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Biochemical and immunochemical characterization of a P-type ATPase from *Leishmania donovani* promastigote plasma membrane

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Abstract

An ATPase on the plasma membrane of *Leishmania donovani* has been characterized. An antiserum, generated against ATPase active bands from native gels, was specific for a 105 kDa protein in promastigotes. However, in plasma membrane preparations a 70 kDa protein is also recognized, suggesting proteolysis of the intact 105 kDa protein or the presence of a second similar ATPase. [γ-^{32}P]ATP phosphorylates two proteins (105 kDa and 70 kDa) in promastigotes and plasma membranes. Both proteins form a transient phosphorylated intermediate, characteristic of a P-type ATPase. Immunostaining of permeabilized parasites shows diffuse staining of the surface of promastigotes and amastigotes, which is consistent with a plasma membrane protein. The antiserum immunoprecipitates a 70 kDa [^{14}C]DCCD binding protein from whole cells and plasma membranes of promastigotes. Furthermore, the antiserum immunoprecipitates a 105 kDa and 70 kDa protein which can be subsequently phosphorylated. These results indicate the presence of a 105 kDa P-type ATPase on the *L. donovani* plasma membrane which is similar to the mammalian and fungal cation pumps.

Keywords: ATPase, P-type; Biochemical/immunochemical characterization; (*L. donovani*)

1. Introduction

The control of cellular ion concentrations is the result of several transport systems within a cell. One important group of transport proteins, the ion motive ATPases, specifically and actively mobilize ions, generating chemical gradients across a membrane. This movement of ions is vital for numerous cellular functions ranging from energy production, motility, nutrient uptake, ionic homeostasis, intracellular signalling, and differentiation, to name a few. The P-type ATPases [1] are a major class of ion motive pumps found in all eukaryotes and some bacteria. These enzymes are characterized by: (1) the formation of a transient phosphorylated intermediate during catalysis (2) sensitivity to vanadate and (3) a major subunit of approx. 100 kDa. ATPases in this class include the Na^+/K^+-ATPase, the Ca^{2+}-ATPase of sarcoplasmic reticulum, and the fungal H^+-ATPases. In addition to their similar catalytic mechanisms, these cation pumps also share identity both at the nucleotide and protein levels.

Cations play an important role in *Leishmania*. Ca^{2+} has been implicated in the differentiation of this organism [2]. That the organism transports protons during nutrient uptake [3,4] and maintains a physiological pH in extreme environments [5] presumes an important role for this ion. As a flagellated promastigote the parasite lives within the gut of a phlebotomine sandfly vector, while the obligate intracellular amastigote resides within macrophages of its mammalian host. Drastic morphological and physiological changes accompany the adaptation of the parasite to its new, yet harsh environment. Consequently, cation pumps must play a major role not only during transition from one parasite form to another but also in survival in its environment.

Thus far two cation pumps have been identified on the plasma membrane of *L. donovani* promastigotes, a

Abbreviations: DCCD, *N,N'*-dicyclohexyl carbodiimide; FITC, fluorescein isothiocyanate; NEM, *N*-ethylmaleimide; PAU-PAGE, phenol-acetic acid-urea polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TNX, Triton X-100.

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H^+-ATPase [6,7], and a Ca^{2+}-ATPase [2,8]. The molecular mass of the H^+-ATPase was reported as 66 000 Da [7] while the Ca^{2+}-ATPase is approx. 50 to 60 000 Da in size [8]. However, these data are difficult to reconcile with the sizes of other evolutionarily conserved cation pumps such as the Na^+/K^+-ATPase [9], and the fungal H^+-ATPases [10,11] which range in size from approx. 100 000 to 110 000 Da. Furthermore, two similar P-type ATPase genes from *L. donovani* have been cloned and sequenced. They encode a protein of 107 000 Da [12,13], a size consistent with other similar cation pumps. This apparent discrepancy between the DNA sequence and protein data may suggest that in *Leishmania*, these proteins may be unique. It could also be that these ATPases are extremely sensitive to proteolysis and the smaller size may in fact be the result of modification by proteolysis.

In this paper we identify a cation ATPase that possesses several characteristics of a P-type ATPase. An antiserum was generated against a plasma membrane ATPase which recognized a 105 kDa protein and a 70 kDa protein which may be similar to a previously characterized 66 kDa protein [7]. One explanation is that the 105 kDa protein and the 70 kDa protein are similar yet distinct ATPases. Alternatively, the 105 kDa phosphoprotein may be prone to proteolytic breakdown giving rise to a truncated 70 kDa product. Nevertheless, to our knowledge this is the first report of a 105 kDa P-type ATPase on the *L. donovani* plasma membrane.

2. Materials and Methods

2.1. Parasite culture and isolation

Promastigotes of *Leishmania donovani* Sudan strain 1S were maintained at 26°C in Medium 199 (Gibco) with 15% fetal bovine serum. Cells were harvested by centrifugation at $1100 \times g$ for 10 min and washed twice in basal salts solution consisting of 17 mM NaCl, 7 mM KCl, 70 mM Na_2HPO_4 (pH 7.0).

