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A High Affinity Ca^{2+}-ATPase on the Surface Membrane of *Leishmania donovani* Promastigote*

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A Ca^{2+}-dependent ATP-hydrolytic activity was detected in the crude membrane ghost of the promastigote or vector form of the protozoal parasite *Leishmania donovani*, the pathogen responsible for kala azar. The Ca^{2+}-ATPase was purified to apparent homogeneity after solubilization with deoxycholate. The enzyme consists of two subunits of M_r = 51,000 and 57,000 and has an apparent molecular weight of 215,000 ± 12,000. The enzyme activity is exclusively dependent on Ca^{2+}, and the pure enzyme can hydrolyze 1.6 μmol of ATP/min/mg of protein. The apparent K_m for Ca^{2+} is 35 nM, which is further reduced to 12 nM in the presence of heterologous calmodulin. The enzyme is sensitive to vanadate, but is insensitive to oligomycin and ouabain. The enzyme is strongly associated with the plasma membrane and has its catalytic site oriented toward the cytoplasmic face. The enzyme spans across the plasma membrane as surface labeling with radioiodine shows considerable radioactivity in the completely purified enzyme. The localization and orientation of this high affinity, calmodulin-sensitive Ca^{2+}-ATPase suggest some role of this enzyme in Ca^{2+} movement in the life cycle of this protozoal parasite.

Calcium ion is now widely recognized as a major intracellular signal through which external stimuli evoke a variety of crucial cellular responses in eucaryotic cells (1, 2). Movement of Ca^{2+}, followed by a transitory rise in its concentration at the site of action has been implicated in such diverse processes that include, among others, muscle contraction, platelet or neutrophil activation, hormone release, cell division, and fertilization (3). There are a few well defined mechanisms for calcium homeostasis that follow this transitory rise in its concentration. Energy-dependent extrusion of calcium from its site of action has been established as a major mechanism of calcium removal in several cellular systems (1, 2). A decade of intense work from several laboratories has clearly established that in sarcoplasmic reticulum and in red blood cells this ATPase acts as a true pump for Ca^{2+}, although the exact molecular mechanism for such a process still remains to be elucidated (4, 5). Since the original discovery of a Ca^{2+}-activated ATPase in red blood cells (6), such high affinity Ca^{2+}-stimulated Mg^{2+}-dependent ATPase activities have been demonstrated and characterized in plasma membranes of various cell types that include skeletal muscle (7), kidney (8), myometrium (9), corpus luteum (10), and rat liver (11, 12). High affinity Ca^{2+}-activated ATPases that do not show any demonstrable dependence on Mg^{2+} have also been reported in plasma membranes of mouse liver (13), rat heart sarcolemma (14), and basolateral membrane of rat kidney cortex (15).

Leishmania donovani is an important member of the kinetoplastida group of protozoal parasites. The organism has a digenic life cycle; a flagellated and elongated promastigote form in the sandfly vector, and a nonflagellated and oval amastigote or pathogenic form in the macrophage systems of the host. The organism is the causative agent for kala azar, a lethal form of visceral leishmaniasis, that is widely prevalent in many parts of the tropical world (16, 17). Like *L. donovani*, all protozoal parasites undergo dramatic morphogenetic transformations either in the host or in the vector in their life cycle, a process closely related to the pathogenicity of the organism. The role of calcium ion, if any, in such transformations or at any other stage of the cellular physiology of the organisms remains to be elucidated. There are occasional reports in kinetoplastida or in other parasitic protozoa that indicate a role for calcium metabolism in the survival and in the life cycle of these pathogens. The malarial parasite, *Palasmodium falciparum* has an obligate calcium requirement for normal intracellular growth (18), and its sensitivity to several antimalarial drugs may be related to their interactions with the parasite calmodulin (19). Among the kinetoplastida, indirect experiments have implicated calcium for the release of variable antigenic surface coat proteins for *Trypansoma brucei* (20). A membrane-associated Ca^{2+}-stimulated ATPase activity and a calmodulin-stimulated cAMP phosphodiesterase activity have been recently reported in *T. rhodesiense* (21) and *T. cruzi* (22), respectively. There has been no report in any species of *Leishmania* that demonstrate clearly the presence of a metabolite or an enzyme specifically related to calcium metabolism. We briefly reported earlier the presence of a plasma membrane-associated Ca^{2+}-ATPase in *L. donovani* promastigotes (23). Philosoph and Zilberstein (24) have recently measured the cytoplasmic pool of free Ca^{2+} in *L. donovani* promastigotes. Further, using permeabilized cells, these authors (24) have obtained preliminary evidences that suggest the presence of two Ca^{2+} uptake systems in unidentified internal organelles of the organism. In short, in spite of its obvious importance, our information regarding any possible role of calcium ion in cellular physiology of parasitic protozoa in general and kinetoplastida in particular is indeed extremely meager. In this paper, we report the purification and initial characterization of a Ca^{2+}-ATPase that does not have a demonstrable requirement for Mg^{2+} for activity and is firmly bound to the plasma membrane of the promastigote

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form of *L. donovani*. The purified enzyme shows an extremely high affinity for Ca^{2+} and is significantly modulated by calmodulin from heterologous systems. Further, in the whole cell, the catalytic site is inaccessible to medium substrates although the enzyme appears to span across the membrane. Taken together, our preliminary characterization of *L. donovani* Ca^{2+}-ATPase suggests a possible role for this enzyme in calcium ion movement across the cell membrane.

