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The β-aspartyl phosphate intermediate in a *Leishmania donovani* promastigote plasma membrane P-type ATPase

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Abstract

The phosphorylated intermediate of a plasma membrane P-type ATPase in *Leishmania donovani* has been further characterized. The formation of the phosphorylated intermediate is sensitive to several ATPase inhibitors including vanadate, dicyclohexyl carbodiimide (DCCD), *N*-ethylmaleimide (NEM), and fluorescein isothiocyanate (FITC). These inhibitors affect purified immunoprecipitated protein as well as total plasma membrane fractions. Oligomycin, an inhibitor of mitochondrial ATPases, and ouabain, an inhibitor of Na^+/K^+-ATPases, had no effect on the formation of the phosphorylated intermediate. The ATPase phosphoprotein was acid stable and dephosphorylated at alkaline pH, indicating the presence of the acyl phosphate chemical linkage. Analysis of the phosphorylated amino acid by reduction with sodium boro[^{3}H]hydride identified the residue as aspartate, confirming the formation of a β-aspartyl phosphate intermediate. These data indicate the presence of a 105 kDa P-type ATPase on *L. donovani* plasma membrane that is mechanistically similar to other P-type enzymes of higher eukaryotes.

Keywords: ATPase, P-type; Phosphoprotein; β-Aspartyl phosphate; (*L. donovani*)

1. Introduction

P-type ATPases are integral membrane proteins which span the surface membrane of the cell and endoplasmic reticulum [1]. These enzymes usually consist of a major subunit of approx. 100 000 daltons and 8 to 10 transmembrane domains. The phosphorylation domains of these proteins contain five amino acid residues which are extremely conserved in animals [2–4], lower eukaryotes [5,6], and bacteria [7]. It is an aspartic acid residue within this site that accepts the terminal phosphate from ATP during hydrolysis, to form a covalent phosphorylated intermediate. This process can be halted by the phosphate analog vanadate, a property that typifies the class of P-type ATPases.

The transient phosphorylation of these enzymes effectively couple the energy of ATP to cation transport, producing conformational changes that mediate ion movement through the membrane. Although DNA sequences for these enzymes have been identified in parasitic protozoa [8–10], much remains to be known about the actual protein and its biochemical attributes. In the parasitic protozoan *Leishmania*, two ATPases have been identified and characterized: a H^+-ATPase [11,12], and a Ca^{2+}-ATPase [13]. However, little information on the catalytic mechanism of these enzymes exist. *Leishmania* has a complex life cycle, living as a flagellated promastigote in the sandfly vector and as a non flagellated amastigote in vertebrate hosts. Specific information on the functional mechanism of these and other ATPases in the cell could provide further insight into their role in the life cycle of the parasite.

In this paper, we further characterize the P-type ATPase of *Leishmania donovani* described in the preceding paper. The enzyme undergoes phosphorylation and this phosphorylation is characteristically inhibited by vanadate. Furthermore, the intermediate is acid stable suggesting the presence of an acyl phosphate linkage. The phosphorylated amino acid has been iden-

Abbreviations: DCCD, *N,N'*-dicyclohexyl carbodiimide; FITC, fluorescein isothiocyanate; NEM, *N*-ethylmaleimide; PAU-PAGE, phenol-acetic acid-urea polyacrylamide gel electrophoresis.

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tified and indicates that the enzyme functions mechanistically similar to other P-type ATPases of higher eukaryotic organisms. To our knowledge, this work provides the first biochemical evidence for the presence of a P-type ATPase in the parasitic protozoan *L. donovani*.

2. Materials and methods

2.1. Parasite culture

Leishmania donovani Sudan strain 1S promastigotes were grown at 26°C in Medium 199 supplemented with 15% fetal bovine serum.

2.2. Plasma membrane preparations

Plasma membranes were prepared by the method of Gottlieb and Dwyer [14] as described in the preceding paper.

2.3. Protein gels

Proteins were phosphorylated with [γ-^{32}P]ATP and resolved on phenol-acetic acid-urea (PAU-PAGE) gels [15].

2.4. Immunoprecipitation

An antiserum generated previously against a P-type ATPase in *L. donovani* was used to immunoprecipitate ATPase protein for further study, employing the method of Roditti et al. [16].

