Protonmotive force-driven active transport of \( \Delta \text{Glc} \) and \( \Delta \text{L-proline} \) in the protozoan parasite *Leishmania donovani* (promastigotes/\( \Delta \mu_m^+ \))

Dan Zilberstein and Dennis M. Dwyer

Cell Biology and Immunology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Building 5, Room 112, Bethesda, MD 20205

Communicated by William Trager, November 5, 1984

**ABSTRACT**

Midlogarithmic phase *Leishmania donovani* promastigotes accumulate 2-deoxy-D-glucose (2-dGlc) and L-proline, maintaining concentration gradient factors across the surface membrane of 78.7 and 60, respectively. Cyanide (1 mM) and iodoacetate (0.5 mM) inhibited the transport of both substrates. L-proline uptake was also inhibited by 2-dGlc (10 mM). Transport of neither substrate was affected by Na\(^+\), phlorizin, or ouabain, indicating the sodium-independent transport of both systems. However, N',N'-dicyclohexylcarbodiimide (DCCD; 20 \( \mu \text{M} \)) significantly inhibited the transport of both 2-dGlc and L-proline (70% and 90%, respectively). The ionophores valinomycin (1 \( \mu \text{M} \)) and nigericin (5 \( \mu \text{M} \)) each partially inhibited the uptake of both substrates. In parallel experiments, nigericin and valinomycin were added concomitantly to promastigotes, each at a concentration that individually inhibited the transport of 2-dGlc and L-proline by <30%. Under such conditions, the transport of 2-dGlc and L-proline was inhibited by 69% and 78%, respectively. However, these ionophores had no significant effect on the promastigotes cellular ATP level. Carbonylcyanide p-(trifluoromethoxy)phenylhydrazone (FCCP; 1 \( \mu \text{M} \)) inhibited 2-dGlc (79%) and L-proline (85%) transport, whereas ATP levels of such cells were diminished by only 20%. Symport of \( \Delta \text{Glc}/H^+ \) and \( \Delta \text{L-proline}/H^+ \) was measured directly in cells pretreated with KCN and DCCD. Upon addition of \( \Delta \text{Glc} \) to such cells, a rapid movement of protons into the organisms occurred and was reversed upon addition of FCCP. Conversely, no proton movement was observed when \( \Delta \text{Glc} \) was added to such cells. L-proline, as \( \Delta \text{L-proline} \), caused a rapid influx of protons into the promastigotes, indicating that both substrates were cotransported with protons. We conclude that transport of \( \Delta \text{Glc} \) and \( \Delta \text{L-proline} \) in *L. donovani* promastigotes is protonmotive force-driven and is coupled to both \( \Delta \text{pH} \) and \( \Delta \psi \).

Species of the parasitic protozoan *Leishmania* are the causative agents of a wide variety of human cutaneous, mucocutaneous, and visceral diseases. One member of this group, *Leishmania donovani*, is the etiologic agent of kala azar, a chronic and usually fatal form of human visceral leishmaniasis. This trypomastigot protozoan has a digenetic life cycle, assuming an extracellular flagellated promastigote form in the alimentary tract of its insect vector and an obligate intracellular amastigote form within the phagolysosomal system of mammalian macrophages (1-3). The interface between these hosts and the parasite occurs ultimately at the level of the parasite surface membrane. All physiologic and biochemical interactions occur, at least temporally, at or across such membranes.

A paucity of studies have been done concerning the physiologic functions of the *Leishmania* sp. cell surface membrane. Previously, carrier-mediated transport systems were demonstrated in several different *Leishmania* species for the uptake of D-glucose (4), L-proline (5-7), L-methionine (8), \( \alpha \)-aminoisobutyric acid (9). Recently, we have shown that promastigotes of *L. donovani* possess a transport system for D-glucose (10). The transport in all of these systems was active in nature.