MATERIALS AND METHODS

All reagents used for this work were of highest purity grade available. All biochemicals including calmodulin and phenothiazines were purchased from Sigma. For routine colorimetric assays during purification, disodium salt of ATP was used. In other cases, triethanolamine salt of ATP or Tris-ATP and in special cases vanadate-free ATP were used. [γ-^{32}P]ATP (3 mCi/mmol) and $Na^{125}I$ were purchased from Bhaba Atomic Research Centre, India.

Organism—The organism used for this work is a clinical isolate of *Leishmania donovani*, originally designated as strain UR6 and presently renamed as *L. donovani* MHOM/IN/1978/UR6 (25). The organism was grown on a solid blood-agar medium that has been described elsewhere (26). For all works, cells grown for 72 h on slants were harvested by scraping and transferred to phosphate-buffered saline (PBS),[1] pH 7.4. Viability of these cells was checked under the microscope for motility and then used for further processing.

Assay of Ca^{2+}-ATPase Activity—Ca^{2+}-ATPase activity was determined either colorimetrically by measuring the Ca^{2+}-dependent release of inorganic phosphate or by measuring the liberation of ^{32}P from [γ-^{32}P]ATP. For colorimetric assay, the final assay mixture contained in a total volume of 1 ml, 100 μmol of Tris-HCl buffer, pH 7.4, 200 nmol of EGTA or CDTA with or without $CaCl_2$, and 0.5 μmol of Tris-ATP. The reaction was initiated by the addition of the requisite amount of the enzyme. $CaCl_2$ concentrations were varied to give the required free Ca^{2+} concentrations as described by Pershadsingh and McDonald (27) based on the experimental value obtained earlier by Sillen and Martell (28). The amount of contaminating Ca^{2+} was determined by atomic absorption to be 8–10 μM. This value was included in the calculation for concentration of free Ca^{2+}. Unless otherwise indicated, the incubation period was 30 min at 28 °C. After the incubation period, the reaction was terminated by the addition of 50 μl of 20% trichloroacetic acid. After removing the precipitate, where necessary, the released inorganic phosphate was measured following the method of Lowry and Lopez (29). Ca^{2+}-stimulated ATPase activity was determined by subtracting values with chelator alone from those with calcium plus chelator.

For isotopic assay, the method of Bais (30) was followed with some modifications. In this case, total volume of the assay mixture was reduced to 0.1 ml. The composition and the concentration of all the ingredients of the assay mixture remained as before. In this case, 50 nmol of [γ-^{32}P]ATP containing 3×10^5 cpm was used. After 10 min of incubation at 28 °C, the reaction was terminated by the addition of 5 μl of 20% trichloroacetic acid followed by the addition of 10 μl of 100 mM KH_2PO_4. Then, 0.1-ml suspension of 50% activated charcoal (Norite) was added. After mild stirring for 10 min, charcoal was precipitated by centrifugation. The process was repeated once more, and finally a 100-μl aliquot of supernatant was transferred to 5 ml of aqueous scintillation fluid (Triton-based) containing 35% Triton X-100. The count was taken in an LKB 1217 Rackbeta liquid scintillation counter.

One unit of the enzyme activity is defined as the amount of enzyme required to release 1 nmol of inorganic phosphate from ATP in the presence of Ca^{2+} in 1 min at 28 °C under appropriately controlled conditions. The agreement between the two types of assay was within ±15%. Mg^{2+}-dependent ATP hydrolytic activity was measured by using CDTA as the chelator. In this case, free metal ion concentration was varied by keeping a fixed CDTA concentration although no attempt was made to calculate the exact concentration of free Mg^{2+}. Concentration of Mg^{2+} in control blank was determined to be 2–3 μM by atomic absorption and was neglected for our calculations. 3′-Nucleotidase activity was measured according to the method of Dwyer and Gottlieb (31).

[1] The abbreviations used are: PBS, phosphate-buffered saline; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid; SDS, sodium dodecyl sulfate.

Surface Labeling of the Promastigote Cells—Surface labeling of the promastigote form of the organism by ^{125}I was performed by basically following the enzymatic iodination method of Hubbard and Cohn (32) by employing the lactoperoxidase-glucose oxidase system. Ten μmol of glucose, 0.1 unit of glucose oxidase, and 0.1 unit of lactoperoxidase were added to a 1-ml suspension of *L. donovani* cells in PBS that contained 2×10^9 cells. The reaction was initiated immediately by the addition of 200 μCi of $Na^{125}I$. After incubation at 4 °C for 10 min with very gentle stirring, eight such tubes were pooled and diluted to 150 ml with RPMI media (RPMI 1640 from GIBCO). The cells were then centrifuged at 3000 × *g*, and the collected cell pellet was washed three times with 150 ml of PBS. The cell pellet was finally suspended in 15 ml of PBS, and a suitable aliquot was taken for count after precipitation with trichloroacetic acid. The recollected cells were then used as starting material for obtaining highly purified Ca^{2+}-ATPase samples.