2.5. Phosphorylated intermediate quantitation

Protein, either 1 μg of plasma membrane protein or 10 μl of precipitated ATPase protein (approx. 500 ng protein) coupled to Affigel-10 beads, was phosphorylated with the addition of 20 μM [γ-^{32}P]ATP (approx. 50 nCi) for 10 s at 4°C in a 50 μl reaction mixture also containing 2 mM $MgSO_4$ in 50 mM Tris-HCl (pH 7.0). The reaction was stopped with stop solution (0.3 M perchloric acid, 1 mM ATP, 1 mM Na_2HPO_4, 1 mM NaH_2PO_4). Phosphorylated protein was recovered by rapid filtration through a 0.45 micron methyl cellulose filter (Millipore), and washed twice with 5 ml each of stop solution. The filter was dissolved with 0.25 ml dimethyl formamide and counted. The inhibitors were incubated with the enzyme at 4°C for 20 min before phosphorylation. Protein was quantified by the method of Lowry et al. [17]. Controls without enzyme and denatured enzyme were run simultaneously and subtracted as background. Assays were performed three to five times. Each point represents an average of these values with a standard deviation of less than 15%.

2.6. pH analysis and hydroxylamine digestion

Determination of pH dependence of precipitated enzyme was performed in buffers at pH: 2, 4, 5, 7, 8, 9.5, and 11 [18]. Briefly, [γ-^{32}P]ATP labeled immunoprecipitated protein was washed twice in 1 ml and incubated for 30 min at 4°C in buffer at a specific pH. The reaction was stopped and the amount of phosphoprotein quantified by filtration as described above or analyzed by PAU-PAGE. Hydroxylamine (NH_2OH) analysis was performed as described [18], except that the [^{32}P]phosphoprotein was incubated with NaCl or NH_2OH (pH 5.3) solutions for 15 min at 30°C. The reaction was stopped and the phosphoprotein quantified by filtration analysis.

2.7. Identification of phosphorylated amino acid

L. donovani plasma membranes and precipitated protein were phosphorylated with [γ-^{32}P]ATP as described above. After 10 s at 4°C the reaction was stopped with 0.75 ml 0.3 M perchloric acid. The pel-

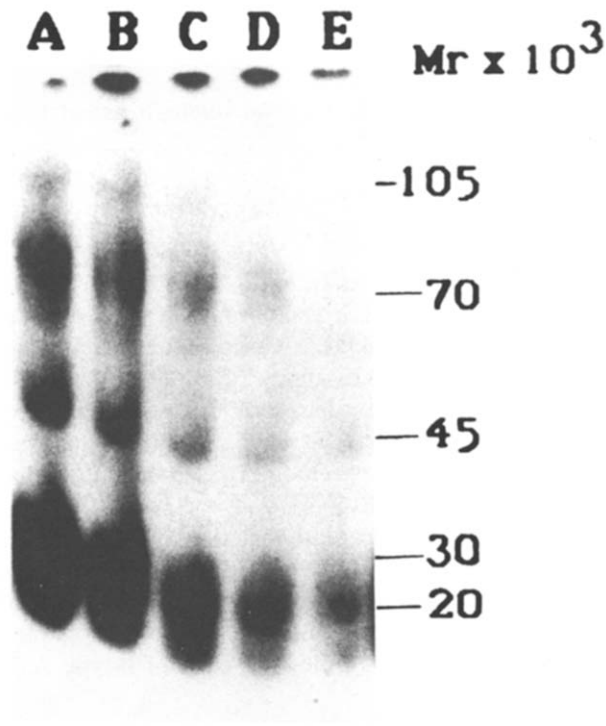


Fig. 1. Vanadate inhibition of plasma membrane phosphorylation. Approx. 50 μg of protein was preincubated with increasing sodium orthovanadate at 4°C for 20 min. Membrane protein was phosphorylated with [γ-^{32}P]ATP. After 10 s, the reaction was stopped and analyzed by PAU-PAGE. (A) Control membranes with no vanadate. (B) 10 μM. (C) 50 μM. (D) 100 μM. (E) 250 μM sodium orthovanadate.

leted protein was washed twice and resuspended in 100 μl dimethylsulfoxide (DMSO). A control sample consisting of non-phosphorylated precipitated protein was also dissolved in DMSO in preparation for reduction. To initiate the reduction reaction 100 μl of 20 mM [^{3}H]$NaBH_4$ (3 mCi) was added to an aliquot of protein and the reaction was performed as described [19]. The samples were stored at 4°C until analyzed as described [20]. A 5 μl sample was spotted onto a 20 × 20 cm silica gel G plate along with 1 μl each of (10 mg/ml) of L-homoserine, homoserine lactone, and hydroxy ornovaline as standards. The plate was developed in one direction using a mixture of n-butanol/acetic acid/H_2O (2:2:1). When finished the plate was dried and developed in the ascending direction with 75% phenol. The plate was sprayed with ninhydrin, and developed at 50°C for 10 min. This mix resulted in seven visible spots which were scraped from the TLC plates and counted.

2.8. Materials

Sodium boro[^{3}H]hydride was purchased from ICN. All other reagents were obtained from Sigma.

