# Identification of a surface membrane proton-translocating ATPase in promastigotes of the parasitic protozoan *Leishmania donovani*

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ATPase activities were measured in surface membranes and mitochondria isolated from promastigotes of the parasitic protozoan *Leishmania donovani*. The two enzymes were differentiated on the basis of pH optima, inhibitor sensitivity and by immunochemical methods. The surface-membrane (SM-) ATPase had an activity of 100 nmol/min per mg of protein, which was optimal at pH 6.5. The enzyme was $Mg^{2+}$-dependent, partially inhibited by $Ca^{2+}$, and unaffected by $Na^+$ or $K^+$. The SM-ATPase was inhibited by orthovanadate, *NN'*-dicyclohexylcarbodi-imide, and *N*-ethylmaleimide [$IC_{50}$ (concentration causing half-maximal inhibition) 7.5, 25 and 520 $\mu$M respectively]; however, it was unaffected by ouabain, azide or oligomycin. The SM-ATPase demonstrated a $K_m$ of 1.05 mM and a $V_{max.}$ of 225 nmol/min per mg of protein. Moreover, fine-structure cytochemical results demonstrated that the SM-ATPase was localized to the cytoplasmic lamina of the parasite SM. A method was devised for the isolation of SM-derived vesicles. These were used to demonstrate the proton-pumping capacity of the SM-ATPase. Cumulatively, these results constitute the first demonstration of a surface-membrane proton-translocating ATPase in a parasitic protozoan.

## INTRODUCTION

Species of the parasitic protozoan *Leishmania* are the causative agents of a wide variety of human cutaneous, mucocutaneous and visceral diseases. These organisms have a digenetic life cycle in which both developmental stages reside in hydrolytic environments, i.e., as extracellular flagellated promastigote forms within the alimentary tract of their sandfly vector and as obligate intracellular amastigote forms within secondary lysosomes of human macrophages (Chang & Dwyer, 1978; Berman *et al.*, 1979; Dwyer, 1979; Tim *et al.*, 1980; Bordier *et al.*, 1982).

Previously we demonstrated that *L. donovani* promastigotes possess active transport systems which are protonmotive-force (pmf)-driven (Zilberstein & Dwyer, 1985), i.e., a proton electrochemical gradient is created across the parasite surface membrane (SM) which is coupled to transport systems by maintaining symport translocation of specific substrates with protons. Such plasma-membrane activities have been described in bacteria (Zilberstein *et al.*, 1979; Kaback, 1983) and fungi (Eddy & Nowacki, 1971; Goffeau & Slayman, 1981; Perlin *et al.*, 1984) as well as in membranes of various cell organelles (Oshumi & Anraku, 1981; Fishkes & Rudnick, 1982; Reeves, 1983).

In a variety of cells and organelles, membrane-bound proton-translocating ATPases ($H^+$-ATPases) have been demonstrated to be energy transducers which utilize the energy of ATP hydrolysis to generate a proton electrochemical gradient (Goffeau & Slayman, 1981; Oshumi & Anraku, 1981; DuPont *et al.*, 1982; Galloway *et al.*, 1983; Reeves, 1983; Al-Awqati, 1986; Pederson & Carafoli, 1987). As pmf drives energy-transduction processes across the leishmanial SM (Zilberstein & Dwyer, 1985), it was anticipated that an $H^+$-ATPase acting as a primary proton pump should be present in the plasma membrane of this organism. Previously, it was suggested that the leishmanial SM might contain an ATPase (Dwyer & Gottlieb, 1983). In the present study we have identified and characterized a vanadate-sensitive $Mg^{2+}$-dependent ATPase activity in isolated SMs of *L. donovani* promastigotes and demonstrated that this enzyme is a proton pump.

Part of this work has already been presented in abstract form (Zilberstein & Dwyer, 1987).

## MATERIALS AND METHODS

### Materials

[$\gamma$-$^{32}$P]ATP was from ICN, Boston, MA, U.S.A.; $^{125}$I-protein A and Aquasol were from New England Nuclear, Boston, MA, U.S.A.; Acridine Orange, activated charcoal, leupeptin, ATP, ADP and Iodonitrotetrazolium Violet (INT) were from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were of analytical grade.

### Parasite maintenance and growth conditions

A cloned line of *L. donovani* promastigotes, strain 1-S (Dwyer, 1977), was used in all experiments. The organisms were maintained and grown in Medium 199 supplemented with 20% (v/v) fetal-calf serum.

Abbreviations used: PAGE, polyacrylamide-gel electrophoresis; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; DCCD, *NN'*-dicyclohexylcarbodi-imide; pmf, protonmotive force; SM, surface membrane; $IC_{50}$, concentration causing half-maximal inhibition; $H^+$-ATPase, proton-translocating ATPase; SM-ATPase, surface-membrane ATPase; INT, Iodonitrotetrazolium Violet; SCB, 0.2 M-sodium cacodylate buffer, pH 7.0.

### Isolation of SMs

Parasites were harvested at a concentration of (2–4) $\times 10^7$ cells/ml (6000 *g*, 30 min, 4 °C), washed once in Hanks Balanced Salt Solution and then twice in 10 mM-Tris/HCl/145 mM-NaCl, pH 7.5. SMs were isolated as previously described (Gottlieb & Dwyer, 1981*a*), except that leupeptin (25 $\mu$g/ml) was included throughout.