2.2. Purification of surface membranes

Surface membranes were isolated using the method of Gottlieb and Dwyer [14] with a few modifications. The discontinuous gradients were centrifuged for 2 h at $100\,000 \times g$ in an SW27 rotor at 4°C. Purified plasma membranes were stored in 20% glycerol, 100 mM Tris-HCl, pH 7.0, at −20°C.

2.3. Enzyme assays

ATPase activity was monitored by measuring the release of inorganic phosphate (P_i) using either gamma [^{32}P]ATP [6] or the method of Lanzetta et al. [15]. Reaction mixtures were composed of 200 mM Tris-HCl, pH 6.7, 5 mM $MgCl_2$, 4 mM ATP, and approx. 1 μg of plasma membrane protein in a final volume of 100 μl. After 10 min incubation at 37°C, 50 μl of the mixture was removed for analysis of P_i. Control values from tubes containing the reaction mixture plus an equivalent amount of bovine serum albumin (BSA) were subtracted as background from experimental values. Tubes containing inhibitors were pre-incubated with plasma membranes for 10 min at 37°C. Experiments were performed in duplicate and each value represents an average of at least three determinations. Activity is expressed as nmoles of inorganic phosphate released per mg protein. Protein was determined by the method of Lowry et al. [16] using a BSA standard.

2.4. Non-dissociating gel electrophoresis

Non-dissociating gel electrophoresis was performed with a 7.5% separating and a 3% stacking gel using the Laemmli system [17] in the absence of sodium dodecyl sulfate (SDS). Plasma membranes were solubilized by suspending in Zwittergent 3–14 (1 mg membrane protein: 1 mg detergent) for 2–4 h at 4°C. After a 10 min spin at $11\,000 \times g$, the supernatant containing solubilized material was loaded onto the gel. ATPase activity was localized by incubating the native gel overnight in a mixture containing: 10 mM Tris-HCl, pH 7.2, 5 mM ATP, 20 mM $CaCl_2$, 20 mM $MgCl_2$, 20 mM KCl, 2% sucrose, 4 mM dithiothreitol, and 40 mg/ml BSA.

2.5. SDS-polyacrylamide gel electrophoresis

SDS gel electrophoresis was performed on plasma membranes and promastigote lysates. Since proteinase activity was a significant problem, whole cell lysates were prepared under conditions that minimized protein degradation. Approx. $(1–2) \cdot 10^8$ cells suspended in 400 μl basal salts were mixed with an equal amount of 2 × SDS sample buffer (4% SDS, 60% glycerol, 5% 2-mercaptoethanol, and 100 mM Tris-HCl, pH 7.2). The sample was heated at 95°C for 2 min and immediately loaded onto the gel and the rest frozen to minimize proteinase activity. Protein bands were visualized with Coomassie blue staining.

2.6. Generation of antiserum

A single band with ATPase activity was excised from the native gel, loaded and run on SDS-PAGE gels. The 70 kDa dissociated protein was excised from SDS-PAGE gels, and mixed with 1 ml Freund's complete adjuvant. Approx. 250 μl of the antigen mixture was injected intradermally in four locations on the back of a female New Zealand White rabbit. The rabbit was

boosted 3 weeks later, bled after 1 week, and the serum titered.

2.7. Western blot analysis

SDS-PAGE gels were transferred to nitrocellulose [18] for 5.5 h at 60 V. Blots were then incubated in a solution of Tris-buffered saline (TBS), 0.05% Tween 20, and 0.75% bovine serum albumin for 1.5 h, washed, and incubated with a 1:500 dilution of the ATPase antiserum for 1.5 h. The blots were then incubated with a 1:5000 dilution of alkaline phosphatase conjugated anti-rabbit IgG (Promega Biotec). The blots were washed and developed according to the manufacturer's suggestions.

2.8. Indirect immunofluorescence

L. donovani promastigotes and amastigotes were placed on coverslips for 2 h at room temperature to allow the cells to adsorb to the surface. Cells were incubated with 3.7% formalin in Tris-buffered saline (TBS) for 20 min to fix the parasites and subsequently washed three times for 5 min with TBS. To permeabilize the parasites, fixed parasites were then incubated with 100 μg/ml saponin for 30 min and washed as before. Background fluorescence was reduced by the method of Beall and Mitchell [19]. The permeabilized parasites were incubated with the purified rabbit antiserum at a dilution of 1:25 for 1.5 h and anti-rabbit rhodamine at a 1:75 dilution. Coverslips were washed thrice, mounted on slides, and viewed in a microscope with a fluorescent attachment to visualize the fluorescence.

2.9. Immunoprecipitation

A method used to immunoprecipitate the *Trypanosoma brucei* procyclin fusion protein [20] was adapted and used to precipitate *L. donovani* ATPase protein. 200 μl of *L. donovani* plasma membranes (2 mg/ml) were solubilized with 400 μg of Zwittergent 3–14 for 4 h at 4°C. The antiserum used in the experiments was first bound to Affigel 10 beads (Bio-Rad) by mixing an equal amount of serum to beads in 4 volumes of 0.1 M Mops according to the manufacturer's instructions. The supernatant was preadsorbed by incubating 1 h at 4°C with 25 μl of pre-immune rabbit Affigel beads. The mixture was centrifuged and the procedure repeated. Subsequently, the supernatant was incubated with 50 μl of immune serum/Affigel beads overnight at 4°C with shaking. Following centrifugation the beads were washed according to Roditi and coworkers [20].