3. Results

3.1. Phosphorylation in presence of inhibitors

Plasma membrane protein when phosphorylated with [γ-^{32}P]ATP, yielded several phosphorylated proteins ranging from approx. 20 kDa to 105 kDa (Fig. 1, lane A). These phosphoproteins may represent ATPase proteins, their breakdown products, or may be the result of other enzymes such as kinases. However, the speed at which the phosphorylation reaction was performed (10 s at 4°C) suggests that these phosphoproteins represent for the most part, P-type ATPase(s) and not other less efficient phosphorylation enzymes.

Vanadate is elementally similar to phosphate and competes for the site of phosphorylation on the enzyme thus preventing transfer of phosphate to the protein. Treatment of the *Leishmania* plasma membrane fraction with vanadate inhibited phosphorylation in a concentration dependent fashion (Fig. 1). However, even at a concentration of 250 μM (lane E), vanadate did not totally block protein phosphorylation. The smaller proteins around 20 kDa were still phosphorylated indicating that they may not be ATPases but other proteins resistant to the effects of vana-

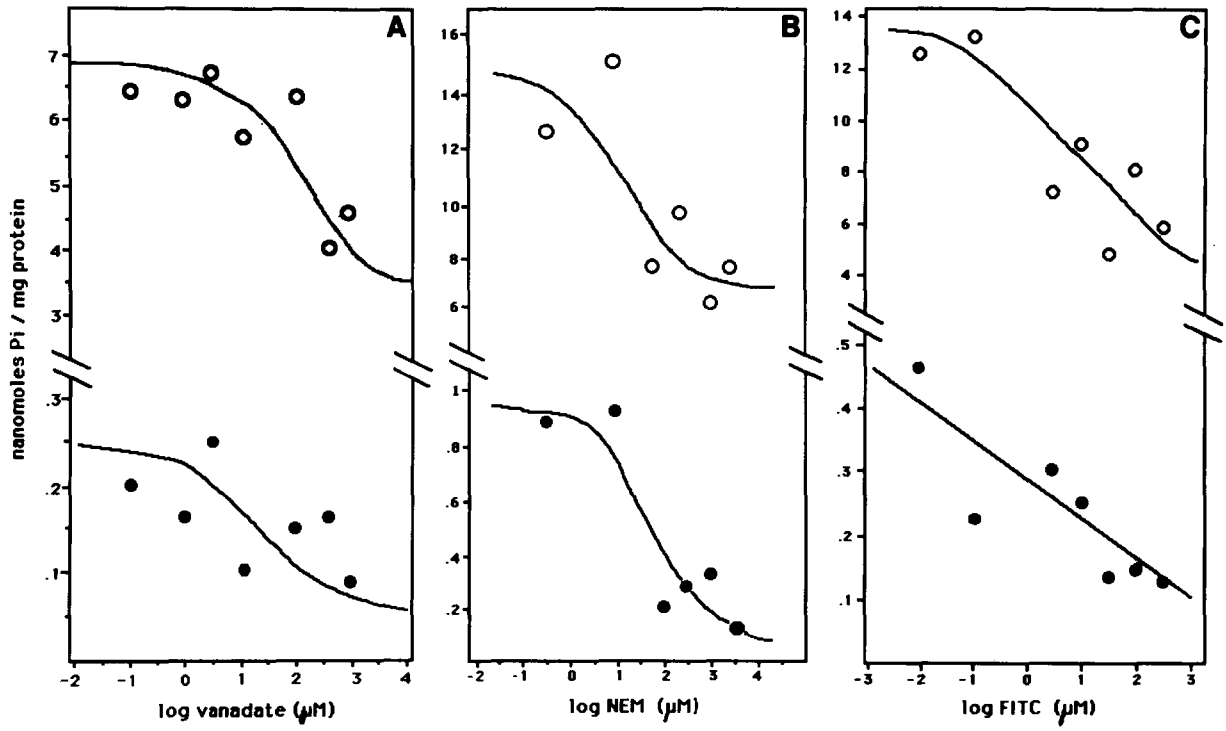


Fig. 2. Filtration analysis of phosphorylation inhibition by Vanadate, NEM and FITC. Immunoprecipitated protein (○) (approx. 500 ng) coupled to Affi Gel-10 beads or plasma membranes (1 μg) (●) were preincubated with inhibitor for 20 min at 4°C. Phosphorylation was initiated by the addition of [γ-^{32}P]ATP. After 10 s, the reaction was stopped and quantified by rapid filatration as described in Materials and methods. A control with denatured enzyme was run simultaneously and subtracted as background. Each point represents an average value of 3–5 experiments with a standard deviation of less than 15%. (A) Vanadate. (B) NEM. (C) FITC.

date. Vanadate insensitive phosphoproteins have been observed in phosphorylation studies of other ATPases [21].