To date, neither the mechanism for the uptake of glucose or amino acids in *Leishmania* promastigotes nor the nature of the driving force responsible for their transport has been elucidated. In the current study, we investigated the energy transduction mechanism for transport of both D-glucose and L-proline in *L. donovani* promastigotes. The cumulative results demonstrated that carriers for both of these compounds maintained proton symport translocation and that a proton electrochemical gradient was requisite for transport maintenance.

**MATERIALS AND METHODS**

Parasite Maintenance and Growth Conditions. A cloned strain of *L. donovani* promastigotes (11) was used in all experiments. The organisms were maintained and grown in chemically defined RE-IX medium (12). Unless indicated, cells at midlogarithmic phase (3-4 \( \times \) 10^7 cells per ml) were harvested and subsequently washed twice at 6000 \( \times \) g for 15 min at 4°C in phosphate buffer (0.1 M, pH 7.1) containing 5 mM MgSO_4_. After the last wash, cells were resuspended in the same buffer to a final concentration of 1 mg of cell protein per ml (3.6 \( \times \) 10^9 cells per ml).

Transport Assays. All transport assays were conducted as described (10). The substrates used and their final concentrations were 0.1 mM 2-deoxy-D-[1,2-3H]glucose (2-[3H]Glc; 62.5 mCi/mmole; 1 Ci = 37 GBq) and 0.05 mM L-[3H]proline (123 mCi/mmole) in the presence of 25 mM cycloheximide.

**Determination of Cellular ATP Levels.** Measurements of adenosine triphosphate levels in *L. donovani* promastigotes were made by using a modification of the method described by Maloney and Wilson (13). Cells were treated as in the transport assays above. At given times, 25 \( \mu \text{l} \) of cold (4°C) 3 M perchloric acid was added to each of the 100-\( \mu \text{l} \) cell suspensions and kept in ice for 30 min. Subsequently 60 \( \mu \text{l} \) of 1 M KOH were added to neutralize these extracts, and they were maintained for an additional 30 min on ice. Prior to assay, 25 \( \mu \text{l} \) of these cell extracts were added to 1 ml of 45 mM glycylglycine (pH 7.4). Aliquots (250 \( \mu \text{l} \)) of a reagent solution containing luciferase (*Pho- nisin pyralis*) at 1.6 \( \mu \text{g} \)/ml and 0.7 \( \mu \text{M} \) D-luciferin (ATP Bioluminescence CLS kit, Boehringer Mannheim) were each mixed with an equal volume of cell extract in glycylglycine above in a BioVial scin-
tillation vial (Beckman). Ten seconds after mixing, each vial was placed in the well of a scintillation spectrophotometer (model LB9000, Beckman) that had been set to maximum sensitivity, and radioactivity was measured for 30 sec. Background radioactivity with no added ATP was 30–60 cpm and with 1 pmol of added ATP was 60,000 cpm. The rate of luminescence decay was 3% per min.

**Direct Measurement of H⁺ Fluxes.** A modification of the method described by West and Mitchell (14) was used to measure proton fluxes into *L. donovani* promastigotes.

Midlogarithmic-phase *L. donovani* promastigotes were washed twice at 6000 × g for 15 min at 4°C in a solution containing 150 mM choline chloride, 40 mM KSCN, 1 mM KCl, and 5 mM MgSO₄. The cells were resuspended to 1 mg of cell protein per ml in the same solution. Subsequently, 1 mM KCN was added to the cells, and they were incubated for 15 min at room temperature. After this, 0.2 mM N,N'-deccycleohexylcarbodiimide (DCCD) was added to these cells, and they were incubated for an additional 15 min. Subsequently, 2 ml of this cell suspension was placed in a pH electrode cell, where 1 μM of valinomycin was added and the pH was adjusted to 7.1 by the addition of either 10 mM HCl or 10 mM KOH, both made in 100 mM KCl solution. After pH equilibration, 10 μl of either 0.5 M 2-dGlc, D- or L-glucose, or L-proline was added to change the cell suspensions. Changes in pH were monitored by using a combination pH electrode (model 39595, Beckman).