Purification of Ca^{2+}-ATPase—Unless otherwise stated, all operations during the purification were carried out at 4 °C. Cells of about 72 h of growth were collected and washed twice with cold PBS. Cells were checked for full motility. Seven g wet weight of washed cells were processed batchwise by suspending in cold 5 mM Tris-HCl buffer, pH 7.4, in long glass tubes in ice (dilution 1 g wet weight of cells/50 ml of buffer). The tubes were vortexed on a cyclomixer six times at an interval of 10 min. Vortexing time in each case was 2 min. This controlled hypotonic shock resulted in the release of several marker enzymes from the internal milieu. The leaky cells were then collected by centrifugation at 6,000 × *g* for 20 min. The pelleted ghosts were resuspended in 20 ml of 50 mM Tris-HCl buffer, pH 7.4, and homogenized briefly twice in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 17,000 × *g* for 20 min, and the pellet was washed twice in the same buffer. This fraction is designated as the membrane fraction.

The washed membrane fraction was resuspended in 18 ml of 0.4% deoxycholate solution containing 50 mM Tris-HCl buffer, pH 7.4. Homogenization was carried out in a Potter-Elvehjem homogenizer three times with two upward and downward strokes each time at a 5-min interval. The homogenate was centrifuged at 17,000 × *g* for 20 min. The supernatant that retained more than 80% of the total Ca^{2+}-ATPase activity in the solubilized form was designated as the solubilized extract. This fraction was slowly treated with a solution of protamine sulfate (0.5% w/v) until the volume of added protamine sulfate was 10% of the original volume of the supernatant. After standing for 5 min, the precipitate was centrifuged off. Solid ammonium sulfate (56 mg/ml) was now slowly added to the supernatant with constant stirring. After standing for 10 min, the precipitate was rejected, and the supernatant was further treated with solid ammonium sulfate (280 mg/ml). The precipitate obtained at this stage was collected by centrifugation at 17,000 × *g* for 20 min and was finally dissolved in 4 ml of 50 mM Tris-HCl buffer, pH 7.4. The dissolved fraction was dialyzed against the same buffer containing 20% glycerol (v/v) for 5 h. The presence of glycerol was necessary for stabilizing the enzyme activity. The dialyzed enzyme was again centrifuged at 17,000 × *g* for 20 min to remove denatured proteins that often precipitated at this stage. The supernatant containing the enzyme was then subjected to further purification on a DEAE-cellulose column (22 × 1.2 cm) previously equilibrated with 50 mM Tris-HCl buffer, pH 7.4, that contained 20% glycerol (v/v). After absorbing the enzyme fraction on the column, the column was washed with 40 ml of the above buffer containing glycerol. Batchwise elutions were finally carried out with 40 ml each of the same buffer containing 0.1%, 0.5%, 0.7%, and 1% sodium deoxycholate, respectively. Fractions of 2 ml were collected in each tube. Ca^{2+}-ATPase activity was eluted in the final solution buffer (1% deoxycholate). Fractions containing the highest specific activity and showing a single band on native polyacrylamide gel electrophoresis were pooled (two to three tubes). The pooled purified enzyme in 1% deoxycholate on the average retained 70% of its activity at the end of 2 weeks when kept at −20 °C.

For few experiments where deoxycholate had to be removed, the deoxycholate-eluted enzyme was passed through a column of Bio-Beads SM2 according to Niggli *et al.* (33). In this case, the column was previously equilibrated with 50 mM Tris-HCl buffer, pH 7.4, containing 10% glycerol.

Other Procedures—Protein was determined either by the method of Lowry *et al.* (34) or where necessary by the method of Bradford (35), using in both cases bovine serum albumin as the standard. Polyacrylamide gel electrophoresis and SDS-gel electrophoresis were carried out following the methods of Davis (36) and Laemmli (37),

respectively. Silver staining was performed according to Merril *et al.* (38).

RESULTS

Purification of Ca^{2+}-ATPase—Results of a typical purification procedure are summarized in Table I. Exposure of *L. donovani* promastigotes to hypotonic shock considerably facilitated the purification procedure for the enzyme. Membrane vesicles along with the detached flagella could be obtained in comparatively large quantities within a short time by simple centrifugation techniques.

For solubilization of the enzyme activity, several detergents were tried but deoxycholate (0.4%) proved to be the most effective. A nearly 2-fold increase in total activity was consistently noted after treatment with protamine sulfate (Table I). The possible presence of an endogenous protein inhibitor that might have been removed at this stage was not further explored. The purified enzyme can hydrolyze 1.6 μmol of ATP/min/mg of protein. This rate is quite comparable with Ca^{2+}-ATPase obtained from other sources (33). Based on the maximum total activity reached after the protamine sulfate step and from the final specific activity of the purified enzyme, 0.94 mg of Ca^{2+}-ATPase was calculated to be present in 190 mg of protein present in the crude membrane ghosts (Table I). Thus, Ca^{2+}-ATPase appears to constitute approximately 0.5% of the total membrane protein of the parasite.