The effect of vanadate on phosphorylation was further corroborated by the filtration method. Plasma membranes were preincubated with vanadate and phosphorylated (Fig. 2A). As with the PAU-PAGE gels, vanadate inhibited the phosphorylation of plasma membrane protein. But, the inhibition rarely exceeded 50%. This may be due to the presence of vanadate insensitive phosphoproteins as seen in Fig. 1, lane E or possibly a second site of phosphorylation on the protein. Protein immunoprecipitated by the *Leishmania* ATPase antiserum also exhibited sensitivity to vanadate when determined by the filtration assay.

Phosphorylation of the precipitated protein was markedly decreased (about 60%) by the ATPase inhibitors NEM (Fig. 2B) and FITC (Fig. 2C). Similar results were obtained when these inhibitors were tested for their effects on total plasma membranes; more than 80% inhibition of phosphorylation was observed at inhibitor concentrations above 1 mM.

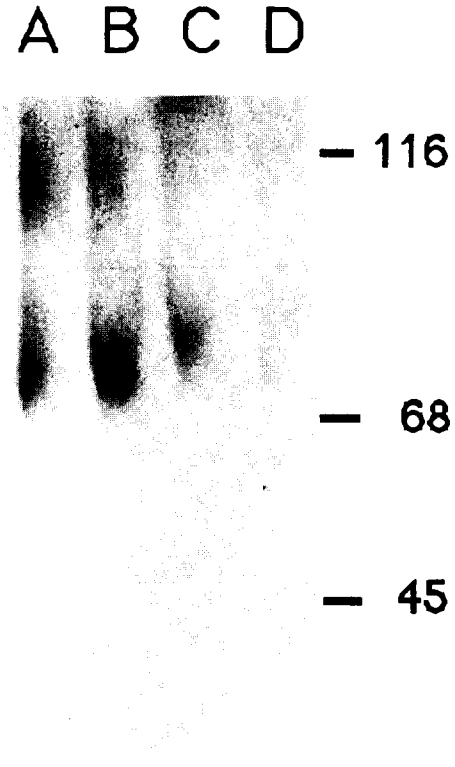


Fig. 3. PAU-PAGE analysis of DCCD inhibited phosphorylation of immunoprecipitated ATPase. Immuno-precipated protein (~ 2 μg) was preincubated with increasing DCCD for 20 min at 4°C. The protein was phosphorylated for 10 s and analyzed by PAU-PAGE. (A) Control with no DCCD. (B) 10 μM. (C) 100 μM. (D) 250 μM DCCD.

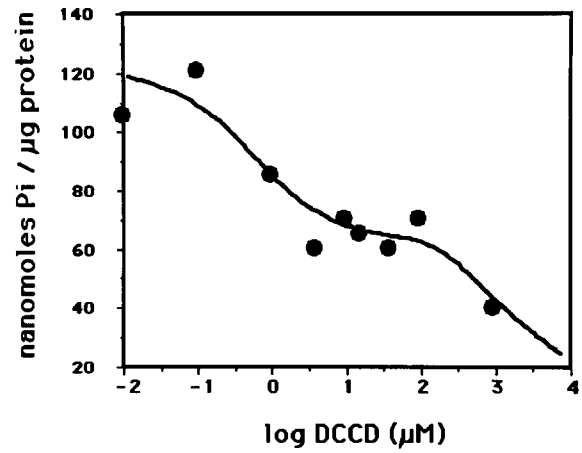


Fig. 4. PAU-PAGE analysis of phosphorylation in presence of DCCD. Immunoprecipitated protein (~ 500 ng) coupled to Affi Gel-10 beads was preincubated with DCCD, phosphorylated with [γ-^{32}P]ATP and analyzed by filtration as described in Materials and methods.

PAU-PAGE analysis showed that the formation of the phosphoryl intermediate was also inhibited by another ATPase inhibitor DCCD (Fig. 3). Protein precipitated with *Leishmania* ATPase antiserum, was incubated with various concentrations of DCCD, and phosphorylated. At lower concentrations of DCCD (10 μM), little effect was noticed (Fig. 3, lane B). However, at a concentration of 500 μM (Fig. 3, lane D) the formation of the intermediate was clearly inhibited. This inhibition was noticeable in both the 105 and the 70 kDa proteins. The effect of DCCD on phosphorylation of precipitated protein was also confirmed by filtration assays (Fig. 4). As the amount of inhibitor is increased so is the inhibition of phosphorylation. This mirrors the effect obtained with the PAU-PAGE analysis and confirms that DCCD does affect phosphorylation.

