase inhibitor oligomycin and DCCD are absolutely insensitive to growth inhibition of amastigote cells. Thus, we may conclude that ATP synthesis in mitochondria is independent of F_oF_1 - ATP synthase.

Table 1: Evaluation of susceptibilities of *Leishmania donovani* AG83 axenic amastigotes to mitochondrial electron transport and ATP synthase inhibitors

Compounds tested	(IC_{50}±SD, n=3) µM [a]
	Axenic AG83 amastigote
Rotenone	1.4±0.2
TTFA	>$4x10^3$ [b]
Antimycin A	0.8±0.2
Oligomycin	>200 [b]
DCCD	>200 [b]

[a] Assays are described in Materials and Methods. All datas are mean ± SD for three experiments.

[b] No inhibition was observed at the indicated concentration.

The lack of ability of *L. donovani* amastigote mitochondria to carry out energy linked functions such as respiration coupled with phosphorylation, proposes the hypothesis that amastigote form of *Leishmania* is less dependent on respiratory energy (Table 1). This might be the reason for the survival of amastigote cells within the phagolysosomes where apparent hypoxic condition persists [24,25]. Amastigotes have poorly developed mitochondria [26] and the spleen which is parasitized by *L. donovani* amastigotes is also poorly oxygenated [27].

Table 2 shows the formation of ATP in presence of various electron donors and acceptors in terms of elimination of inorganic phosphate exclusively in presence of ADP. Wild promastigote and amastigote cells differ substantially with respect to the rate and nature of electron donors and acceptors. In promastigote cells, the highest rate was observed for NADPH-fumarate couple, whereas in amastigote cells, succinate-pyruvate couple showed the highest rate. NADPH-fumarate couple in promastigote cells showed 2.3 times higher rate, compared to succinate-pyruvate couple in amastigote. NADH-fumarate couple failed to synthesize ATP in amastigote, whereas the same couple in promastigote showed rate of 130 nmol/min/mg protein. Similarly NADH-pyruvate couple in promastigote cells showed rate of 45 nmol/min/mg protein, whereas the same couple showed zero rate in amastigote cells. Succinate-pyruvate couple showed the highest rate in amastigotes, whereas NADH-fumarate and NADH-pyruvate couples failed to show any rate in amastigote cells. Here it is noteworthy that in amastigote cells only NADPH acts as electron donor, whereas NADH did not act as electron donor. However, in promastigote cells both NADH and NADPH act as electron donors. Other electron donors in amastigote cells are succinate, lactate and dihydroorotate. From these observations, it appears that bioenergetic adaptations in amastigote cells have changed and that could be exploited for potential drug target.

Table 2: Evaluation of ATP formation in presence of electron acceptors and donors in digitonin permeabilized *L. donovani* AG83 promastigotes and amastigotes [a]

Substrate added	Axenic promastigote		Axenic amastigote	
	Rate (mean ± SD, n=3) (nmoles / mg protein / min)	Relative Rate	Rate (mean ± SD, n=3) (nmoles / mg protein / min)	Relative Rate
NADPH + Fumarate + ADP +Pi	232±28	100	66±8	66
NADH + Fumarate + ADP + Pi	130±20	56	0±0	0
Lactate + Pyruvate + ADP + Pi	94±12	40.36	34±5	34
DHO + Fumarate + ADP + Pi	79±12	34.11	27±4	27
Succinate + Pyruvate + ADP + Pi	71±10	30.73	101±16	100
Lactate + Fumarate + ADP + Pi	68±8	29.29	49±8	49
NADPH + Pyruvate + ADP + Pi	65±7	28.12	78±12	77
NADH + Pyruvate + ADP + Pi	43±6	18.75	0±0	0

[a] Assays are described in Materials and Methods. All datas are mean ± SD for three experiments.

Anaerobic metabolism of amastigote suggests that fumarate may serve as electron sink of the reducing equivalents generated during metabolism. This is evident from the amount of succinate formed during the growth of amastigote [18]. A CO_2 requirement for amastigote growth often reflects the involvement of phoshoenolpyruvate carboxylase or pyruvate carboxylase in the synthesis of succinate. Martin *et al.* [28] demonstrated that in the amastigote cell pyruvate carboxylase was the principal enzyme catalyzing heterotrophic CO_2 fixation to produce oxaloacetate from phoshoenolpyruvate. Evidence also suggests that the activity of these enzymes are comparatively higher in the amastigotes. Such a pathway could provide dicarboxylic acids for biosynthetic processes and also contributed to $NADP^+$ recycling.

Amastigote cells continuously excrete relatively large quantities of succinic acid and other organic acids into their environment [12]. Michels *et al.* [29] in their "energy recycling model" proposed that electrogenic efflux of these organic end products via ion symport systems might lead to the generation of an electrochemical ion gradient across the

cytoplasmic membrane, thus providing metabolic energy to the cell. Amastigote cells would also be able to utilize succinate excretion as a metabolic energy. It appears that aberrant amastigote mitochondrial functions cause these cells to produce inordinate amount of succinic acid and other acids. An analogous observation for the cancer cells was the inactive cytochrome c oxidase that led to preferential utilization of glycolysis over aerobic respiration to produce ATP [30].

The differences between parasite and host energy metabolism described in our work hold great promise as targets for chemotherapy. For example, most of the active antileishmanial compounds tested on promastigotes failed to inhibit amastigotes [31,32]. If an agent that can specifically inhibit the amastigote fumarate reductase and pyruvate reductase is found, it is expected to be extremely effective and selective as an antileishmanial agent.

Mitochondrial metabolism in amastigote form of wild *L. donovani* is less understood so far. However, Rainey and MacKenzie showed that succinate and lactate were the major excretory products of catabolism in amastigote [12]. They also showed that amastigotes were relatively less affected by lack of oxygen which was supported by Singh *et al.* [33]. Constou *et al.* showed that cell growth of trypanosomatid procyclic *T. brucei* was unaffected by high oligomycin concentration and ATP level of procyclic *T. brucei* was maintained at the normal level in presence of high concentration of oligomycin [34]. They showed that cytosolic substrate level phosphorylation was essential for the growth of procyclic trypanosomes. In view of their conclusions, we may also postulate that survival of amastigotes of wild *L. donovani*, a trypanosomatid, may require substrate level phosphorylation. Substrate level phosphorylation and inhibition of synthesis and excretion of succinate could be a potential chemotherapeutic strategy for drug development in leishmaniasis.

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