### Isolation of SM vesicles

Parasites were harvested and washed as described above and resuspended to $1 \times 10^9$ cells/ml in lysis buffer, which contained 3 mM-EDTA and 10 mM-Tris/HCl, pH 8.2. Cell suspensions were frozen in liquid $N_2$ and then thawed at room temperature. Subsequently these were homogenized with 10–15 strokes in a tight-fitting Dounce-type homogenizer (Kontes Glass, Vineland, NJ, U.S.A.) and frozen and thawed as described above. This procedure was repeated until > 90% of the cells were lysed. These lysates were made 10 $\mu$g/ml with respect to leupeptin and centrifuged in a swinging-bucket rotor (HB4; Sorval Instruments, Newtown, CT, U.S.A.) at 15900 *g* for 30 min at 4 °C. The supernatants were removed and re-centrifuged (SS-34 rotor; Sorval) at 48000 *g* for 45 min at 4 °C. Pellets were resuspended and washed once in 20 mM-Tris/HCl/3 mM-$MgCl_2$, pH 8.2, containing 10 $\mu$g of leupeptin/ml (TM buffer) by centrifugation as described above. Subsequently these pellets were resuspended in 12 ml of TM buffer. Aliquots (2 ml) of this suspension were overlayed on to step gradients consisting of 2 ml each of 2.5 M-, 1.0 M- and 0.5 M-sucrose in TM buffer. These were centrifuged in a SW-27 swinging-bucket rotor (Beckman Instruments, Palo Alto, CA, U.S.A.) at 75000 *g* for 4 h at 4 °C. Fractions from these gradients were assayed for SM and mitochondrial enzyme marker activities as described below. Aliquots of SM-derived vesicles were washed twice in 10 mM-Tris/HCl/3 mM-$MgCl_2$, pH 6.5, containing 10 $\mu$g of leupeptin/ml by centrifugation (48000 *g*, for 30 min at 4 °C), resuspended in this buffer at a concentration of 1 mg of protein/ml and frozen and stored at −70 °C.

### Iodination of intact cells

Promastigotes of *L. donovani* were surface-iodinated by previously described methods (Gardiner & Dwyer, 1983). SM vesicles were derived from these surface-labelled cells as outlined above.

### Enzyme markers

The following enzymes were used as markers of the subcellular fractions: tartarate-resistant acid phosphatase (Gottlieb & Dwyer, 1981*b*) for SMs (i.e. plasma membranes containing attached subpellicular microtubules) and succinate:INT reductase for mitochondria (Morre, 1971).

### ATP-hydrolysis assays

ATPase activity was measured by determining the release of $P_i$ from [$\gamma$-$^{32}$P]ATP as described previously (Zilberstein *et al.*, 1986*a*). The reaction medium at a final volume of 0.2 ml contained: 10 mM-Tris/Mes at pH 6.7, 10–40 $\mu$g of membrane protein, 2 mM-MgATP and [$\gamma$-$^{32}$P]ATP ($2.5 \times 10^6$ c.p.m.). The assays were carried out at 37 °C for 5 min and terminated by the addition of 0.9 ml of activated-charcoal suspension (10%, w/v, in 50 mM-$KH_2PO_4$, pH 4.0). Samples were vortex-mixed and incubated for 10 min at 4 °C and vortex-mixed again to facilitate adsorption of unhydrolysed ATP on to the activated charcoal. Subsequently, these samples were centrifuged in an Eppendorf microcentrifuge for 5 min and 0.6 ml of the supernatant mixed with 4 ml of Aquasol and counted for radioactivity in a liquid-scintillation spectrometer.

### pH measurements

Acidification of membrane vesicles was measured by using Acridine Orange as described by Cidon *et al.* (1983). Reaction mixtures contained 100 $\mu$g of membrane vesicle protein in 1 ml of 10 mM-Tris/Mes (pH 6.5)/2 $\mu$M-Acridine Orange. Assays were initiated at 30 °C by adding MgATP to a final concentration of 2 mM. Changes in absorbance at 492–520 nm were measured in an Aminco dual-beam spectrophotometer (American Instrument Co., Silver Spring, MA, U.S.A.).

### Electron microscopy

**(1) Fine structure.** Subcellular vesicle fractions isolated by density-gradient centrifugation were fixed and processed for thin-section electron microscopy as previously described (Dwyer, 1980).

**(2) Fine-structure cytochemistry.** The basic incubation medium used in these experiments was modified from that used in previous studies on mammalian cells and subcellular fractions (Marchesi & Palade, 1967; Wagner *et al.*, 1972; Windell, 1972; Farquhar *et al.*, 1974; Cheng & Farquhar, 1976). This incubation medium was composed of 0.1 M-Tris/maleate, appropriate cations (added to 10 mM as chlorides), 4 mM-adenosine nucleotides (see below) and 0.5 mM-$Pb(NO_3)_2$, adjusted in various experiments from pH 6.8 to 8.0. The adenosine nucleotides, ATP (magnesium or sodium salt as desired) and ADP (sodium salt) were made as 10 mM stocks in distilled water immediately before use and neutralized. These stocks were added dropwise to a 2-fold-concentrated stock buffer solution with vortex mixing. Similarly, $Pb(NO_3)_2$ was added last to the reaction mixtures in 10 $\mu$l aliquots. The incubation media were micro-filtered (Millex-GS filter; 0.22 $\mu$m pore size; Millipore Corp., Bedford, MA, U.S.A.) immediately before use. All media were pre-equilibrated to 37 °C for 10 min before the addition of cells or subcellular fractions.

Live promastigotes were resuspended to $5 \times 10^7$ cells/ml in 5 ml of the various reaction media and incubated at 37 °C for 30 min with continuous gentle rocking agitation. Reactions were terminated by the addition of 5 ml of ice-cold 4% (v/v) glutaraldehyde (EM grade; Polysciences Inc., Warrington, PA, U.S.A.) in 0.2 M-sodium cacodylate buffer, pH 7.0 (SCB), immediately mixed and kept on ice for 20 min. The samples were centrifuged at 6000 *g* for 10 min at 4 °C and the pellets were resuspended in fresh 2% (v/v) glutaraldehyde in 0.1 M-SCB and kept on ice for 60 min. Subsequently, the samples were washed three times in 0.1 M-SCB by centrifugation as described above and stored overnight at 4 °C. After an additional SCB wash, samples were transferred to 1.5 ml polyethylene Microfuge tubes and centrifuged in a Microfuge (Beckman Instruments, Palo Alto, CA, U.S.A.) for 3–5 min. These samples were post-fixed in 1% (v/v) $OsO_4$ (Polysciences) in 0.1 M-SCB, pH 7.4, for 60 min on ice, washed three times in SCB, dehydrated, embedded, sectioned, stained and observed

with a Joel-100C electron microscope as previously described (Dwyer, 1980).