3.2. Turnover of the phosphorylated intermediate

A pulse chase experiment in which precipitated *Leishmania* ATPase protein labeled with [γ-^{32}P]ATP was diluted with more than a 100 fold excess of unlabeled ATP (Fig. 5). Upon addition of the excess ATP the ^{32}P was rapidly lost from the enzyme. Decrease in the [^{32}P]phosphoprotein was completed within 15 s and probably occurred within 1 s as observed with other P-type enzymes [22]. With the *Leishmania* ATPase (Fig. 5), more than 80% of the ^{32}P was removed from the protein leaving about 15% residual phosphoprotein. These results indicate that the phosphorylated intermediate is transient and the P_i on the protein undergoes rapid turnover on a continuous basis.

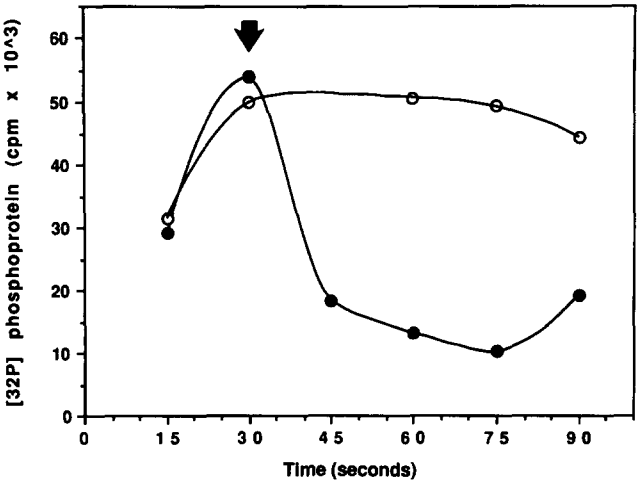


Fig. 5 Turnover of the phosphorylated intermediate. Precipitated protein coupled to Affi Gel-10 beads was incubated in 100 mM Mops, 20 mM KCl, 30 mM sucrose and 2 mM $MgCl_2$ (pH 6.7) at 4°C. Phosphorylation was initiated by the addition of [γ-^{32}P]ATP at time zero. Aliquots were removed at 15 s intervals, reaction stopped with an equal amount of 10% TCA and analyzed by filtration. Additions made at 30 s (indicated by arrow) were: 5 mM ATP (●) or an equal amount of H_2O (○). Counts from an aliquot of denatured and subsequently phosphorylated protein beads were subtracted as background.

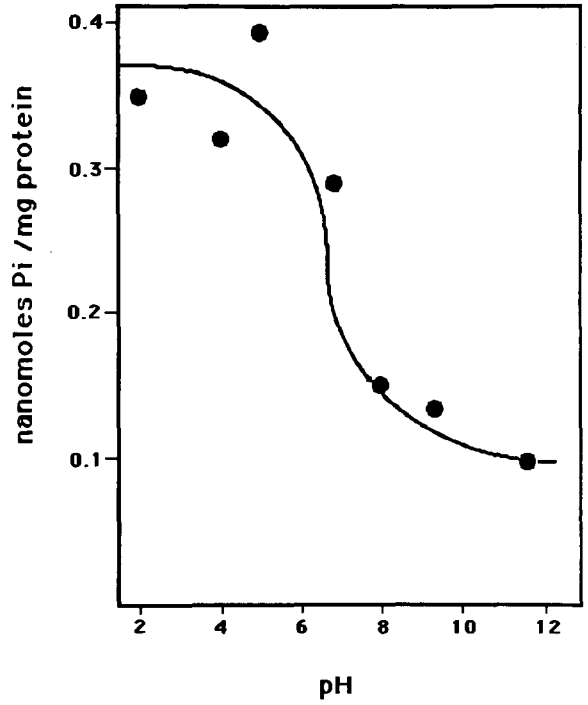


Fig. 6. Filtration analysis of the pH dependent phosphorylated intermediate. Precipitated protein was phosphorylated with [γ-^{32}P]-ATP for 10 s at 4°C and subsequently incubated for 30 min in buffers at pH 2–11.5. Phosphoprotein was quantified by filtration analysis as described in Materials and methods.

3.3. pH dependence of phosphoprotein formation

The pH profile of the phosphoryl intermediate formation provides valuable insight into the chemical nature of the phosphate-enzyme bond. The question is whether the phosphoryl bond is an acyl phosphate bond linked to the carboxy group of an amino acid residue within the enzyme, as seen in other cation ATPases. To address this question immunoprecipitated protein was washed and resuspended in buffered solutions ranging from pH 2 to 11.5 as described in Materials and methods. After incubation the amount of phosphoprotein formed was quantified by rapid filtration. The results are summarized in Fig. 6. Phosphoprotein was maintained maximally at pH 2 to approx. pH 7 indicating that the phosphate bond was stable at acidic pH. However, above pH 8, the phosphoprotein decomposed suggesting that the bond is alkaly labile. Acid stability of the phosphorylated intermediate is an earmark of P-type ATPases which possess an aspartylphosphate linkage.