For the [^{14}C]DCCD labeled protein, approx. 5 μCi of [^{14}C]DCCD was added after solubilization and incubated for an additional 4 h at 4°C. The labeled protein was then processed according to the immunoprecipitation procedure or run directly on 10% SDS-PAGE gels. The gels were impregnated with ENTENSIFY (New England Nuclear), dried, and exposed to film for 8–12 days.

The precipitated protein was phosphorylated by incubating at 30°C for 10 sec with 50 μM [γ-^{32}P]ATP (0.2 μCi; 3000 Ci/mmol) in 2 mM $MgSO_4$/50 mM Tris-HCl (pH 7.0). After 10 s the reaction was stopped by the addition of 0.3 M perchloric acid/1 mM ATP solution. The phosphorylated intermediate was visualized on phenol-acetic acid-urea polyacrylamide (PAU-PAGE) gels according to the procedure of Gallagher and Leonard [21]. The gel was run overnight and exposed, usually 3–12 h, on X-Omat AR film.

2.10. Chemicals

[^{14}C]DCCD was purchased from Research Products. Zwittergent 3–14 was purchased from Calbiochem. All other reagents used were obtained from Sigma (St. Louis, MO).

3. Results

3.1. Biochemical characterization of surface membrane ATPase activity

Surface membranes were assayed for ATPase activity in the presence of several potential substrates. Activity was greatest with ATP with a specific activity of 485 nmol/min per mg protein (100%), while ADP (22%), UTP (3%), and GTP (2%) elicited less activity. Analysis of cation requirements of enzyme activity indicated a dependence upon Mg^{2+}. Maximal activity occurred at 5 mM Mg^{2+} (485 nmol/min per mg protein) but significant activity was observed with Co^{2+} (90%) and Mn^{2+} (73%) as well. Appreciably less activity was seen with Ca^{2+} (26%) and very little activity was observed with Zn^{2+}, Cu^{2+} or Ni^{2+}. Optimal activity occurred at pH 6.7.

Some ATPase inhibitors act specifically on certain ATPases and are diagnostic for a particular class or type of enzyme. Dicyclohexyl carbodiimide (DCCD), a general inhibitor of ATPases [22–25] inhibited activity maximally at 40 μM (53% inhibition). Vanadate sensitivity is indicative of P-type ATPases, which form phosphorylated intermediates, In *Leishmania* vanadate inhibits 50% activity at a concentration of 7.5 μM. This vanadate sensitivity suggests the presence of a P-type ATPase on the *L. donovani* plasma membrane. Fluorescein isothiocyanate (FITC) is another ATPase inhibitor [9] that optimally inhibits *Leishmania* membrane activity 51% at 500 μM. All three of the above

inhibitors maximally affect activity near their IC_{50} values (Table 1). On the other hand, the sulfhydryl reagent NEM inhibits activity to a greater extent, 72% at 200 μM.

Oligomycin, an inhibitor of mitochondrial ATPases [22], had little effect (less than 10%) on activity which indicates, the absence of the mitochondrial enzyme. Similarly, ouabain, a known inhibitor of mammalian Na^+/K^+-ATPases [9] had no effect even at 10 mM. This would suggest that the Mg^{2+} dependent plasma membrane ATPase of *L. donovani* is not a Na^+/K^+-ATPase.

3.2. Polyacrylamide gel electrophoresis

Solubilized plasma membrane proteins were run on non-dissociating gels and stained for enzyme activity. Fig. 1 (lane A) shows a prominent band of activity near the top of the gel. Radiolabeled inhibitors bind strongly to ATPases and this phenomenon was exploited to localize these proteins in native gels. [^{14}C]DCCD bound to proteins in *Leishmania* surface membrane fractions (lane B). To get an estimate of the size of these proteins the upper bands of ATPase activity were excised and analyzed by SDS-PAGE (Fig. 2, lane A). The excised native ATPase bands were comprised predominantly of a 70 kDa protein. The band of activity was analyzed several different times by SDS-PAGE and each time the 70 kDa protein was present. The lowest band near the middle of the gel, which did not possess activity, was also excised, analyzed by SDS-PAGE, and found to contain some 70 kDa protein (data not shown). However, several proteins including transporters and channel proteins may bind DCCD and would not necessarily hydrolyze ATP.