In several experiments lysed cell ghosts were also examined. Washed promastigotes were resuspended at $5 \times 10^7$ cells/ml in ice-cold 10 mM-Tris/HCl, pH 7.5, and vortex-mixed vigorously for 30 s and then pelleted by centrifugation at 8000 *g* for 30 min at 4 °C. The pellet was resuspended, then washed three times in Tris/maleate buffer by centrifugation as described above. These lysed cell ghosts were prepared for cytochemical experiments as described above for intact cells.

In other cytochemistry experiments, isolated SM fractions were sedimented at 48000 *g* for 30 min at 4 °C, subsequently resuspended in the Tris/maleate buffer described above, passed through a 22-gauge needle in the various incubation media used for the cytochemical assays described above at approx. 300 $\mu$g of total membrane protein/ml. These samples were treated as described for intact cells; however, all centrifugation steps before post-fixation were at 25000 *g* for 15 min at 4 °C.

In several preliminary experiments, living cells, lysed cell ghosts and isolated SMs in Tris/maleate were fixed in suspension (i.e. $5 \times 10^7$/ml and isolated SMs 300 $\mu$g of protein/ml respectively) with an equal volume of ice-cold 4% (v/v) glutaraldehyde in 0.1 M-SCB, pH 7.4, for various periods (i.e., 10, 20 and 40 min respectively) on ice. Subsequently, these samples were centrifuged as described above and washed extensively (i.e., seven changes of 1 h each in 100 times their volume) in Tris/maleate on a platform rocker at 4 °C. After an overnight wash at 4 °C and one additional wash in this buffer, pelleted samples were treated as described above for unfixed samples.

### Immunochemical assays

Isolated SMs were solubilized in Laemmli (1970) sample buffer, separated by SDS/PAGE (Laemmli, 1970) and transferred to nitrocellulose (Towbin *et al.*, 1979) for analysis with various antibodies. Monospecific rabbit antibodies against the $\beta$-subunit of the *Escherichia coli* $F_0F_1$-ATPase were kindly provided by Dr. Nathan Nelson of the Roche Institute of Molecular Biology, Nutley, NJ, U.S.A. Nitrocellulose blots were blocked, incubated with primary antibody, washed, incubated with $^{125}$I-protein A and autoradiographed at −70 °C with Kodak X-Omat AR film all as previously described (Zilberstein *et al.*, 1986*a*). Control samples were treated as described above, except that normal rabbit serum was used.

### Protein assays

Protein was determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

## RESULTS

### Purity of the isolated SMs and mitochondria

SMs and mitochondria of *L. donovani* promastigotes were isolated from cell lysates by using sucrose-density-gradient methods (Gottlieb & Dwyer, 1981*a*). The purity of the isolated SM and mitochondria fractions were assessed on the basis of the distribution of two characteristic enzymes: (1) SM-bound acid phosphatase, which was previously described and demonstrated to be a useful marker for such membranes (Gottlieb & Dwyer, 1981*b*; Dwyer & Gottlieb, 1983), and (2) succinate:INT reductase activity, for mitochondria (Morre, 1971). The isolated mitochondrial fraction contained 83% of the total succinate:INT reductase activity which was applied to the gradient, whereas only 8% of this activity was present in the isolated SM fraction. In contrast, acid phosphatase activity was present in both the surface membrane (53%) and mitochondrial fractions (39%), indicating that the latter was contaminated with surface membranes. The mean distribution of these markers in the several subcellular fractions is given in Table 1. An average purification factor of 18.3 for the surface membranes was calculated from the results in this Table. The SM fraction contained less than 8% of the succinate:INT reductase activity compared with that in the mitochondrial fraction (Table 1), indicating the relatively high purity of the isolated SMs. The mitochondrial fraction demonstrated an average purification factor of 7.8. However, this fraction also contained a significant amount of SMs (i.e. 50% of the acid phosphatase specific activity compared with that of the SM fraction).

**Table 1. Distribution of enzyme markers in the subcellular fractions of *L. donovani* promastigotes**

SMs and mitochondria were separated on a sucrose density gradient as described in the Materials and methods section. Acid phosphatase and succinate:INT reductase activities were used as SM and mitochondrial markers respectively. Each value represents the mean ± S.D. for six experiments.

| Fraction | Enzyme activity ($\mu$mol/min per mg of protein) | |
|---|---|---|
| | Acid phosphatase | Succinate: INT reductase |
| Cell lysate | 0.014 ± 0.003 | 0.220 ± 0.07 |
| Mitochondria | 0.127 ± 0.025 | 1.720 ± 0.09 |
| SMs | 0.256 ± 0.009 | 0.133 ± 0.01 |

### pH optimum

The ATP-hydrolysis activities in the isolated SM and mitochondrial fractions as a function of pH are shown in Fig. 1. For these experiments, both of these fractions were derived from the same promastigote cell preparations.

The SMs demonstrated optimal activity at pH 6.5, hydrolysing 100 nmol of ATP/min per mg of protein (Fig. 1). In contrast, maximal mitochondrial ATPase activity occurred at pH 8.0, with a rate of 127 nmol/min per mg of protein. The latter showed 76.3% less activity at pH 6.7, indicating that the mitochondrial ATPase had insignificant activity at the pH optimum of the SM enzyme.