To further verify this conclusion, precipitated protein was treated as above and analyzed by PAU-PAGE electrophoresis (Fig. 7). As with the filtration assays the phosphoprotein is indeed acid stable with maximal phosphorylation observed at pH 4 and 5 (lanes A and B). As the pH rises into the alkaline range, pH 9 and 11.5 (lanes E and F), decomposition of the phosphoprotein is clearly noticeable. These data suggest that the phosphorylated protein of *L. donovani* ATPase

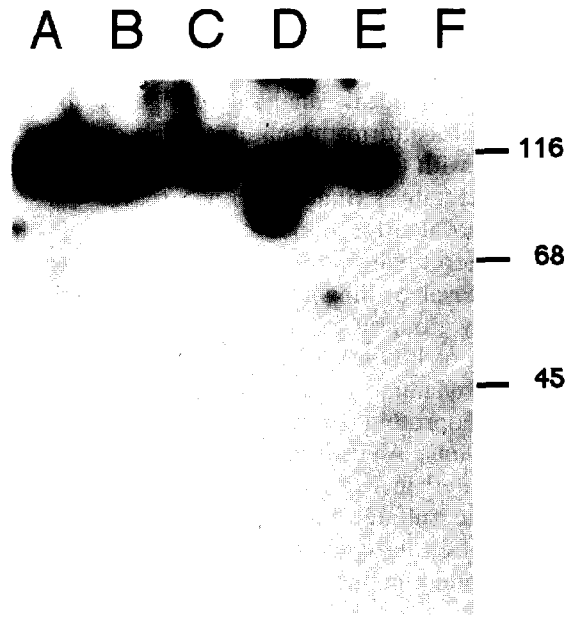


Fig. 7. PAU-PAGE analysis of the pH dependent phosphorylated intermediate. Determination of pH dependence of precipitated enzyme was performed in buffers at various pH values. (A) pH 4. (B) pH 5. (C) pH 7. (D) pH 8. (E) pH 9.5. (F) pH 11.5. [γ-^{32}P]ATP-labeled immunoprecipitated protein was incubated in buffer for 30 min at 4°C. Subsequently, phosphoprotein was quantified by filtration analysis as described in Materials and methods.

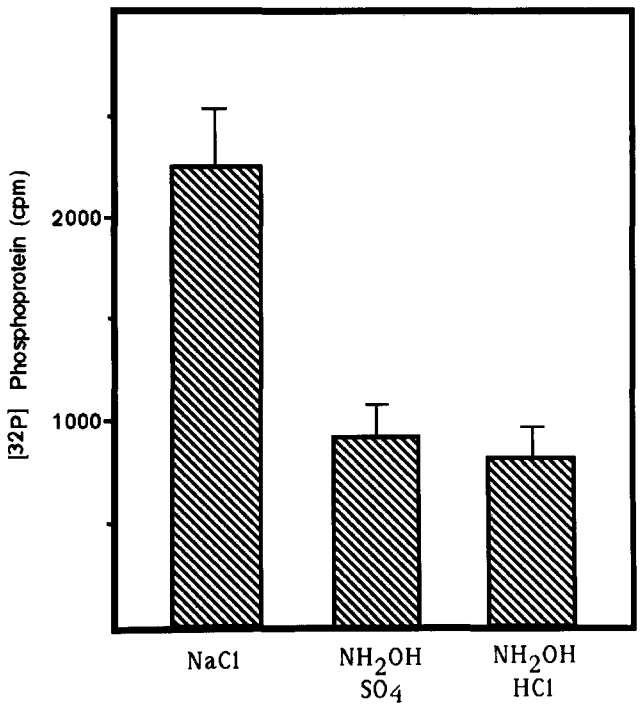


Fig. 8. Hydroxylamine hydrolysis of the acyl linkages of the phosphoprotein. [^{32}P]Phosphoprotein was incubated separately with NaCl, NH_2OH-HCl and NH_2OH-SO_4 solutions for 15 min at 30°C. The reaction was stopped and the phosphoprotein quantified by filtration analysis. The average of three experiments is represented.

forms an acyl phosphate, i.e., a phosphate linked to the carboxy portion of the amino acid.