We further analyzed the proteins in whole cells and promastigote plasma membranes that bound the ATPase inhibitor DCCD. Fig. 2, lane B shows a promastigote lysate that was incubated with [^{14}C]DCCD. lane C shows promastigote plasma membranes that were similarly incubated with [^{14}C]DCCD. In both cases the 70 kDa band was labeled by the inhibitor. However, there

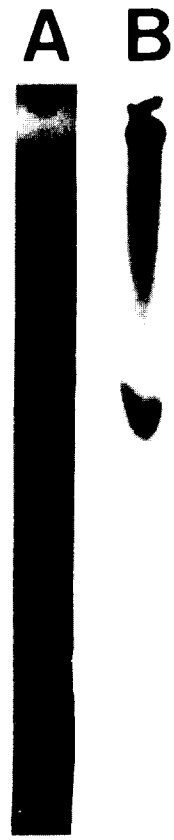

Fig. 1. Identification of ATPase by non-dissociating gel electrophoresis. Solubilized plasma membranes were run on a 7.5% non-dissociating gel. Lane A is a gel slice that was incubated in ATPase activity stain overnight as described in Materials and methods. In lane B, solubilized plasma membranes were incubated with [^{14}C]DCCD for 2–4 h.

was no labeling in the region corresponding to 100 kDa. It is possible that during the long incubation time with the inhibitor, the intact protein suffered proteolytic break-down to the 70 kDa fragment. From the SDS-PAGE analysis and [^{14}C]DCCD labeling it would appear that the enzyme is composed, entirely or in part, of a 70 kDa protein.

Table 1
Effect of ATPase inhibitors on total ATPase activity of *L. donovani* promastigote plasma membranes

Inhibitor	IC_{50} (μM)
DCCD	38
FITC	475
NEM	53
Vanadate	7.5
Oligomycin	N.I.
Ouabain	N.I.

Inhibitors were preincubated with plasma membranes for 10 min. Assay incubations were performed for 10 min at 37°C. N.I. indicates no inhibition.

3.3. Immunoblotting

The 70 kDa SDS protein was excised and used to generate an antiserum. This serum recognized a 105 kDa protein and a 70 kDa protein in most promastigote lysates (Fig. 3, lane A). However, in most membrane preparations only the 70 kDa protein was identified (lane B). This apparent discrepancy suggested that the 70 kDa protein might be a proteolytic breakdown product of the larger 105 kDa protein or a second ATPase. Preparations of plasma membranes in the presence of several proteinase inhibitors including aprotinin A, leupeptin, trypsin inhibitor, phenylmethanesulfonyl fluoride, EDTA, and 2-mercaptoethanol, failed to eliminate the effect. That most of the

P-type ATPases in eukaryotes are composed of a 100–115 kDa protein [9–11] would support the hypothesis that the 105 kDa protein represents an intact *L. donovani* ATPase.

3.4. Indirect immunofluorescence

To be useful in further analysis of the protein the antiserum must recognize the native conformation of the protein. This was tested using an immunofluorescence assay. With immunofluorescence it is possible to localize the protein and determine its general location and distribution within the parasite. Promastigotes permeabilized with saponin and followed by treatment with our rabbit antiserum and an anti-rabbit rhodamine antibody showed strong staining (Fig. 4A) The staining was diffuse over the entire surface of the parasite including the flagellum. Cells incubated in a similar manner with preimmune rabbit serum showed no fluorescence (Fig. 4B). Amastigotes (Fig 5A) purified from infected hamsters and treated in a similar fashion also exhibited intense staining over the entire surface when treated with the fluorescent antibody. This diffuse staining pattern is consistent with a plasma membrane protein. This and the fact that the antiserum was raised against plasma membrane proteins confirm its specificity for a plasma membrane protein. Amastigotes treated in a similar manner with preimmune rabbit serum showed slight background fluorescence (Fig. 5B). Non-permeabilized cells showed very weak surface staining with the antiserum. This may be because P-type ATPases are integral membrane proteins and only less than 4% of the molecule is exposed on the surface. Since the antibody specifically recognizes the native, non-denatured form of the protein it could be a useful tool in precipitation and eventual purification of the protein.

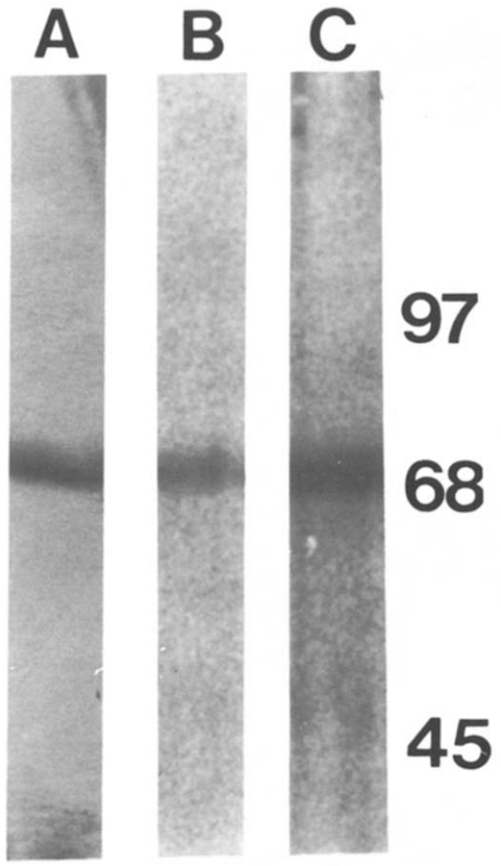

Fig. 2. Analysis of ATPase activity and [^{14}C]DCCD binding by SDS-PAGE. The band with ATPase activity was excised from the non-dissociating gel and run on a 10% SDS-PAGE gel and silver stained (lane A). Lanes B and C represent fluorograms of lysed promastigotes and plasma membranes respectively labeled with [^{14}C]DCCD and processed as described in Materials and methods.

