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A novel sucrose/H^+ symport system and an intracellular sucrase in *Leishmania donovani*

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ABSTRACT

The flagellated form of pathogenic parasitic protozoa *Leishmania*, resides in the alimentary tract of its sandfly vector, where sucrose serves as a major nutrient source. In this study we report the presence of a sucrose transport system in *Leishmania donovani* promastigotes. The kinetics of sucrose uptake in promastigotes are biphasic in nature with both high affinity K_m (K_m of ~75 μM) and low affinity K_m (K_m ~ 1.38 mM) components. By contrast the virulent amastigotes take up sucrose via a low affinity process with a K_m of 2.5 mM. The transport of sucrose into promastigotes leads to rapid intracellular acidification, as indicated by changes in the fluorescence of the pH indicator 2′,7′-bis-(2-carboxyethyl)-5-(6) Carboxyfluorescein (BCECF). In experiments with right side-out plasma membrane vesicles derived from *L. donovani* promastigotes, an artificial pH gradient was able to drive the active accumulation of sucrose. These data are consistent with the operation of a H^+-sucrose symporter. The symporter was shown to be independent of Na^+ and to be insensitive to cytochalasin B, to the flavonoid phloretin and to the Na^+/K^+ ATPase inhibitor ouabain. However, the protonophore carbonylcyanide P- (trifluromethoxy) phenylhydrazone (FCCP) and a number of thiol reagents caused significant inhibition of sucrose uptake. Evidence was also obtained for the presence of a stable intracellular pool of the sucrose splitting enzyme, sucrase, in promastigote stage parasites. The results are consistent with the hypothesis that *L. donovani* promastigotes take up sucrose via a novel H^+–sucrose symport system and that, on entering the cell, the sucrose is hydrolysed to its component monosaccharides by an intracellular sucrase, thereby providing an energy source for the parasites.

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1. Introduction

Leishmaniasis, a vector-borne parasitic disease, is transmitted by the female phlebotomine sandfly and affects 10–15 million people worldwide. The primary etiological agent of life-threatening visceral leishmaniasis (kala-azar) is *Leishmania donovani*; however, *Leishmania tropica* has also been implicated in some cases of kala-azar from India (Desjeux, 1996; Sacks et al., 1995). The *Leishmania* parasite passes through two morphological states during its life cycle. While residing in the gut of the sandfly vector, the parasite exists in the flagellated promastigote form. After injection into the human host it transforms into the infective amastigote stage. A critical aspect of leishmanial biology is the ability of the parasite to adapt to two completely different microenvironments encountered in the sandfly gut and in the phagolysosomes of human macrophages. The temperature, pH and availability of nutrients in the two environments are radically different. Thus the occurrence of selective and controlled transport of nutrients and ions into and from the parasite cell is important for the maintenance of intracellular homeostasis.

Sandflies are known to survive largely on plant sap rich in sucrose (Bray, 1983; Schlein, 1986). Survival and growth of *Leishmania* has been found to be entirely dependent on the plant sap meal of the insect vector (Schlein and Jacobson, 1999, 2001). The presence of the disaccharides, sucrose and maltose, as well as the monosaccharides, glucose and fructose, has been reported in the gut of sandflies from a region where leishmaniasis is endemic (Anez et al., 1994). The question therefore arises of whether the parasite possesses specific transporters for the internalisation of sucrose for utilisation. It is this that is the major focus of this study.

Elegant studies from several groups have shown that sugar and amino acid transport is coupled to the influx of H^+ in *Leishmania* spp. (Vieira and Cabantchik, 1995). Sucrose/proton symport systems have been described in other eukaryotes (Santos et al., 1982; Slee and Tanzer, 1982; Riesmeier et al., 1994; Stambuk et al., 1998) and one such system has been shown to contribute to virulence in a plant pathogenic fungus (Talbot, 2010). Biochemical studies have established glucose as a major nutrient of *Leishmania* (Schaefer and Mukkada, 1976) and glucose transporters

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from different species of trypanosomes and *Leishmania* have been characterised in some detail (Mukkada et al., 1974; Zilberstein and Dwyer, 1985; Burchmore and Hart, 1995; Landfear, 2008). Nearly 50 years ago, sucrose was reported to be important in the metabolism of *Leishmania* promastigotes (Chatterjee and Ghosh, 1960). Blum and Opperdoes (1994) reported the occurrence of sucrase in both permeabilised *L. donovani* cells and in culture media. Gontijo et al. (1996) subsequently purified the extracellular sucrase to homogeneity. However there has not, to date, been any reported description of a plasma membrane sucrose transporter or of an intracellular sucrose metabolizing enzyme in *Leishmania*.

In the current study we describe a sucrose transport system in *L. donovani*. The transporter is highly specific for sucrose and is distinct from the previously-described glucose transport system. The transport of sucrose via this system is coupled with transmembrane movement of protons. We further demonstrate the presence of an intracellular sucrase in *L. donovani* promastigotes. Further detailed characterisation of the H^+–sucrose symporter and the intracellular sucrase will contribute to our understanding of the parasite's physiology and may provide a foundation for the design of new anti-leishmanial drugs that will target these proteins.