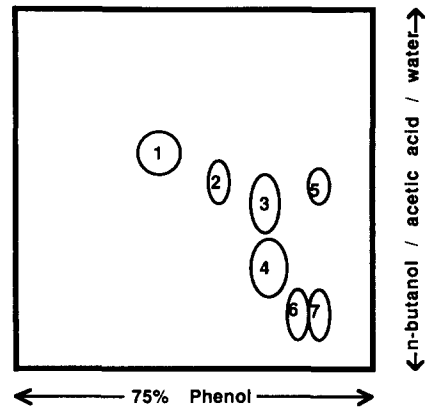
3.4. Phosphoprotein formation and hydroxylamine

Further chemical analysis using hydroxylamine cleavage will confirm the nature of the chemical bond associated with the phosphoryl intermediate. Although the intermediate is transient in nature, the phosphoryl group is covalently bound to the protein. Hydroxylamine preferentially cleaves these acyl linkages, in this case, the covalent linkage between the phosphate and the carboxy group of an amino acid within the protein. Incubation of P-type ATPase phosphoryl intermediate with hydroxylamine would result in the breakdown of the phosphoprotein. Protein immunoprecipitated with the *Leishmania* ATPase antiserum was phosphorylated and incubated in hydroxylamine hydrochloride or hydroxylamine sulfate (Fig. 8). When incubated in the presence of either type of hydroxylamine, approx. 60% of the protein bound phosphate was released compared to control phosphoprotein incubated with an equivalent amount of NaCl. Longer incubation periods with hydroxylamine did not release any more phosphate from the enzyme. This may be due to the enzyme-antibody bead complex which may not allow all of the bound enzyme to undergo the necessary conformational change to release the bound phosphate. These data strongly suggest that, for the precipitated *Leishmania* ATPase, the phosphate group is covalently linked to the carboxy group of the protein.

3.5. Identification of the phosphorylated residue

The phosphorylated amino acid was identified by boro[^{3}H]hydride reduction of affinity purified phosphorylated ATPase. The method involved a reductive cleavage of the phosphate bond with boro[^{3}H]hydride resulting in a ^{3}H-labeled amino acid residue [19]. Acid hydrolysis of the resulting material, followed by separation of the hydrolyzed amino acids using thin layer chromatography, with homoserine, homoserine lactone, and hydroxy ornovaline standards, was used to identify the labeled amino acid.

There are two possible acidic amino acids (aspartate and glutamate) which could be phosphorylated during ATP hydrolysis. In the P-type ATPase it is an aspartate



SPOT		RADIOACTIVITY (cpm)		
		C	PRO	MEMB
1		42	37	24
2		31	17	8
3	OH-Ornovaline	0	16	7
4	Homoserine lactone	0	0	8
5		0	10	7
6	Homoserine	0	52	165
7		0	64	251

Fig. 9. Phosphoamino acid analysis of *Leishmania* ATPase. Three 5 μl samples of sodium boro[^{3}H]hydride treated samples were spotted onto a 20×20 cm silica gel G plate along with standards as described in Materials and methods. C represents precipitated plasma membrane protein (~ 5 μg) treated only with sodium boro[^{3}H]hydride. PRO indicates precipitated promastigote lysate and MEMB indicates precipitated plasma membrane protein. Both were phosphorylated with [^{32}P]ATP for 10 s followed by sodium boro[^{3}H]hydride (3 mCi) treatment for 15 min at 23°C. The reaction was quenched with 0.1 M HCl and evaporated in a Sorvall speed vac with heat until dry. Samples were resuspended in 100 μl dH_2O for analysis. The seven visible spots after developing were scraped from the TLC plates and counted.

residue that is phosphorylated during ATP hydrolysis. Reductive cleavage of aspartate with boro[^{3}H]hydride would yield a [^{3}H]homoserine. [^{3}H]homoserine lactone is another product that is readily formed upon reductive cleavage of an aspartyl phosphate. However, this lactone is easily converted back to homoserine by incubation with NaOH or by long term storage of the hydrolysate. Phosphorylated glutamate residues would generate hydroxyornovaline upon reductive cleavage. Using the solvent system that we did, hydroxyornovaline could be distinctly separated from homoserine and homoserine lactone. After visualization of the amino acid standards with ninhydrin, seven spots were observed on the TLC plate (Fig. 9). The seven spots were scraped from the plate and counted for radioactivity. The table (Fig. 9) is a summary of the results from *Leishmania* ATPase immunoprecipitated from promastigote lysates and plasma membranes. For the boro[^{3}H]hydride reduced protein precipitated from plasma membranes the spots corresponding to homoserine (spots 6 and 7) had the highest number of counts, 10 to 30-fold higher than the other spots. Although the difference in counts from protein precipitated from promastigote lysates was significantly less, the trend was similar. Homoserine (spots 6 and 7) still contained the highest radioactivity. Control hydrolysates of non-phosphorylated reduced ATPase, and phosphorylated non-reduced ATPase were also analyzed by TLC which showed no labeled product. These data confirm that the principal phosphorylated amino acid is indeed an aspartate residue. This further corroborates the conclusion that the *L. donovani* ATPase is a P-type ATPase which forms the characteristic aspartyl phosphate during ATP hydrolysis.