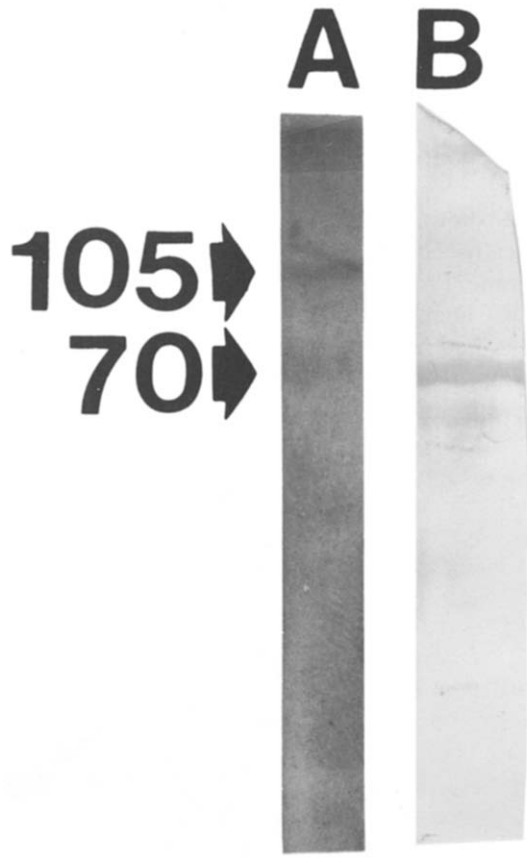

Fig. 3. Western blot of promastigotes and plasma membranes. Promastigotes were lysed and run on a 10% SDS-PAGE gel (lane A), while solubilized plasma membranes were run in lane B. The gel was blotted onto nitrocellulose and probed with *Leishmania* ATPase antiserum at a 1:500 dilution.

3.5. Immunoprecipitation

Direct proof of antibody specificity lies in its ability to precipitate the protein of interest. Thus far, we have established that a 70 kDa protein can be labeled by the inhibitor DCCD. Also, an antiserum generated against native bands possessing ATPase activity similarly rec-

ognized the 105 and 70 kDa proteins on immunoblots. Although there is a strong correlation suggesting that the antiserum is specific for an ATPase, precipitation of a DCCD binding phosphoprotein with ATPase activity would confirm that it is in fact specific for a P-type ATPase.

Data shown in Fig. 6 confirm that the antiserum specifically recognizes a DCCD binding protein in the membrane. Promastigote membranes were labeled with [^{14}C]DCCD and pre-adsorbed with non-immune serum as described in Materials and methods. In lane A, labeled, pre-adsorbed plasma membranes were precipitated with immune serum. It shows that only one protein of approx. 70 kDa was labeled by [^{14}C]DCCD. Lane B represents labeled plasma membrane protein incubated with non-immune serum and shows no non-specific precipitation of any [^{14}C]DCCD labeled protein. This experiment clearly shows that the antiserum is specific for a [^{14}C]DCCD binding protein on the plasma membrane which, presumably, is an ATPase.

A prominent characteristic of the major cation transporting ATPases is the rapid formation of a phosphorylated intermediate during the hydrolysis of ATP [1,25]. With this in mind, plasma membranes and promastigote lysates were phosphorylated in the presence of [γ-^{32}P]ATP and the reaction immediately stopped