**Oxygen Consumption Assays.** Oxygen consumption by *L. donovani* promastigotes was measured by using an oxygen electrode system (Yellow Springs Instrument).

**Protein Assays.** Total cell protein was determined as described by Lowry et al. (15).

**Cell Volume Measurements.** Promastigotes cell volume was determined by using [³⁵Cl]ulinin (0.5 mM, 3 μCi/ml) and [³⁵H]H₂O (10 μCi/ml) as described (10).

2-³⁵H[dGlc, [³¹Cl]ulinin, and [³⁵H]H₂O were from New England Nuclear; L-[³¹]proline was from Amersham; carbonyl-cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP) was from Aldrich; valinomycin, DCCD, ouabain, phlorizin, and cycloheximide were from Sigma; nigericin was from Eli Lilly.

**RESULTS**

Previously we have shown that 2-dGlc and D-glucose share a common surface membrane carrier and thus the transport of 2-dGlc in *L. donovani* promastigotes was representative of the glucose transport system of this organism (10). Moreover, we also observed that 70% of the intracellular 2-dGlc was freely exchanged with externally added 2-dGlc or D-glucose (10). Further, in the presence of 25 μM cycloheximide, less than 1% of the transported L-proline was present in the trichloroacetic acid precipitate of these cells (not shown).

Thus, under these conditions, the measured intracellular 2-dGlc and L-proline concentrations were representative of the net accumulations of these compounds.

**Active Transport of L-Proline and 2-dGlc.** Midlogarithmic-phase *L. donovani* promastigotes rapidly accumulated both 2-dGlc (Fig. 1A) and L-proline (Fig. 1B) and maintained steady-state levels for these compounds of 34 and 13 nmol, respectively, per mg of cell protein. In parallel experiments, the mean volume of the promastigotes was determined to be 4.32 ± 0.92 μl/mg of cell protein. Thus, the calculated intracellular concentrations of 2-dGlc and L-proline were 7.87 ± 1.7 mM and 3.0 ± 0.63 mM, respectively. It was assumed that all intracellular space was available for the accumulated substrates and, therefore, that their concentrations were 78.7 and 60 times higher than that of the external medium.

The effect of metabolic inhibitors on the transport and respiration rates of promastigotes is shown in Table 1. Washed untreated control *L. donovani* cells maintained a respiration rate of 145 nmol of O₂/mg of protein per min. Addition of low concentrations of iodoacetate completely inhibited both cellular respiration and the uptake of both L-proline and 2-dGlc. Cell respiration and transport of L-proline were also affected by 2-dGlc, which like iodoacetate significantly inhibits cellular glycolysis. Cells treated with cyanide had no respiratory activity, yet they maintained 20% of their transport activity for both substrates. These results indicated that both 2-dGlc and L-proline were actively transported by *L. donovani* promastigotes. Further, they also indicated that both glycolysis and respiration provided the energy for the transport activity of these systems.

Addition of 20 mM NaCl to the promastigote cells did not affect the steady-state level of either 2-dGlc or L-proline uptake (Table 2). Further, neither phlorizin nor ouabain, both of which inhibit sodium-coupled transport systems (16, 17), in the presence of NaCl had any effect on the uptake of either substrate. These results indicated that both transport systems were sodium independent. The latter observations are in agreement with those recently described by Bonay and Cohen (7), showing that L-proline uptake in *Leishmania* sp. was also sodium independent. Strikingly, promastigotes incubated in low concentrations of DCCD (20 μM) demonstrated a decreased uptake of both 2-dGlc (10.5 nmol/mg of protein).