### Inhibitors

The effect of various inhibitors on the SM and mitochondrial ATPases was assessed at their respective pH optima and the results are summarized in Table 2. The mitochondrial $F_0F_1$-ATPase inhibitors azide and oligomycin were used to further differentiate between the *L. donovani* mitochondrial and the SM-ATPase activities.

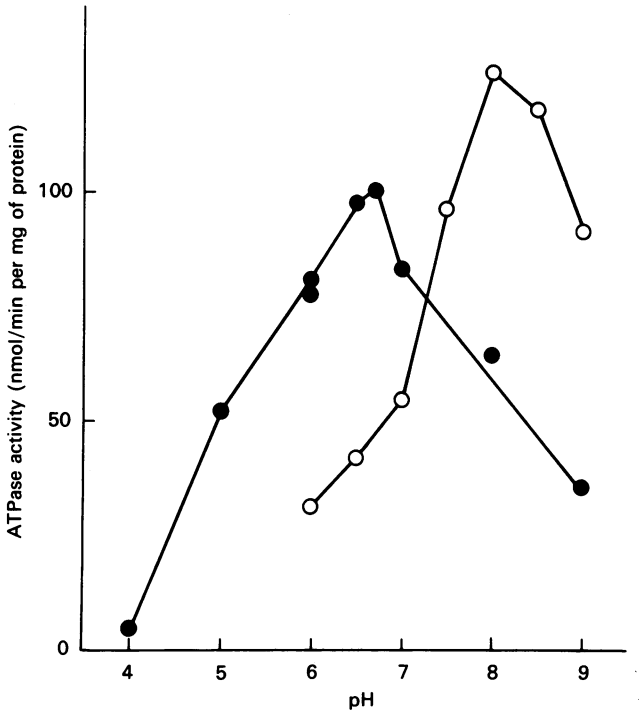


**Fig. 1. pH profile of ATPase activity in isolated SM and mitochondria of *L. donovani* promastigotes**

The reaction mixture (0.2 ml) contained 10 mM-Tris/Mes (pH adjusted as indicated), 2 mM [$\gamma$-$^{32}$P]ATP (8 Ci/mmol), 2 mM-$MgCl_2$ and 20 $\mu$g of membrane protein. The assays were carried out at 37 °C for 5 min, terminated by the addition of 0.9 ml of activated charcoal (10%, w/v) and the released phosphate assayed as described in the Materials and methods section. The isolated SMs were assayed in the presence of 0.1 mM-$NaN_3$ (●) and the mitochondria with 10 $\mu$M-orthovanadate (○).

**Table 2. Effect of various inhibitors on the SM- and mitochondria ATPase activities of *L. donovani* promastigotes**

SMs and mitochondria were assayed at pH 6.7 and at pH 8.0 respectively. Abbreviation: N.I., not inhibited.

| Inhibitor | $IC_{50}$ ($\mu$M) SMs | Mitochondria |
|---|---|---|
| Azide | N.I. | 25 |
| Oligomycin | N.I. | 0.5† |
| DCCD | 33 | 25 |
| *N*-Ethylmaleimide | 520 | > 1000 |
| Orthovanadate | 7.5 | N.I. |
| Ouabain | N.I. | N.I. |

† Given in $\mu$g/ml.

ATPase activity in the mitochondrial fraction was significantly affected by azide, a 50% inhibition at 25 $\mu$M being demonstrated. The mitochondrial enzyme was also sensitive to oligomycin, reaching a 50% inhibition at 0.5 $\mu$g/ml. However, both azide and oligomycin had no apparent effect on ATPase activity in the SM fraction (Table 2). DCCD inhibited the activity of both the SM- and mitochondrial ATPases. *N*-Ethylmaleimide decreased the SM-ATPase activity by 50% at 520 $\mu$M (Table 2) and by 100% at 1 mM (results not shown). By contrast, the mitochondrial ATPase was less sensitive to *N*-ethylmaleimide, a 50% inhibition only being demonstrated at concentrations above 1 mM. Ouabain, an inhibitor of the mammalian $Na^+/K^+$-ATPases, at concentrations of up to 1 mM had no deleterious effect on the SM-ATPase activity.

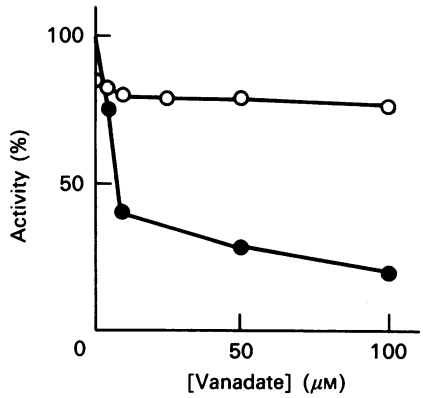


**Fig. 2. Effect of orthovanadate on the ATPase activities of isolated SMs and mitochondria of *L. donovani* promastigotes**

SM- and mitochondrial ATPase activities were assayed at pH 6.5 and 8.0 respectively. The assays were performed as described in Fig. 1. ●, SMs; ○, mitochondria.

**Table 3. Cation requirements of the *L. donovani* promastigote SM-ATPase**

| Substrate* | Additions | ATPase activity (nmol/min per mg of protein) |
|---|---|---|
| $Na_2ATP$ | None | 0 |
| | KCl + NaCl (10 mM) | 0 |
| MgATP | None | 135 |
| | EDTA (3 mM) | 3.8 |
| | NaCl (10 mM) | 156 |
| | KCl (10 mM) | 148 |
| | $CaCl_2$ (5 mM) | 124 |
| | $CaCl_2$ (10 mM) | 93 |

* ATP was added at final concentration of 3 mM.