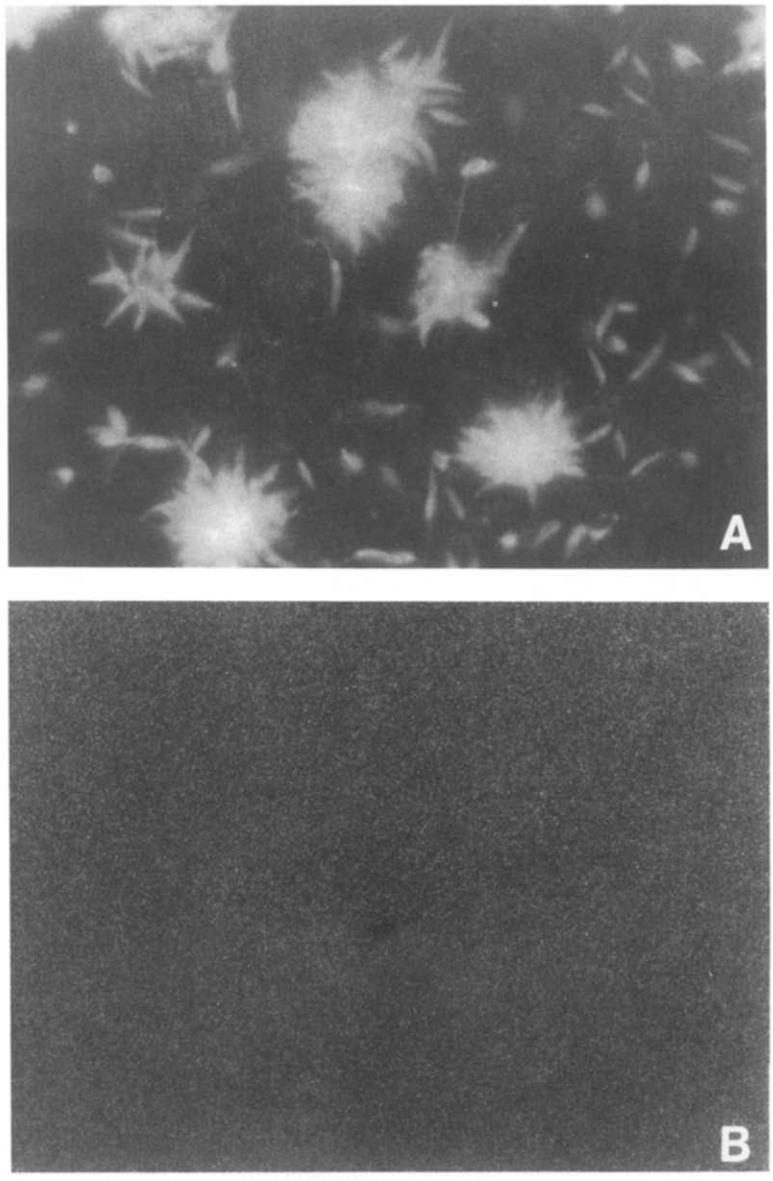

Fig. 4. Binding of antiserum to the surface of *L. donovani* promastigotes as shown by indirect immunofluorescence. (A) Promastigotes were fixed with formaldehyde and permeabilized with saponin. Parasites were incubated with immune serum (1:500). (B) Parasites were incubated with preimmune serum (1:500). Bound antibody was visualized with a rhodamine secondary antibody (1:50) and viewed for fluorescence at a 450 × magnification.

by the addition of trichloroacetic acid (TCA). The phosphoryl intermediate formed by P-type ATPases during hydrolysis is stable at acid pH and thus better visualized on an acidic polyacrylamide gel. For these experiments the PAU-PAGE gel system [21] was used. Besides acetic acid, the gels included phenol and urea which are extremely strong denaturants. Under these conditions if the phosphoryl intermediate was indeed sensitive to acid, it could not be visualized. These strong denaturants facilitate the solubilization of integral membrane proteins, such as ATPases, which may not resolve well with more conventional SDS-PAGE gels because of poor protein solubility. In our hands the plasma membrane ATPase was resolved into visible, distinct bands (Fig. 7). On normal Laemmli SDS-PAGE gels, which resolve proteins at pH 8.9, little phosphoprotein was visualized.

With respect to phosphorylation of immunoprecipitated material, promastigote lysates and plasma membranes were incubated with immune serum, precipitated by PANSORBIN Protein A-Sephadex beads, followed by phosphorylation. Precipitated material from promastigote lysates as well as plasma membranes contained protein that was phosphorylated with [γ-^{32}P]ATP. Fig. 7 shows that three proteins were phosphorylated (105, 70 and an approx. 180 kDa protein) from plasma membranes (lane A) and whole cells (lane C). Phosphorylation of precipitated plasma membranes (lane B) and whole cells (lane D) with non-immune serum failed to recognize any labeled proteins. Precipi-