Purity, Molecular Weight, and Subunit Structure of the Enzyme—The pooled enzyme fractions after the final purification step on a DEAE-cellulose column revealed a single band of protein on polyacrylamide gel electrophoresis (Fig. 1*A*). The apparent molecular weight of Ca^{2+}-ATPase was determined to be 218,000 (±10,000) on a calibrated Sephadex G-200 column by using lactate dehydrogenase, alcohol dehydrogenase, hemoglobin, and cytochrome *c* as marker proteins (Fig. 2*A*). The void volume was determined with blue dextran. This column was equilibrated with 50 mM Tris-HCl buffer, pH 7.4, containing 20% glycerol (v/v). The molecular weight was separately determined on a Sephadex G-200 column equilibrated with 50 mM Tris-HCl buffer, pH 7.4, containing 0.8% deoxycholate. In this case, approximately the same molecular weight of 215,000 (±12,000) was obtained. In both cases, the enzyme as applied to the column contained 1% deoxycholate. The possibility that the enzyme remained associated with deoxycholate micelles and its exclusion reflects a radius of both the enzyme and the associated detergent cannot be altogether ruled out. A detailed study involving other physical techniques may be necessary to make a more definitive molecular weight determination.

The enzyme consists of two subunits. This was revealed when sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol was carried out with the purified enzyme (Fig. 1*B*). The subunit molecular weights of 51,000 and 57,000 were determined using β-galactosidase, albumin, γ-globulin, and ovalbumin as marker proteins (Fig. 2*B*).

TABLE I
Purification of Ca^{2+}-ATPase from L. donovani

Step	Total protein	Total activity	Specific activity	Purification	Yield
	mg	*units*	*units/mg*	*-fold*	*%*
Membrane fraction	190	895	4.56	1	100
Solubilized enzyme	133	840	6.32	1.4	94
Protamine sulfate	78	1520	19.5	4.3	169
Ammonium sulfate	40	1300	32.5	7.12	145
Dialysis	22	967	44.0	9.64	108
DEAE-cellulose column	0.048	77	1604	352	8.6

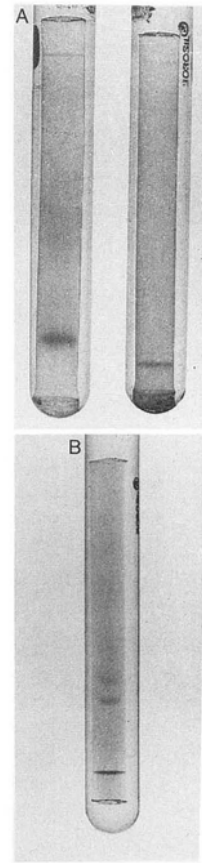

FIG. 1. **Polyacrylamide gel electrophoresis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of Ca^{2+}-ATPase.** In *A*, *tube 1* (*left*), polyacrylamide gel electrophoresis in 5% gel and *tube 2* (*right*), polyacrylamide gel electrophoresis in 7.5% gel. *B*, sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The amount of protein applied in each tube for polyacrylamide gel electrophoresis was 18 μg. For sodium dodecyl sulfate-gel electrophoresis, the amount of protein applied was 30 μg. The stain used was Coomassie Brilliant Blue. The *lowermost band* represents the tracking dye. In *tube A*, the migration was from *bottom* (cathode) to *top* (anode). The migration was from *top* (cathode) to *bottom* (anode) in *tube B*.

Catalytic and Kinetic Properties of the Enzyme—The nucleotide specificity of Ca^{2+}-ATPase was tested with different nucleotides at a concentration of 0.5 mM (Table II). As shown in the table, except for GTP, no other di- or triphosphate could act as substrate for this enzyme. Further, the enzyme did not show any nonspecific phosphatase activity. With ATP as substrate, the pH dependence of the enzyme was tested in presence of Tris buffer of varying pH. The enzyme showed an optimum pH of 7.4 (data not shown). The enzyme showed a strictly hyperbolic kinetics with ATP as the varying substrate at optimum pH and with saturating concentrations of free Ca^{2+} (data not shown). The apparent K_m for ATP was calculated to be 0.20 mM..

Metal Ion Specificity of ATP Hydrolyzing Activity—Table III shows that the purified ATPase can hydrolyze ATP exclusively in the presence of free Ca^{2+} ion. Even when CDTA was used to chelate the endogenous Mg^{2+} ion present in the incubation medium, hydrolysis of ATP was found to proceed quite efficiently. In fact, the rate of ATP hydrolysis in this case was generally comparable with the rate of hydrolysis obtained with the EGTA buffering system. None of the metal ions, including Mg^{2+}, either alone or in combination, could

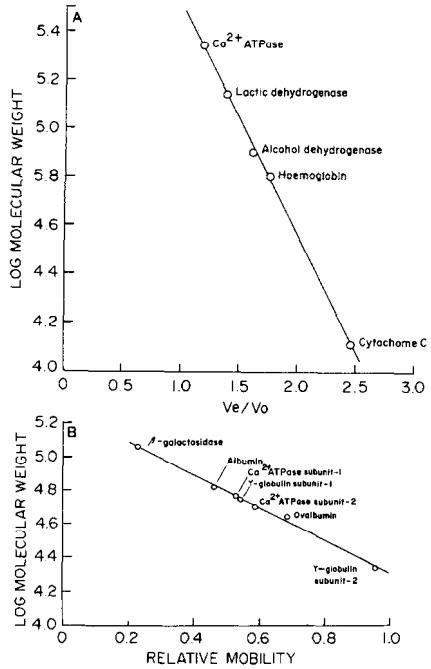

FIG. 2. *A*, determination of molecular weight of Ca^{2+}-ATPase on a Sephadex G-200 column. V_e is the elution volume of proteins and V_o is the void volume of the column. *B*, determination of subunit molecular weight of the enzyme by SDS-polyacrylamide gel electrophoresis.