![Fig. 1. Uptake of 2-dGlc (A) and L-proline (B) by *L. donovani* promastigotes. Cells were washed twice and resuspended in 0.1 M potassium phosphate (pH 7.4) containing 5 mM MgSO₄, at a final concentration of 1 mg of cell protein per ml. The above cells (100 μl) were equilibrated at 30°C for 10 min, and the assays were initiated by the addition of 0.1 mM 2-[³¹]dGlc (62.5 μCi/mmol) or 50 μM L-[³¹]proline (123 μCi/mmol) in the presence of 25 μM cycloheximide.](image)

<p>| Table 1. Effect of metabolic inhibitors on respiration rate and the transport of 2-dGlc and L-proline in <em>L. donovani</em> promastigotes |</p>
<table>
<thead>
<tr>
<th>Additions</th>
<th>Respiration rate, nmol of O₂ atoms/min per mg of protein</th>
<th>Uptake, nmol/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>145 ± 18</td>
<td>13.0 ± 1.73</td>
</tr>
<tr>
<td>KCN (1.0 mM)</td>
<td>0</td>
<td>3.0 ± 0.43</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>0</td>
<td>0.35 ± 0.15</td>
</tr>
<tr>
<td>(0.5 mM)</td>
<td>2-dGlc (10.0 mM)</td>
<td>44 ± 7.8</td>
</tr>
</tbody>
</table>

Transport assays were carried out as described in Fig. 1. KCN was added 2 min prior to the assay and iodoacetate and 2-dGlc were added 15 min prior to the assay.
Transport assays were carried out as described in Fig. 1. Phlorizin was added to the cells 5 min prior to the assay. The cells were preincubated with either ouabain or DCCD for 30 min.

*Respiration rate of the DCCD-treated cells was 107 nmol of O atoms/min per mg of protein.

DCCD is a lipophilic reagent that blocks protonated carboxyl groups and inhibits proton translocation ATPase activities (18, 19). Hence, the effect of DCCD on L. donovani may imply that an electrochemical gradient of protons (ΔμH+) across the parasite surface membrane is required to maintain active accumulation of both 2-dGlc and L-proline. Such a gradient is composed of the chemical gradient (ΔpH) and the membrane potential (Δψ) according to the equation:

\[ Δμ_{H^+} (mV) = Δψ - RT/FΔpH \]

(where \( F \) is the Faraday constant and Δ values are expressed as \( X_m - X_w \)). As was previously shown, ΔμH+ drives endergonic processes such as active transport of solutes across the surface membrane of prokaryotes (20, 21) and eukaryotes (22, 23). Experiments in the following sections were done to assess whether such a mechanism also drives the transport of 2-dGlc and L-proline in L. donovani promastigotes.

**Effect of Ionophores.** The individual effects of the ionophores valinomycin and nigericin on promastigotes cellular ATP levels are shown in Fig. 2 A and B and Table 3. The results demonstrated that the cellular ATP levels were diminished by ~10% with nigericin (3.4 μM) and unaffected by valinomycin (Fig. 2 A and B). FCCP, when added at a concentration of 1 μM, reduced the cellular ATP by ~21% (Table 3). Respiration rates of the promastigotes were not affected by any of these ionophores (data not shown). Thus, any effect that these ionophores might have on cellular transport activity would be due to changes in either the ΔpH or the Δψ across the parasite surface membrane.

L-proline transport was not affected in the presence of low concentrations of nigericin (2 μM). However, such transport was slightly inhibited (19%) at 3 μM nigericin (Fig. 2A and Table 3) and further inhibited (up to 46%) by 5 μM nigericin. Valinomycin, when added in low concentration (e.g., 0.1 μM), inhibited the cellular transport of L-proline by 28%. Inhibition of L-proline transport to 50% occurred at a valinomycin concentration of 1 μM; however, no further inhibition of cell transport was observed, even at valinomycin concentrations of up to 5 μM (Fig. 2B). Similar inhibition results were obtained with these ionophores on transport of 2-dGlc by the promastigotes (e.g., see Table 2).