Orthovanadate caused significant inhibition of the SM-ATPase activity, demonstrating an $IC_{50}$ of 7.5 $\mu$M and almost complete inhibition (84%) at 100 $\mu$M (Table 2 and Fig. 2). However, the mitochondrial ATPase activity was not affected by this compound, even at concentrations of up to 100 $\mu$M. The slight apparent inhibition of ATPase activity observed in the mitochondrial fraction with orthovanadate presumably reflects the ATPase activity of SMs which contaminate this fraction.

### Cation requirements

The cation requirements of the SM-ATPase are summarized in Table 3. Membranes incubated in buffer containing $Na_2ATP$ as a substrate showed no ATP-hydrolysis activity. Addition of $K^+$ to these preparations

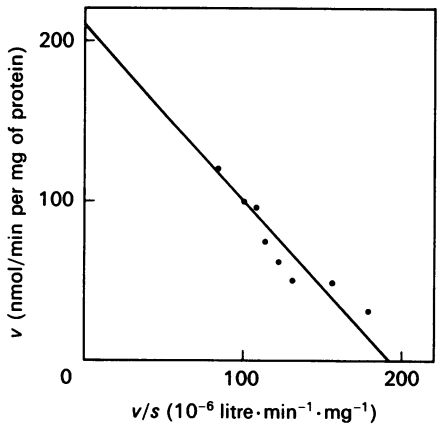


**Fig. 3. Eadie–Hofstee plot of the *L. donovani* SM-ATPase activity**

Initial rates of ATPase activity (1 min) were carried out as described in Fig. 1.

caused no enhancement of the ATPase activity. On the other hand, membranes incubated with MgATP had a significant rate of ATP hydrolysis (135 nmol/min per mg of protein). In parallel experiments we determined that 1 mol of $Mg^{2+}$ was required/mol of ATP in order to maintain optimal membrane ATPase activity (results not shown). Addition of EDTA to 3 mM to such mixtures completely inhibited the ATPase activity. Further, neither NaCl nor KCl had any significant effect on the $Mg^{2+}$-dependent ATP hydrolysis by this enzyme. However, $CaCl_2$ caused a slight inhibition at 5 mM and a 40% inhibition at 10 mM. These results indicate the $Mg^{2+}$-dependency of this ATPase.

### Kinetic analysis

The kinetic properties of the *L. donovani* SM-ATPase were estimated by using the Eadie–Hofstee plot (Fig. 3). The straight line obtained under all experimental conditions demonstrates that Michaelis–Menten kinetics apply to this enzyme. The calculated apparent $K_m$ is 1.05 mM and the $V_{max.}$ is 225 nmol/min per mg of protein.

### Fine-structure localization of surface membrane ATPase activity

No detectable enzyme reaction products were observed in samples in which intact living cells were incubated in any of the cytochemical reaction media. These results indicated that the ATPase activity was not externally oriented in the SM of intact cells. This is in contrast with both the previous biochemical and cytochemical results concerning the leishmanial SM acid phosphatase and 3′- and 5′-nucleotidase (Gottlieb & Dwyer, 1981*b*; Dwyer & Gottlieb, 1984) activities, all of which are externally oriented enzymes and thus would not contribute to the cytochemical results detailed below. Similar uniform negative results were also obtained with intact cells, lysed cell ghosts and isolated SM preparations which were pretreated with glutaraldehyde (i.e., regardless of the fixation span or washing period) before incubation with substrates for cytochemistry. These results demonstrated the sensitivity of the ATPase activity to aldehyde fixation, similar to that of the

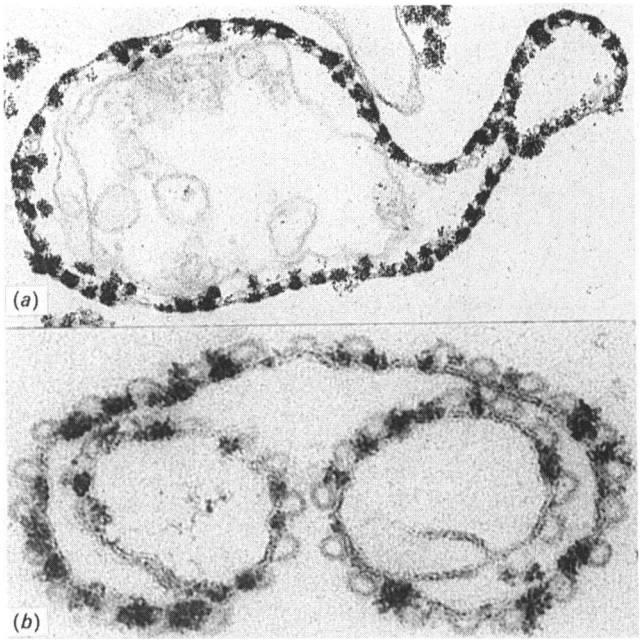


**Fig. 4. Fine-structure localization of *L. donovani* SM-ATPase activity**

(*a*) Cross-tangential section through a lysed (ghost) *L. donovani* promastigote incubated with MgATP as substrate. An electron-dense enzyme reaction product is deposited between the attached microtubules on the cytoplasmic surface of the plama membrane of this cell. Magnification ×57000. (*b*) Cross-section through an isolated *L. donovani* promastigote SM incubated in MgATP as a substrate. Attached microtubules serve as a marker for the cytoplasmic surface of these membranes. Note that the electron-dense enzyme-reaction product is distributed between these microtubules on the cytoplasmic surface of this membrane. Magnification ×127000.

parasite's two nucleotidases (Dwyer & Gottlieb, 1984), but in contrast with the SM acid phosphatase, which retains or regains activity after glutaraldehyde treatment (Gottlieb & Dwyer, 1981*a*). Further, preparations of living intact cells, lysed cell ghosts and isolated SMs incubated in control reaction mixtures lacking ATP substrates were devoid of any enzyme reaction products (results not shown). Similar negative cytochemical results were obtained with cells and subcellular fractions incubated in reaction media containing ADP as substrate.