TABLE II

Substrate specificity of Ca^{2+}-ATPase

The reaction mixture contained in a total volume of 1 ml, 100 μmol of Tris-HCl buffer, pH 7.4, 0.5 μmol of different substrates as indicated, and 1.7 μM free Ca^{2+} which was maintained by EGTA (0.2 mM)/Ca^{2+} (0.2 mM) buffering system. The reaction was started by addition of a suitable aliquot of the enzyme. Incubation was for 30 min at 25 °C. Details of the assay procedure have been described under "Materials and Methods."

Substrate	Nanomoles P_i released (30 min)
ATP	154.0
UTP	0.0
CTP	0.0
GTP	39.0
ADP	6.0
UDP	0.0
CDP	0.0
AMP	0.0
UMP	2.0
p-Nitrophenyl phosphate	0.0

activate the ATPase. Interestingly, Mg^{2+} ion, at higher concentrations, significantly inhibited the Ca^{2+}-ATPase activity both in presence of EGTA and in the presence of CDTA as chelating agents.

Affinity of the Purified ATPase for Ca^{2+}—When a detailed kinetics was carried out with varying free Ca^{2+} ion concentrations and keeping the concentration of ATP at the saturating level, a strictly hyperbolic kinetics was observed (Fig. 3). The apparent K_m for Ca^{2+} was calculated to be 35 nM. Obviously, the enzyme has a high affinity for Ca^{2+} in the presence of ATP. As expected, Mg^{2+} ion alone, even at high concentrations, failed to activate the enzyme.

Effect of Inhibitors and Modulators on Ca^{2+}-ATPase Activity—Ion-motive ATPases respond differentially to a variety of inhibitors and effectors depending on their molecular mechanism of catalysis and their metabolic functions (39, 40). Table IV shows the effect of a series of such compounds on

TABLE III

Metal ion specificity of Ca^{2+}-ATPase from L. donovani

Part A of the table represents ATP-hydrolyzing activity of the highly purified enzyme with various metal ions in the presence of EGTA (0.2 mM) as the chelator. Concentration of added metal ions are indicated in the second column. The next column provides the available concentrations of free metal ions as calculated from published values (Ref. 28). Part B of the Table measures the same enzymatic activity with CDTA (0.2 mM) as the chelator.

Metal ion	Concentration added	Concentration free ion present (calculated)	Nanomoles P_i released (30 min)
A. None			0.0
Ca^{2+}	190 μM	273 nM	156.0
Ca^{2+}	200 μM	1.7 μM	184.0
Mg^{2+}	200 μM		0.6
Mg^{2+}	2 mM		1.2
Ca^{2+}	190 μM		80.0
$+Mg^{2+}$	100 μM		
Zn^{2+}	200 μM		0.0
Na^{+}	2 mM		1.3
K^{+}	2 mM		0.7
Na^{+}	2 mM		0.0
$+K^{+}$	2 mM		
$+Mg^{2+}$	2 mM		
B. Ca^{2+}	175 μM	710 nM	148.0
Ca^{2+}	200 μM	4.5 μM	173.0
Mg^{2+}	100 μM	352 nM	0.0
Mg^{2+}	200 μM	8.2 μM	1.5
Mg^{2+}	3 mM		1.1

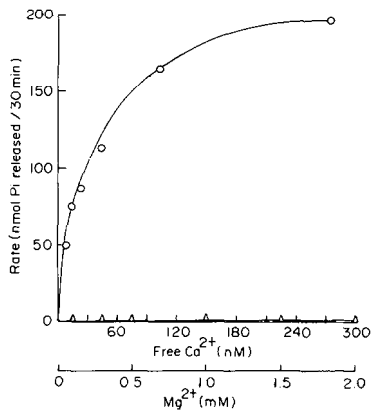

FIG. 3. **Ca^{2+} dependence of Ca^{2+}-ATPase for ATP hydrolysis.** The rate of Ca^{2+}-dependent ATP hydrolysis was estimated colorimetrically by measuring the inorganic phosphate liberated in the presence of Ca^{2+}. An appropriate control was run in the absence of Ca^{2+}. No other cation was added to the assay mixture, and the free Ca^{2+} concentration was maintained by EGTA (0.2 mM)/Ca^{2+} buffering system. Other compositions of assay mixture and conditions of assay were the same as described under "Materials and Methods." To test the Mg^{2+} dependence of the enzyme, Mg^{2+} was used instead of Ca^{2+}, and all other conditions were the same as with Ca^{2+}. For this experiment, highly purified enzyme was used. The same experiment was performed by measuring the liberation of ^{32}P from [γ-^{32}P]ATP, and the similar results were obtained. O——O and Δ——Δ represent the rate of ATP hydrolysis by the enzyme in the presence of Ca^{2+} and in the presence of Mg^{2+}, respectively.

the catalytic activity of the enzyme. Ouabain and oligomycin, the two classic inhibitors for Na^{+}, K^{+}-ATPase and mitochondrial Mg^{2+}-ATPase, respectively, did not have any inhibitory effect on the enzyme. In contrast, vanadate was found to strongly inhibit the enzyme. Vanadate is regarded as the transition state analogue of phosphate and inhibits those ATPases that have a phosphorylated aspartyl residue in the reaction pathway (40). Calmodulin, the endogenous modulator for numerous Ca^{2+}-dependent phenomena in many other systems, had a distinct stimulatory effect on the enzyme.