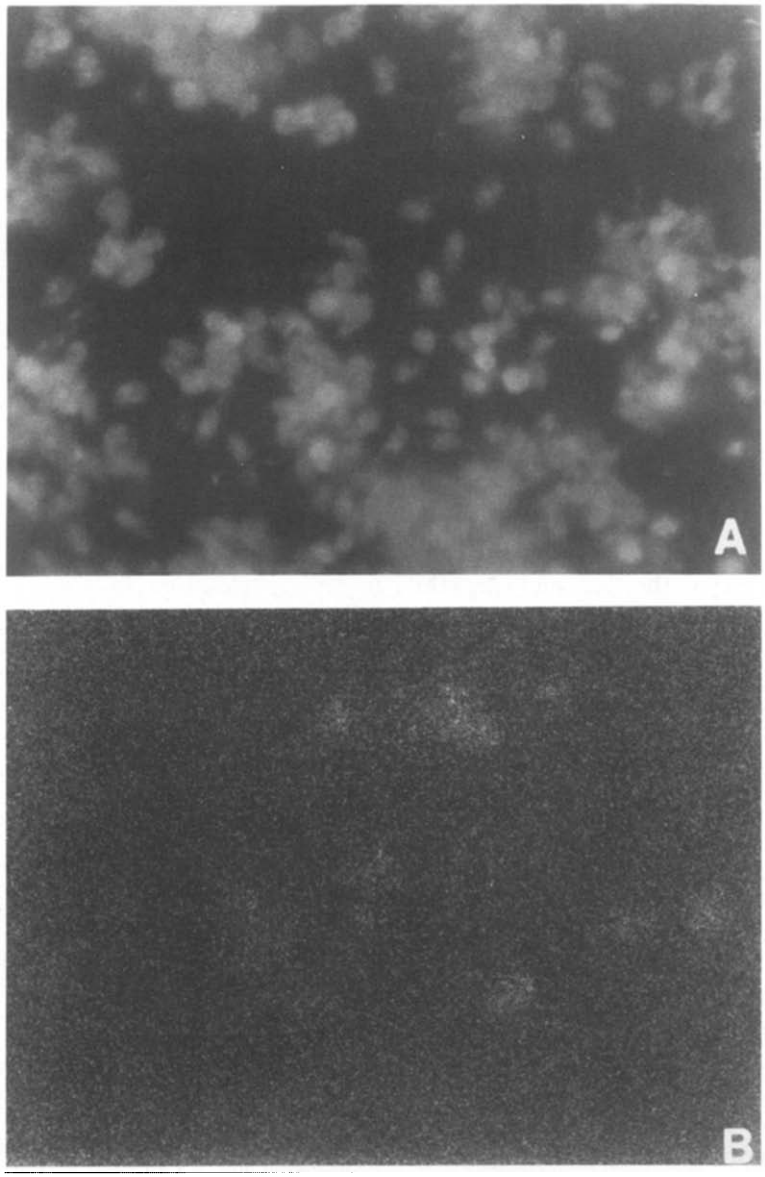

Fig. 5. Indirect immunofluorescence of *L. donovani* amastigotes. Parasites were treated in the same manner as in Fig. 4. (A) Amastigotes treated with immune serum (1:500). (B) Amastigotes treated with preimmune serum.

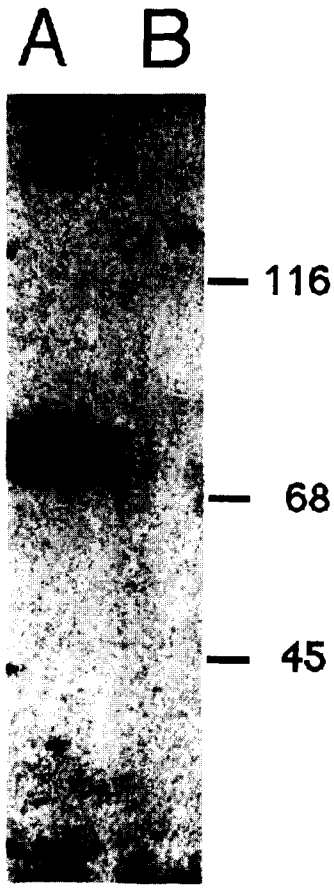

Fig. 6 Immunoprecipitation of ATPase with antiserum. Approx. 100 mg of plasma membrane protein was incubated with [^{14}C]DCCD for 4 h. Lane A contains 50 mg of the labeled membrane protein that was precipitated with immune serum. Lane B represents a 100 mg aliquot of labeled membrane protein precipiated with non-immune serum.

tation and phosphorylation of the 105 and 70 kDa proteins are consistent with our conclusion that the native ATPase is a 105 kDa protein and the 70 kDa protein is a breakdown product. It is possible that the large 180 kd protein may be a high molecular mass aggregate of ATPase(s).

4. Discussion

Two ATPases, a H^+-ATPase [6] and a Ca^{2+}-ATPase [8] have been identified and partially characterized from plasma membranes of *Leishmania*. Our results on the general properties of the membrane ATPase with reference to cation requirements, pH optimum, and sensitivity to inhibitors are in agreement with those of Zilberstein and Dwyer [6]. Activity is resistant to ouabain suggesting that the Na^+/K^+-ATPase present in many animal cells, is not present in *L. donovani* as was also reported by others [6,7]. To date, no Na^+/K^+-ATPase has been found in either yeast or *Neurospora*, nor is it present in *Trypanosoma cruzi* [28, 29]. In its sensitivity to vanadate, FITC, NEM and DCCD, the *L. donovani* enzyme resembles the cation pumps in animals (Na^+/K^+-ATPase, K^+/H^+-gastric ATPase) and the fungal H^+-pumps. This shared sensitivity suggests that the sites for many of these inhibitors are conserved and are probably associated with or are near regions of the protein that are important for enzyme activity (e.g., ATP binding site).