Lysed ghosts incubated in reaction media containing MgATP demonstrated an electron-dense lead phosphate–ATPase reaction product distributed over the entire cytoplasmic face of the SM (Fig. 4*a*). As the subpellicular microtubules remained attached to the membranes, the deposition and orientation of the substrate reaction product permitted unequivocal localization of the enzyme. In that regard, the enzyme reaction product was deposited on the inner lamina of the membrane but not apparently on the attached microtubules. Further, in five separate experiments, the ATPase reaction product was never observed on the external surface of any of the lysed-cell-ghost preparations. Isolated SMs incubated in the presence of MgATP have an electron-dense ATPase reaction product distributed on their cytoplasmic lamina (Fig. 4*b*). Again, the presence of attached microtubules

permitted the unequivocal identification of the surface origin of these membranes and assigned the localization of the enzyme activity to their cytoplasmic surface. Deposition of the ATPase reaction product appeared to be restricted to the membrane inner lamina and not on the free faces of the attached microtubules.

No apparent quantitative differences in the amount of ATPase reaction product was evident in samples incubated in reaction mixtures at pH values between 6.8 and 7.6 (in 0.2 pH unit increments); however, the overall structural integrity of the membranes was best preserved at pH 7.0 (e.g. Figs. 4*a* and 4*b*). Samples incubated at pH 8 had enzyme reaction product deposited on their membranes, albeit qualitatively less than those treated at pH 7.0 (results not shown). No differences were observed in either the amount or extent of enzyme reaction product among samples incubated in MgATP alone or with the addition of NaCl, KCl or $CaCl_2$. However, samples incubated with MgATP in the presence of EDTA (5 mM) lacked any detectable enzyme reaction products. Further, lysed cell ghosts and isolated SM incubated in $Na_2ATP$ alone or in the presence of KCl were devoid of ATPase reaction products. However, addition of $MgCl_2$ (10 mM) to such preparations resulted in demonstrable enzyme-reaction-product deposition. No diminution or inhibition of ATPase reactivity was observed in samples incubated in MgATP in the presence of either $NaN_3$ or ouabain. However, ATPase reactivity was completely abolished in samples incubated in MgATP in the presence of orthovanadate (results not shown). The cumulative ATPase cytochemical results obtained with both lysed cell ghosts and isolated SMs are in overall agreement with the enzymic data presented above.

### Characterization of SM-derived vesicles

The foregoing results were obtained by using isolated SM which possessed attached subpellicular microtubules (Dwyer, 1980). Such membranes were previously shown to assume a stable open sheet-like configuration, presumably owing to their attached microtubules (Dwyer, 1980). In order to demonstrate proton-pumping capacity, sealed membrane vesicles are required. Therefore we devised a procedure for the isolation of SM-derived vesicles from *L. donovani* promastigotes.

SM vesicles were isolated from frozen–thawed cell lysates by sucrose-density-gradient methods. These vesicles sedimented to the interface between the 0.5 M- and 1.0 M-sucrose layers in such gradients, having an apparent density of 1.127 g/cm$^3$. The typical yields from such preparations were low, representing 0.1–0.2% of the total cell-lysate protein. Fig. 5 shows the fine structure of a typical gradient-isolated vesicle preparation. Such preparations appeared uniform in nature and were devoid of any apparent mitochondrial contamination. As shown in Fig. 5, these vesicles lacked any attached cytoskeletal elements.

Previously, we reported (Zilberstein *et al.*, 1986*b*) that such vesicle preparations were highly enriched in all of the enzyme markers described for isolated *L. donovani* SMs (i.e. tartarate-resistant acid phosphatase, 3′- and 5′-nucleotidases) and demonstrated that they contained the SM D-glucose transporter. Currently the isolated membrane vesicles demonstrated a high specific activity for membrane-bound acid phosphatase, but virtually none for succinate:INT reductase (Table 4). To demonstrate

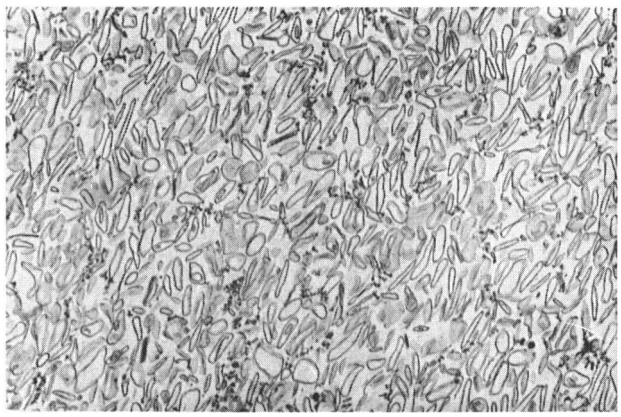

**Fig. 5. Electron micrograph of ultrathin sections through sucrose-density-gradient-isolated SM vesicle preparations from *L. donovani* promastigotes**

Magnification ×16900.

**Table 4. Distribution of marker enzymes in cell lysates and isolated SM vesicles of *L. donovani* promastigotes**

The enzymic assays were carried out as in Table 1. Each value represents the mean ± S.D. for four experiments.

| | Activity (nmol/min per mg of protein) | |
|---|---|---|
| Fraction | Acid phosphatase | Succinate:INT reductase |
| Lysates | 16 ± 3 | 326 ± 28 |
| Membrane vesicles | 514 ± 36 | Not detected |

further their cell-surface origin, membrane vesicles were prepared from [$^{125}$I]Iodogen-surface-labelled intact cells. Sucrose-density-gradient fractions of these were analysed for the distribution of both $^{125}$I-labelled material and tartarate-resistant acid phosphatase activity. The results of such an experiment are shown in Fig. 6, which demonstrates that both the membrane-bound acid phosphatase activity and the radiolabelled activity were coincident in fraction 4 containing the membrane vesicles. Such vesicles were also analysed for the presence of ATPase activity. As shown in Table 5, the vanadate-sensitive ATPase was co-purified with the vesicles and contained virtually no azide-sensitive ATPase activity, indicating the lack of mitochondrial contamination in this fraction. In parallel experiments, the sidedness of these vesicles was assessed by measuring the membrane-bound acid phosphatase activity (which is externally oriented on the surface of promastigotes) in intact and Triton X-100-permeabilized preparations. The results showed that permeabilized vesicles possessed twice the specific activity of intact vesicles, suggesting that at least 50% of this fraction had an inside-out orientation.