2. Materials and methods

2.1. Materials

All chemicals, unless otherwise mentioned, were purchased from Sigma (Saint Louis, MO, USA). [U–^{14}C]-Sucrose (500 mCi/mmoles) and [U–^{14}C]-2-deoxy-D-glucose (330 mCi/mmoles) were obtained from Amersham Pharmacia Biotech, UK.

2.2. Cell lines and growth conditions

The strain MHOM/IN/1978/UR6 used in this study is a clinical isolate cell line of *L. donovani*, isolated from a patient with confirmed visceral leishmaniasis (kala-azar). The UR6 promastigotes were cultured in solid blood-agar medium, at pH 7.4 (Saha et al., 1986). Hemin (10%) was used to replace blood from media, whenever needed. Promastigotes were also grown in synthetic liquid media, (Chaudhuri et al., 1982) in which glucose was replaced with the alternative potential energy source viz sucrose, glycerol or proline. Parasites in the exponential phase of growth were used for this study; cells were collected at different time points while checking growth rates. For the study of growth rate in synthetic liquid media as well as on solid agar media, 2×10^6 promastigotes were used as inoculum and the parasites were incubated at 22 °C. At designated time points, an aliquot (100 μl) of cell suspension from liquid culture was sampled for cell counting using a haemocytometer. Promastigotes from solid agar media were collected and washed with PBS before counting for growth rate determination. All of the steps were performed aseptically.

2.3. Preparation of sealed ghosts

The plasma membrane of a *L. donovani* promastigote has a characteristic pellicular microtubular structure that protects the membrane against hypotonic shock. Sealed ghosts were prepared from *L. donovani* promastigotes as described by Mukherjee et al. (2001) with a few modifications. Complete sealing of the ghost membranes was achieved by adding 140 mM KCl and 10 mM $CaCl_2$ in 25 mM HEPES–Tris buffer at pH 8.0.

2.4. Amastigote purification

Amastigotes were isolated from the spleen of a Syrian hamster 12 weeks p.i. with *L. donovani* (AG83). The infected spleens were chopped and then macerated in-between two wire meshes (mesh size 200) in ice cold PBS (pH 6.8) to release the intracellular parasites from ruptured spleen cells. Centrifugation of whole cell suspension at 388*g* for 5 min using a Sorvall SS34 rotor under cold conditions allowed the separation of amastigotes (in the supernatant) from the host cell debris (in the pellet). The above step was repeated twice. Finally, amastigotes from the supernatant were purified by Percoll density gradient centrifugation (Meade et al., 1984).

Animals use for experiments was approved by the Animal Ethics Committee of the Indian Institute of Chemical Biology, West Bengal, India, which is regulated by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India. National guidelines on human use of animals in experimentation were followed.

2.5. Oxygen consumption

Harvested cells were resuspended at a concentration of 0.5×10^8 cells/ml after washing with ice cold PBS. The oxygen consumption of promastigotes was measured using an Oxygraph (Gilson 5/6 Oxygraph) with a Clark type oxygen electrode (Santhamma and Bhaduri, 1995) in a final volume of 2 ml cell suspension. The electrode was calibrated with air-saturated water and sodium metabisulphite. To perform the experiment, cells were 'stressed' by keeping cells in PBS for 150 min at 28 °C, prior to experimentation, in order to reduce the endogenous respiration rate. Oxygen consumption was monitored in both control cells (that had not been subjected to the pre-incubation in PBS) and stressed cells for 10–15 min after the addition of either 5 mM sucrose or glucose.

2.6. Transport assays

Promastigotes were washed twice by centrifugation and resuspension in ice cold PBS. Washed cells were finally resuspended at a final density of 2×10^8 cells/ml in buffer A (130 mM NaCl, 10 mM KCl and 10 mM K_2PO_4, pH 7.4) for the purpose of transport assays. The uptake of radiolabelled substrate was measured using a rapid filtration technique (Saha et al., 1986; Mukherjee et al., 2001). Transport was initiated by transferring 2×10^8 cells/ml to an equal volume of buffer A containing radioactive (2 μCi/ml) [U–^{14}C]-sucrose and supplemented with varying concentrations of non-radioactive sucrose. Assays were carried out at 28 °C. For most of the uptake experiments, at designated time points 200 μl aliquots of the suspension were sampled and rapidly filtered through nitrocellulose filters of 0.8 μm pore size (Millipore), then the cells were washed immediately with 10 ml of ice-cold buffer A. Accumulation of radioactivity within cells trapped on membrane filter was measured using a scintillation counter. In a number of cases rapid short-term transport assays (10–15 s) were carried out using a procedure in which samples of the radiolabelled suspension (200 μl) were centrifuged through 100 μl of 95% Dibutylthalate plus 5% Paraffin oil mixture and the resulting cell pellets were lysed in 100 μl of 1% Triton X-100 for measurement of radioactivity (Ghosh and Mukherjee, 2000). Where appropriate, alternative sugars or inhibitors were added to the assay prior to the addition of [U–^{14}C]-sucrose to initiate the transport. The ionic composition of buffer A was varied by replacing NaCl or KCl with either 140 mM N-methyl-D-glucamine (NMDG) or 140 mM NaCl, as appropriate. All assays were done in triplicate.

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