TABLE IV

Effect of various potential modulators on the Ca^{2+}-ATPase activity

For this experiment, the Ca^{2+}-ATPase activity was monitored by estimating the ^{32}P liberated from [γ-^{32}P ATP]. The complete system contained in a total volume of 0.1 ml, 10 μmol of Tris-HCl buffer, pH 7.4, and 0.05 μmol of ATP. The Ca^{2+} concentration was maintained at 1.7 μM by EGTA (0.2 mM)/Ca^{2+} buffering system. The reaction was initiated by addition of requisite amounts of the enzyme. After 10 min of incubation at 25°C, the reaction was terminated by adding 5 μl of 20% trichloroacetic acid and the liberated ^{32}P was estimated as described under "Materials and Methods."

Addition to the complete system	Activity
	%
None	100
NaN_3 (2 mM)	103
Ouabain (2.5 mM)	100
Ouabain (5 mM)	108
Oligomycin (7.5 μg)	103
Arsenazo III (20 μM)	104
Ruthenium red (100 μM)	97
Vanadate (10 μM)	36
Vanadate (20 μM)	11
Calmodulin (1 μM)	148
Chlorpromazine (200 μM)	65
Trifluoperazine (200 μM)	54
Calmodulin (1 μM) + chlorpromazine (200 μM)	86
Calmodulin (1 μM) + trifluoperazine (200 μM)	97

Thus, calmodulin, from a heterologous bovine brain system, could activate the enzyme by nearly 50%, even when the enzyme was assayed at a saturating concentration of free Ca^{2+}. Phenothiazines, such as chlorpromazine and trifluoperazine, are well known antagonists for calmodulin. The stimulatory effect of calmodulin could be effectively reversed with these drugs.

Characterization of Stimulatory Effect of Calmodulin—Fig. 4*A* shows that at a fixed and subsaturating concentration of Ca^{2+} (45 nM), the stimulatory effect of calmodulin reaches a plateau at a concentration of 1.5 μM. Further, the nature and extent of activation is effectively the same both with bovine testis and with brain calmodulin. Calmodulin shows a striking effect on the affinity of Ca^{2+} for the enzyme. This became evident when the effect of calmodulin was studied at varying concentrations of Ca^{2+} (Fig. 4*B*). The apparent K_m for Ca^{2+} was reduced from 35 nM to 12 nM in the presence of calmodulin (1 μM). The V_{max} on the other hand increased only by 50%.

Localization and Orientation of Ca^{2+}-ATPase—The Ca^{2+}-ATPase appears to reside exclusively in the plasma membrane of the parasite. For this purpose, we developed a procedure for obtaining an essentially pure plasma membrane fraction from the crude ghost. During the preparation of crude ghost by hypotonic shock treatment, it was found that approximately 60% of succinate dehydrogenase activity was removed in the medium and nearly 40% was retained with the crude ghost preparation. This indicated that significant portions of the mitochondrial-kinetoplast membrane fragments were still associated with the ghost preparation. This contaminating mitochondrial membrane was removed by essentially following a procedure by Scarborough (41). Working with *Neurospora crassa*, he (41) was the first to show that binding with lectins may greatly facilitate isolation of pure plasma membrane in eucaryotic cell types where more conventional methods pose special technical problems. Concanavalin A binding property of *L. donovani* cells is well documented. By some modification of Scarborough's procedure, a plasma membrane fragment was obtained that exclusively contained all Ca^{2+}-ATPase activity. For this purpose, thoroughly washed crude