Recently we demonstrated that the mitochondrion of *L. donovani* promastigotes possessed an $F_0F_1$-type ATPase (D. Zilberstein, V. Liveanu & D. M. Dwyer, unpublished work). In that study we showed that this ATPase contained the highly conserved $\beta$-subunit, since it cross-reacted with antibodies made against the $\beta$-

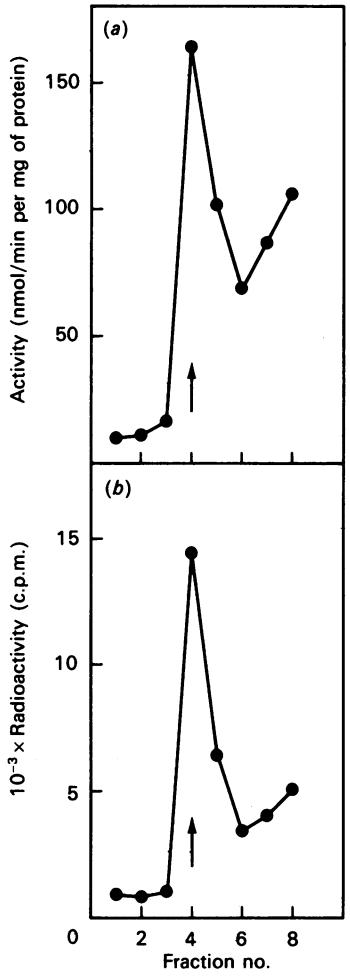


**Fig. 6. Isolation of SM-derived vesicles**

Promastigotes of *L. donovani* were harvested and washed twice in 20 mM-Tris/HCl containing 145 mM-NaCl (Tris/saline buffer) and resuspended to $5 \times 10^8$ cells/ml in the same buffer. A 20 ml aliquot from the promastigote suspension was removed and was surface-labelled with [$^{125}$I]Iodogen as described in the Materials and methods section. The labelled cells were mixed with the rest of the unlabelled suspension and SM vesicles were prepared as described in Fig. 5. Fractions (1 ml each) were collected from the sucrose gradient and were tested for radioactivity (*b*) and tartarate-resistant acid phosphatase activity (*a*).

**Table 5. Purification of SM-derived vesicles from *L. donovani* promastigotes**

Abbreviations: V-ATPase, vanadate-sensitive ATPase activity; A-ATPase, azide-sensitive ATPase activity; AcP, tartarate-resistant acid phosphatase activity.

| Ratio | Lysates | Membrane vesicles |
|---|---|---|
| V-ATPase/AcP | 1.4 | 1.65 |
| A-ATPase/AcP | 1.6 | 0 |

subunits from spinach (*Spinacia oleracea*) chloroplasts, yeast mitochondria and *Escherichia coli* $F_0F_1$-ATPases. The latter antibody was used to differentiate further the mitochondrial ATPase and SM-ATPase. Immunoblot

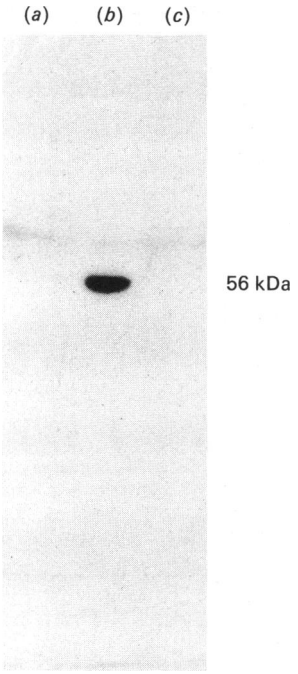


**Fig. 7. Detection of the $F_1$-ATPase β-subunit in subcellular fractions of *L. donovani* promastigotes**

Proteins from SM ghosts (*a*), mitochondria (*b*) and SM vesicles (*c*) were separated by SDS/9%-(w/v)-PAGE and transferred to nitrocellulose paper and further analysed for reactivity with rabbit anti-(*E. coli* β-subunit) antiserum.

analyses demonstrated that the isolated *L. donovani* mitochondria contained a 56 kDa band which corresponds to the β-subunit protein (Fig. 7*b*). However, this antibody showed no reactivity with either the isolated SMs or the membrane-derived vesicles (Figs. 7*a* and 7*c*). None of these fractions reacted with normal rabbit serum. These immunochemical results further demonstrate that the *L. donovani* SM-ATPase is distinct from its mitochondrial $F_0F_1$-ATPase.

### Proton-pumping activity of the SM-ATPase

Isolated membrane vesicles were incubated in the presence of Acridine Orange and ATP to demonstrate the proton-pumping activity of the SM ATPase. ATP-driven acidification of the vesicle internal space is illustrated in Fig. 8(*a*). As shown in this Figure, a decrease in Acridine Orange absorbance occurred upon addition of MgATP, which was reversed by the addition of FCCP. This indicates that the change in absorbance was due to acidification of the vesicle internal space. No acidification of the vesicles was observed when FCCP was added before the addition of MgATP (Fig. 8*b*). Similarly, both DCCD and orthovanadate inhibited vesicle acidification (Figs. 8*c* and 8*d* respectively). These results indicate that the leishmanial SM-ATPase is a proton pump.