Since these ionophores only partially inhibited the transport of 2-dGlc and L-proline, it seemed most likely that both ΔpH and Δψ were required to maintain normal levels of cellular transport. These results also suggested that the concomitant addition of both ionophores would cause the uncoupling of the ΔμH+, resulting in the inhibition of both 2-dGlc and L-proline uptake. The results of such an experiment are illustrated in Table 3. Valinomycin (0.1 μM)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Addition</th>
<th>ATP, nmol/mg of protein</th>
<th>Uptake, nmol/mg of protein</th>
<th>Inhibition of transport, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-dGlc</td>
<td>None</td>
<td>9.0 ± 0.08</td>
<td>33.6 ± 4.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Valinomycin (0.1 μM)</td>
<td>9.2 ± 0.06</td>
<td>26.0 ± 2.1</td>
<td>22.5</td>
</tr>
<tr>
<td></td>
<td>Nigericin (3.0 μM)</td>
<td>8.1 ± 0.14</td>
<td>27.9 ± 3.1</td>
<td>17.0</td>
</tr>
<tr>
<td></td>
<td>Valinomycin (0.1 μM)/nigericin (3.0 μM)</td>
<td>8.34 ± 0.10</td>
<td>10.4 ± 1.3</td>
<td>69.0</td>
</tr>
<tr>
<td></td>
<td>FCCP (1.0 μM)</td>
<td>7.2 ± 0.13</td>
<td>7.1 ± 0.65</td>
<td>79.0</td>
</tr>
<tr>
<td>L-Proline</td>
<td>None</td>
<td>9.0 ± 0.08</td>
<td>11.70 ± 1.84</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Valinomycin (0.1 μM)</td>
<td>9.2 ± 0.06</td>
<td>8.42 ± 0.78</td>
<td>28.0</td>
</tr>
<tr>
<td></td>
<td>Nigericin (3.0 μM)</td>
<td>8.1 ± 0.14</td>
<td>9.47 ± 1.03</td>
<td>19.0</td>
</tr>
<tr>
<td></td>
<td>Valinomycin (0.1 μM)/nigericin (3.0 μM)</td>
<td>8.34 ± 0.10</td>
<td>2.57 ± 0.15</td>
<td>78.0</td>
</tr>
<tr>
<td></td>
<td>FCCP (1.0 μM)</td>
<td>7.2 ± 0.13</td>
<td>1.76 ± 0.12</td>
<td>85.0</td>
</tr>
</tbody>
</table>


discussion
and nigericin (3 μM), when added separately, inhibited the transport of 2-dGlc by 22.5% and 17%, respectively, whereas L-proline transport was inhibited by 28% (0.1 μM valinomycin) and 19% (3 μM nigericin). Promastigotes treated with the valinomycin and nigericin combination at the above concentrations demonstrated an inhibition in both L-proline and 2-dGlc uptake of 78% and 69%, respectively. Cellular ATP levels were unaffected under these conditions. Similar results were obtained when 1 μM of the uncoupler FCCP was added to the promastigotes. The cumulative results indicate that the transport of both substrates is driven by the ΔμH⁺. Further, it seems that both the ΔpH and the Δψ are required to maintain normal levels of transport in these cells.

**Glucose/H⁺ and Proline/H⁺ Symport.** As was previously shown, ΔμH⁺ drives active uptake of solutes via symport translocation with protons (14, 22). To assess whether such a mechanism also occurs in *L. donovani* promastigotes, proline/H⁺ and glucose/H⁺ symport was measured directly by using a modification of the West and Mitchell method (14). Promastigotes were depleted of endogenic energy by sequential treatments with KCN and DCCD. Addition of D-glucose to the nonmetabolizing promastigotes induced an effective movement of protons into the cells (Fig. 3, trace A). Proton influx was instantaneously reversed by the addition of FCCP. Such proton efflux continued until protons were equilibrated across the parasite surface membrane. In parallel experiments L-glucose was added to such nonmetabolizing cells (Fig. 3, trace B). Under these conditions no proton movement was detected, demonstrating the molecular specificity of the D-glucose/H⁺ symport. The latter result further supports our previous observations concerning the stereospecificity of the glucose transport system of *L. donovani* (10). L-Proline, when added to nonmetabolizing promastigotes, also induced a rapid influx of protons into the cells (Fig. 4, trace A). When these cells were pretreated with FCCP prior to the addition of L-proline, no proton influx was detected (Fig. 4, trace B), indicating that protons were transported with this substrate. Further, data shown in Figs. 3 and 4 obviates any nonspecific effects of DCCD on either of the carriers for D-glucose or L-proline.