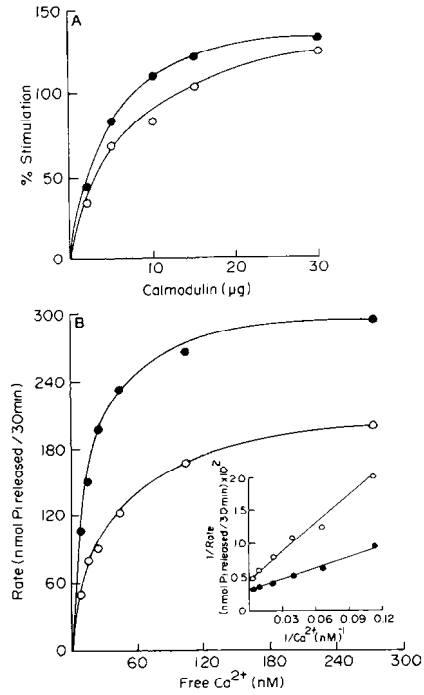

FIG. 4. *A*, stimulation of Ca^{2+}-ATPase activity as a function of calmodulin concentration. Each assay mixture contained in a total volume of 1 ml, 100 μmol of Tris-HCl buffer, pH 7.4, 0.5 μmol of ATP (Tris salt), and varying amounts of calmodulin as indicated. The concentration of Ca^{2+} in the assay mixture was maintained at 45 nM by EGTA (0.2 mM)/Ca^{2+} (0.15 mM) buffering system. The reaction was started by the addition of a requisite amount of the enzyme. After 30 min of incubation at 25 °C, the reaction was terminated by the addition of 50 μl of 20% trichloroacetic acid, and liberated P_i was measured colorimetrically. The same experiment was also performed following the release of ^{32}P from [γ-^{32}P]ATP, and similar results were obtained. ●——● and ○——○ represent the stimulation of enzyme activity by calmodulin from bovine brain and bovine testis, respectively. *B*, effect of calmodulin on the Ca^{2+}-ATPase activity at various concentrations of free Ca^{2+}. Ca^{2+}-ATPase activity was measured in the absence (○——○) and the presence (●——●) of calmodulin (30 μg) from bovine brain. *Inset* shows a Lineweaver-Burk plot of the same data. The assay mixture and conditions of assays were the same as described in *A*. Ca^{2+} concentrations were maintained as indicated in the figure by the EGTA (0.2 mM)/Ca^{2+} buffering system.

ghost pellet (1.6×10^9 cells) was suspended in 10 ml of Buffer A (50 mM Tris-HCl, pH 7.4, 10 mM $MgSO_4$, and 0.25 M mannitol) and centrifuged gently at 1000 × *g* for 6 min in Sorvall HB4 rotor. The resuspended pellet in 5 ml of Buffer A was mixed with 5 ml of concanavalin A solution (0.5 mg/ml in Buffer A) at 25 °C and gently agitated for 10 min. The agglutinated precipitate, collected after centrifugation at 1000 × *g*, was suspended in 6 ml of ice-cold Tris-HCl buffer, pH 7.4, and was extensively homogenized with breaks for 10 min in a Potter-Elvehjem homogenizer. About 3-ml portions of the resulting suspension were layered on 20 ml of Buffer B (100 mM Tris-HCl buffer, pH 7.4, containing 0.5 M mannitol) in 30-ml Corex tubes, and the resulting two-phase system was centrifuged at 656 × *g* for 30 min in a swinging bucket rotor (Sorvall HB4) at 4 °C. After removal of supernatant by aspiration, precipitates were collected and resuspended in 6 ml of Tris-HCl buffer, pH 7.4. The process of homogenization and separation was repeated twice. The final membrane pellet was suspended in 2 ml of 50 mM Tris buffer, pH 7.4, and assayed for marker enzymes.

The resuspended membrane fragments had less than 5% of the succinate dehydrogenase activity that was originally associated with the crude ghost preparation. In contrast, 70% of the 3′-nucleotidase activity and more than 80% of the Ca^{2+}-

TABLE V
Localization and orientation of Ca^{2+}-ATPase activity in L. donovani

Fraction	3′-AMP nucleotidase (units:total)	Ca^{2+}-ATPase (units:total)
1. Whole cell	3,050	⩾100
2. Supernatant after hypotonic shock	126	600
3. Permeabilized crude ghost after hypotonic shock	2,880	10,300
4. Supernatant of homogenized crude ghost	100	1,800
5. Crude ghost membrane	2,650	6,840
6. Deoxycholate extract		9,660

ATPase activity were found to be retained in this fraction. 3′-Nucleotidase was earlier shown by biochemical and cytochemical techniques to be a plasma membrane enzyme with its catalytic site exposed to the outside milieu (31).

The Ca^{2+}-ATPase appears to have its catalytic site on the cytoplasmic face. This became evident when the activity of the enzyme was measured in the whole cell and in the permeabilized crude ghost obtained after hypotonic shock (Table V). Under standard isotonic conditions, no significant Ca^{2+}-ATPase activity could be detected with the whole cell. In contrast, full catalytic activity for 3′-nucleotidase could be demonstrated under identical conditions of assay. After hypotonic shock, when membrane permeability barrier collapses and internal materials freely leak out, both the enzymes show their full activity. Only trace amounts of these activities were released in the supernatant fraction during this hypotonic shock.

Although the catalytic site of the enzyme is faced inward, portions of the enzyme appear to be exposed to outside surface. This was demonstrated by surface iodination of whole cells with ^{125}I and subsequent purification of the enzyme. *L. donovani* promastigotes have been extensively surface-iodinated to identify potential protein antigens of the parasite. A total of approximately 1.6×10^{10} cells was iodinated in batches (see "Materials and Methods") and then used as the starting material for purification of the enzyme. The purification procedure followed was exactly the same as described under "Materials and Methods." The dialyzed ammonium sulfate fraction containing 1.1×10^7 cpm was applied to a DEAE-cellulose column and batchwise eluted with deoxycholate as outlined above. The fractions (two tubes) with highest Ca^{2+}-ATPase specific activity contained 6×10^5 cpm. Gel electrophoresis of this iodinated sample revealed a single band of protein indicating Ca^{2+}-ATPase to be extensively iodinated. This experiment suggested that some tyrosine residues of the enzyme must be exposed to the outside surface.