The present study represents the first instance to generate an antiserum from membrane proteins of *L. donovani* specific for a 105 kDa ATPase. The antiserum was generated against the excised 70 kDa band from SDS-PAGE gels. However, immunoblots identified the 105 and 70 kDa proteins in plasma membrane preparations. In general, plasma membrane preparations revealed the presence of a 70 kDa protein and

Fig. 7. Autoradiograms of immunoprecipitated and phosphorylated *L. donovani* promastigotes and plasma membranes. (A) Plasma membranes immunoprecipitated with immune serum. (B) Plasma membranes precipitated with pre-immune serum. (C) Promastigote lysate immunoprecipitated with immune serum. (D) Promastigote lysate precipitated with pre-immune serum. In all cases whole cell lysates and plasma membranes were pre-adsorbed with non-immune serum conjugated to Protein A-Sephadex beads to reduce non-specific binding of protein. Serum conjugated beads were used to precipitate the protein. Subsequently, the protein was phosphorylated at 30°C for 10 s and analyzed by PAU-PAGE. Approx. 100 mg plasma membrane or cell lysate was used for each precipitation reaction.

relatively smaller amounts of the 105 kDa protein. Occasionally plasma membrane preparations were obtained which showed an abundance of the 105 kDa protein and less of the 70 kDa protein. Proteolysis, which might vary among membrane preparations, could give rise to variation in the amounts of those two proteins. However, this phenomenon was unaffected by a variety of proteinase inhibitors, which may support the presence of two similar yet distinct enzymes.

The estimated size of the 105 kDa protein is consistent with the recent cloning of an ATPase gene from *L. donovani* with its product predicted to have a molecular mass of 107.4 kDa [12,13]. This gene has considerable homology with fungal H^+-ATPase genes. Other cationic ATPases such as the Na^+/K^+-ATPase, the Ca^{2+}-ATPase of the sarcoplasmic reticulum, and the fungal H^+-ATPases all have apparent molecular masses of 100 kDa. It is worth noting that immunoblotting by Liveanu et al. [7], recognized only a 66 kDa protein in *L. donovani* membrane preparations even in the presence of proteinase inhibitors although the antiserum used was that against yeast H^+-ATPase. This 66 kDa protein is so close to our 70 kDa protein that they may in fact be the same. However, we do not believe that this 70 kDa protein is the intact native enzyme. Several arguments favor this view. First, most major cation ATPases including the Na^+/K^+-ATPase and fungal H^+-ATPases are approx. 100 kDa to 110 kDa in size. Second, a major cation ATPase cloned in *L. donovani* also has a predicted molecular mass of 107 kDa [11,12]. Third, and perhaps most importantly, a serum generated against the 70 kDa band recognizes a 105 kDa protein in addition to the 70 kDa protein.

The biochemical and immunochemical data presented here suggest that the 105 kDa plasma membrane ATPase and the 70 kDa enzyme from *L. donovani* may represent one of the ATPases previously described [7,8,12] or it may represent a new, distinct ATPase enzyme. Analysis on nondenuturing gels indicated apparent [^{14}C]DCCD labeling of several proteins (Fig. 1B). One possibility is that these may be breakdown products of ATPase proteins that lack enzyme activity. These proteins could also be other transporters [30] or small noncatalytic subunits of mitochondrial or vacuolar type ATPases. But the 70 kDa protein was precipitated and labeled by [^{14}C]DCCD indicating that it is an ATPase. [^{14}C]DCCD labeling of the 105 kDa protein was not detected. This may be due to the low abundance of the 105 kDa protein or its susceptibility to breakdown. Immunostaining shows localization on the surface of both life stages of the parasite (promastigotes and amastigotes). Vanadate sensitivity and the formation of a phosphoryl intermediate categorize them as P-type ATPases distinct from the vacuolar (V-type) and mitochondrial (F-type) ATPases [1,25]. The most prominent characteristic of P-type ATPases is the formation of a phosphorylated intermediate as part of their catalytic mechanism. Both the 105 and 70 kDa proteins (and even some smaller proteolytic products) are phosphorylated with [γ-^{32}P]ATP. In Fig. 7 we also observed a high molecular mass phosphoprotein (180 kDa) which may be an aggregate of more than one protein. High molecular mass aggregates that are phosphorylated have been observed with other ATPases, especially when the phosphorylation is performed at temperatures greater than 4°C. Willsky [26] observed rapid phosphorylation of a 160 kDa and a 210 kDa protein in yeast. In *Neurospora* a number of phosphoproteins from 110 kDa to 200 kDa were observed [27]. These large proteins were suggested to be the proenzyme form of ATPases. The Ca^{2+}-ATPase in *Leishmania* has a native molecular mass of approx. 215 kDa as determined by size exclusion chromatography [8]. It would seem that the 180 kDa phosphoprotein precipitated by our antiserum may be a proenzyme form of a larger complex of H^+-ATPase or Ca^{2+}-ATPase.

That the enzyme is a P-type ATPase is further indicated by the observations that the protein precipitated by the antiserum can be phosphorylated, and that the protein already phosphorylated and acid denatured can be precipitated by the antiserum. Even more significant is the ability of the native protein to be precipitated and subsequently phosphorylated by [γ-^{32}P]ATP. This implies that the protein is functionally active and capable of hydrolyzing ATP and becoming phosphorylated in the process. *L. donovani* has a tandem pair of genes that encode proteins typical of P-type ATPases [12,13] although a direct link between those genes and a specific ATPase is still lacking. Phosphorylation of an ATPase in this organism identifying it definitively as a P-type enzyme has not been reported until now. Identification of an approx. 105 kDa protein indicates that *Leishmania* does possess a P-type cation ATPase similar to other eukaryotic ATPases.

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