## DISCUSSION

In the current study a proton-translocating ATPase ($H^+$-ATPase) activity was characterized and localized to the SM of *L. donovani* promastigotes. This enzyme was

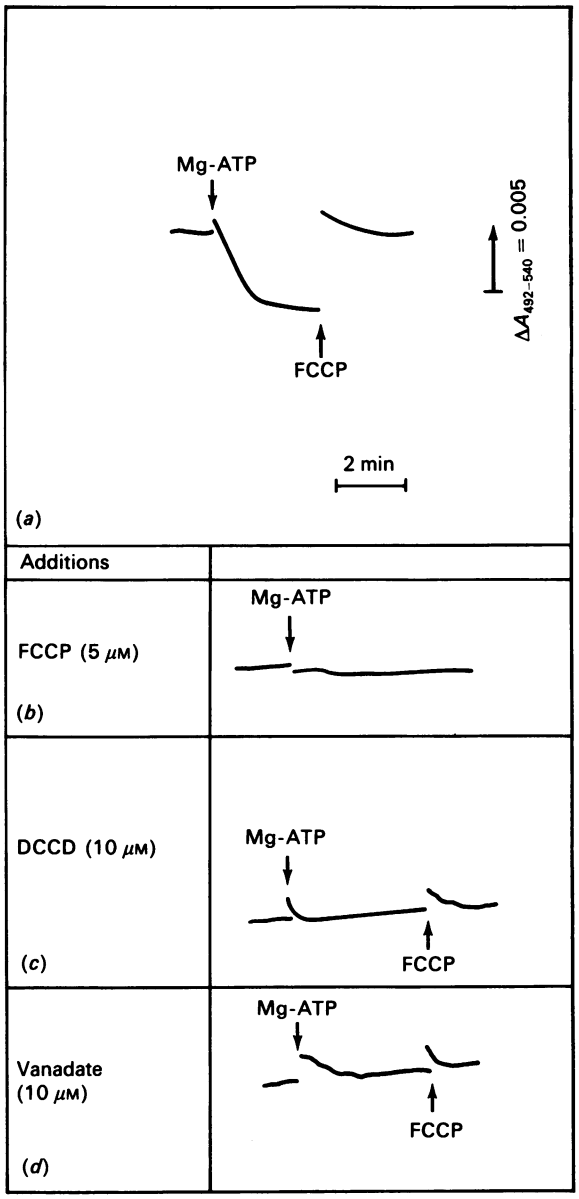


**Fig. 8. ATP-driven changes in absorbance of Acridine Orange in SM vesicles from *L. donovani* promastigotes**

The reaction mixture in a final volume of 1 ml contained vesicles (100 μg of protein), 2 μM-Acridine Orange and 10 mM-Tris/HCl, pH 6.5. Assays were initiated at 37 °C by adding MgATP (2 mM final concn.). Vesicles were treated with the inhibitors 10 min before the assays.

differentiated from the $F_0F_1$-ATPase present in the mitochondrion of this organism (D. Zilberstein, V. Liveanu & D. M. Dwyer, unpublished work). The SM-ATPase has an optimal activity at pH 6.5 opposed to the pH 8.0 optimum shown by the mitochondrial ATPase. Furthermore, this ATPase does not react with antiserum against the β-subunit of the $F_0F_1$-ATPase, and it is insensitive to oligomycin and azide. However, the SM-ATPase activity is significantly inhibited by orthovanadate, DCCD and *N*-ethylmaleimide.

The SM-ATPase activity was shown to be $Mg^{2+}$-dependent and was unaffected by the presence of either $Na^+$ or $K^+$. Furtheremore, ouabain had no inhibitory effect on the leishmanial SM enzyme, indicating that *Leishmania* do not possess a $Na^+/K^+$-ATPase. The latter is in agreement with our previous observations indicating that active-transport processes across the *L. donovani* promastigote SM are not driven by a sodium-motive force (Zilberstein & Dwyer, 1985). However, the SM-ATPase activity was partially inhibited by $Ca^{2+}$. A $Ca^{2+}$-stimulated ATPase was recently reported to be present in the plasma membrane of a related trypanosomatid protozoan, namely *Trypanosoma rhodesiense* (McLaughlin, 1985). The specific activity of that enzyme was about 10-fold lower than that observed in the current study. Further, we were not able to detect any $Ca^{2+}$-stimulated ATPase activity in the isolated leishmanial SMs.

A method for the isolation of membrane vesicles from the SM of *L. donovani* was devised in order to demonstrate the proton-pumping activity of the ATPase. These highly purified membrane vesicles contained all of the SM enzyme markers, but were devoid of attached subpellicular microtubules. Further, at least 50% of this vesicle population had an inside-out orientation. The proton-pumping capacity of these vesicles was assessed by incubating them in Acridine Orange and ATP. Results of these experiments demonstrated the ATP-driven acidification of the vesicle internal space. Such acidification was inhibited by FCCP, DCCD and orthovanadate. Cumulatively, these results indicate that the *L. donovani* SM-ATPase possesses proton-pumping activity.

The foregoing suggests that the *L. donovani* SM-ATPase has characteristics similar to the plasma-membrane proton-translocating ATPases of yeast and fungi (Goffeau & Slayman, 1981; Serrano, 1984). These enzymes are: (1) sensitive to vanadate; (2) stimulated by $Mg^{2+}$; (3) partially inhibited by $Ca^{2+}$; and (4) display their optimal activities over the acidic pH range.

The current study constitutes the first demonstration of a proton-translocation SM-ATPase in a parasitic protozoan. Previously, proton pumps have been demonstrated to play an important role in regulating intracellular pH homoeostasis (Padan *et al.*, 1981; Roos & Boron, 1981; Moolenaar, 1986; Padan & Schuldiner, 1987). It is suggested that the leishmanial $H^+$-ATPase is involved in such activities and thus is essential for parasite survival (Zilberstein & Dwyer, 1984).

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