DISCUSSION

This is probably the first report of purification and characterization of a Ca^{2+}-ATPase from any parasitic protozoal system. Earlier, a cytosolic Ca^{2+}-activated ATPase of M_r = 89,000 with three equivalent subunits, but of unknown function, was described in the free living protozoa, *Tetrahymena pyriformis* (42). Several ATP hydrolytic activities that are dependent on Ca^{2+} and Mg^{2+} or exclusively on Ca^{2+} have been reported from many other eucaryotic sources. Only a few of these have been purified to homogeneity and biochemically characterized. Both the human erythrocyte plasma membrane Ca^{2+}-ATPase and the fast-twitch skeletal Ca^{2+}-ATPase are single chain proteins with molecular weights of 140,000 (43) and 110,000 (44), respectively. Advent of molecular cloning techniques is rapidly revealing the presence of broadly similar cDNA sequences that probably codes for calcium-transporting ATPase of the aspartyl phosphate class from a number of human and other mammalian tissues (45, 46). In contrast, a rat liver plasma membrane Ca^{2+}-ATPase that probably does not act as a pump has been shown to have an approximate M_r of 200,000 with a trimeric quarternary structure (12, 47). Based on apparent M_r of 215,000 and two subunits of M_r = 51,000 and 57,000 (Figs. 1*B* and 2*B*), the leishmanial enzyme appears to have a tetrameric quarternary structure. Whatever be the stoichiometry of assembly, the parasite enzyme seems to be quite distinct from enzymes so far isolated from mammalian sources. Our earlier studies with aspartate transcarbamylase had also shown that significant differences exist in tertiary structure and functional organization between the protozoal enzyme and the mammalian enzyme (48).

We have demonstrated that the parasite enzyme does not need any metal ion other than Ca^{2+} for its activity (Table III and Fig. 3). In fact, Mg^{2+} at a higher concentration was found to inhibit the enzyme. Working with a Ca^{2+}-ATPase from basolateral membrane of rat kidney cortex, Tsukamoto *et al.* (15) has recently observed a similar inhibition of their enzyme with Mg^{2+}. However, unlike their enzyme, leishmanial enzyme failed to show any biphasic kinetic phenomenon over a wide range of free Ca^{2+} ion concentration (5×10^{-7} M to 10^{-3} M, data not shown).

The parasite enzyme may belong to the aspartyl phosphate class of ion-motive ATPase (39). It is fairly sensitive to vanadate but is completely insensitive to oligomycin (Table IV). Using an oligonucleotide probe designed to detect a conserved region of the active site of vanadate-sensitive ATPases, Meade *et al.* (49) have recently identified a tandem pair of genes of a probable cation-transporting ATPase from this parasite that codes for 974 amino acids. The relationship between this finding and our enzymological work remains unclear at the moment.

We classify this enzyme as a high affinity Ca^{2+}-ATPase because of its extremely low K_m for Ca^{2+} (Fig. 3). Interestingly, the K_m is further lowered (10–15 nM) in the presence of calmodulin from heterologous sources (Fig. 4*B*). We have now detected an endogenous cytosolic heat-stable activating protein factor in the parasite that closely resembles properties of calmodulin from other sources.[2] High affinity for Ca^{2+} demonstrated by this enzyme coupled with its modulation by calmodulin clearly makes it a candidate for regulating free Ca^{2+} ion concentration in the cytoplasmic milieu. The location and orientation of the enzyme further strengthens such a postulation. Surface labeling of the enzyme and its association with plasma membrane (text) suggests the enzyme to be an integral membrane protein with its catalytic site exposed to the cytoplasmic face (Table V). This makes the Ca^{2+}-ATPase a candidate for a pump that is involved in extrusion of Ca^{2+}. This, however, will remain speculative until the enzyme is integrated on liposomes and ATP-driven Ca^{2+} movement is demonstrated in such systems.

The role of Ca^{2+} as a component of a biomodulator system in the life cycle and pathogenicity of protozoal parasites in general and kinetoplastida in particular remain speculative at the moment. Evidences are slowly accumulating that suggest active Ca^{2+} metabolism in kinetoplastida. We report here the presence of a high affinity calmodulin-sensitive Ca^{2+}-ATPase on the surface membrane of *L. donovani* that has its catalytic site on the cytoplasmic milieu. McLaughlin (21) had earlier reported the presence of a Mg^{2+}-independent, Ca^{2+}-ATPase in the plasma membrane fraction of *T. rhodesiense*. No attempt was, however, made to solubilize and purify the enzyme.

[2] J. Ghosh, M. Ray, S. Sarkar, and A. N. Bhaduri, manuscript in preparation.

Presence of calmodulin (50) and its tandemly repeated genes (51) have been demonstrated in *T. cruzi*. Interestingly, phenothazine groups of drugs that are known to inhibit processes activated by calmodulin have been shown to have significant leishmanicidal activity both for the vector and for the pathogenic form (52). More significantly, in *T. brucei*, extensive electron microscopic analysis has shown that in presence of such drugs, the pellicular microtubular structure is selectively and completely disintegrated leaving the flagellar microtubular structure entirely unaffected (53). Our present finding, coupled with these and other scattered informations, strongly suggest some role for Ca^{2+} in the physiology of this group of parasites. The polarity of the *L. donovani* Ca^{2+}-ATPase and its high affinity for Ca^{2+} is indicative of a role for this enzyme in Ca^{2+} movement. Moreover, the parasite appears to have one or more uptake systems for Ca^{2+} that transport cytoplasmic Ca^{2+} into internal organelles (24). It is not unlikely, therefore, that a regulated Ca^{2+} current exists in *L. donovani* that has one or more modulatory functions in the life cycle of the organism. Sustained work in this area may shed new light on the pathobiology of these parasites.

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