**Transport Driven by an Artificial Membrane Potential.** To impose an electrical potential across the parasite surface membrane, nonmetabolizing promastigotes as above, preloaded with potassium, were resuspended in a potassium-free medium containing valinomycin. As valinomycin markedly increases membrane permeability to potassium, an electrical potential (negative inside) will be created across the cell surface membrane (13). Thus, such an artificially imposed membrane potential drove the uptake of both L-proline and 2-dGlc (Fig. 5 and 6).

Cells resuspended in potassium-free medium containing valinomycin rapidly accumulated L-proline, reaching a maximum of 3.6 nmol/mg of protein within 1 min. This corresponds to a concentration gradient of 16.7. Subsequently, proline was released from these cells, paralleling the dissipation of the membrane potential (Fig. 5). The uptake of proline was inhibited when cells were resuspended in medium containing FCCP (Fig. 5). Cells resuspended in a potassium-containing medium (Fig. 5) demonstrated a gradual uptake of proline, which reflects equilibration of proline across the cell membrane.

Similar results were obtained for 2-dGlc transport by using the conditions outlined above (Fig. 6). The maximal uptake in valinomycin-treated cells occurred within 0.75 min, reaching a concentration gradient of 9.6. The results shown in Figs. 5 and 6 demonstrate that a Δψ component drives the active transport of both L-proline and 2-dGlc.

Attempts were made to artificially generate ΔpH across the parasite’s membrane. However, under the conditions tested, e.g., pH shift, the cells became freely permeable or lysed immediately. Thus, it was not possible to measure any membrane transport under such conditions.

**Conclusions.** The results described in this report demonstrated the active nature of the transport systems of L-proline and D-glucose in *L. donovani* promastigotes. Both transport systems are electrogenic, driven by the proton electrochemical gradient across the parasite surface membrane by
Fig. 5. $\Delta \phi$ driven uptake of L-proline in L. donovani promastigotes. Promastigotes were washed in 10 mM Tris/2-(N-morpholino)ethanesulfonate (pH 7.2) containing 150 mM KCl and 3 mM MgCl$_2$ and were resuspended to 1 mg of cell protein per ml in the same solution. These cells were incubated with 0.5 mM Na$_3$ and 0.2 mM DCCD for 20 min at room temperature. Subsequently, 3-ml aliquots of the cell suspension were centrifuged, and the pellets were resuspended in 8 ml of 10 mM Tris/2-(N-morpholino)ethanesulfonate (pH 7.2) containing 150 mM NaCl and 5 mM valinomycin (solid) or 150 mM NaCl, 5 mM valinomycin, and 5 mM FCCP (dotted). The assay solutions also contained 50 $\mu$M L-[3H]proline (125 mCi/mmol).

maintaining symport translocation with protons. Such a mechanism has been described in fungi (see ref. 23 for re-

Fig. 6. $\Delta \phi$ driven uptake of 2-deGlc in L. donovani promastigotes. Cell washing and treatment were carried out as in Fig. 5. Pellets of the potassium-preloaded cells were resuspended in 8 ml of 10 mM Tris/2-(N-morpholino)ethanesulfonate (pH 7.2) containing 150 mM NaCl and 5 $\mu$M valinomycin (solid) or 150 mM KCl and 5 $\mu$M valinomycin (dotted) or 150 mM NaCl, 5 $\mu$M valinomycin, and 5 $\mu$M FCCP (dotted). The assay solutions also contained 0.1 mM 2-[3H]deGlc (62.5 mCi/mmol).