4. Discussion

The formation of a phosphorylated intermediate has been the cornerstone of the molecular mechanism of the P-type ATPases [1]. The existence of this intermediate differentiates these ion pumps from the mitochondrial (F-type) and vacuolar (V-type) cation pumps. In theory, the P-type cation pumps undergo a cycle of auto-phosphorylation and dephosphorylation in which ATP is hydrolyzed to achieve ion transport through the membrane. In the present study the phosphorylated intermediate of an *L. donovani* plasma membrane ATPase was analyzed and further characterized. Preliminary studies indicated the presence of a rapidly formed approx. 105 and 70 kDa phosphoenzyme intermediate in immunoprecipitated protein. This phosphorylated intermediate also undergoes rapid turnover in the presence of excess ATP substrate confirming the identity of a catalytic transient intermediate. Although identical membrane preparations were used in various experiments, differences still arose with reference to the presence or absence of the 105 kDa and 70 kDa proteins.

Several ATPase inhibitors bind to the enzyme, inducing conformational changes which in turn modify phosphoenzyme formation. The effect of an inhibitor on enzyme phosphorylation could provide indirect evidence into its specific action in the E_1-E_2 reaction scheme of P-type ATPases. Previous studies have reported binding of vanadate to the E_2 conformation of the catalytic intermediate. This may be accomplished in two ways, one in which vanadate may act in an allosteric manner by reacting with a low affinity ATP binding site inhibiting enzyme activity while phosphorylation remains unaffected as in the Ca^{2+}-ATPase of sarcoplasmic reticulum, the H^+-ATPase of yeast [21], and the Kdp-ATPase of *Escherichia coli* [22]. Conversely, vanadate may directly displace phosphate at the phosphorylation site, inhibiting activity as well as phosphorylation as in the Na^+/K^+-ATPase [23], the gastric H^+/K^+-ATPase [24], and the K^+-ATPase [25]. With respect to *Leishmania*, vanadate specifically displaces ^{32}P from the precipitated phosphoprotein at the site of phosphorylation, placing it with the latter group of enzymes.

Phosphoprotein formation of precipitated *Leishmania* protein was inhibited by other ATPase inhibitors such as DCCD, NEM and FITC. DCCD reacts preferentially to a glutamate residue of the mitochondrial (F-type) ATPase [26] and to a glutamate residue of the *Neurospora* plasma membrane H^+-ATPase [27]. The site of action for DCCD is buried within the hydrophobic environment of the transmembrane portion of the enzyme. Mutational and functional analyses suggest that DCCD may impair ion transport through binding within the channel portion of the mitochondrial ATPase [26] and cytochrome *c* oxidase [28]. DCCD may disrupt phosphoprotein formation by inducing conformational changes resulting in dephosphorylation of the enzyme. In the PAU-PAGE analysis both the 105 kDa and the 70 kDa phosphoproteins were progressively inhibited with increasing concentrations of DCCD. NEM reportedly stalls the enzyme in the E_1 conformation as observed in the yeast H^+-ATPase [29] affecting the phosphorylated intermediate by preventing its phosphorylation or perhaps enhancing its dephosphorylation. FITC reacts with a lysine residue near the ATP binding domain and its binding to the protein can be ablated by the addition of ATP [30]. Both NEM and FITC affect the active site of the protein and subsequent phosphorylation. This may be achieved by locking the enzyme in the E_1 conformation making phosphorylation of the enzyme difficult, or stimulating dephosphorylation of the enzyme. The mode of action of both NEM and FITC within the active site of other P-type ATPase enzymes is consistent with the dephos-

phorylation we observed with precipitated ATPase protein from *Leishmania*. Ouabain and oligomycin had no effect on phosphorylation of the precipitated protein. This was expected since these inhibitors had no effect on ATPase activity in *Leishmania* plasma membrane fractions.

Filtration analysis of phosphorylated *Leishmania* protein in the presence of several inhibitors revealed incomplete inhibition by excess inhibitor. This residual phosphorylation (about 40%) was also observed for the pH dependent phosphorylation and hydroxylamine experiments. One possible explanation is that an aspartyl-phosphate intermediate accounts for only 60–70% of the observed phosphorylation of the *Leishmania* protein. The remaining phosphorylation may be the result of phosphorylation of amino acid residues at other sites on the protein which are unaffected by ATPase inhibitors and hydroxylamine. Such a phenomenon has been observed for the H^+-ATPase of *Saccharomyces cerevisiae* in which multiple serine and threonine residues are phosphorylated by a kinase [31]. Other P-type enzymes are also secondarily phosphorylated in a similar manner as well [32,33].

During hydrolysis the terminal phosphate of ATP is covalently bound to the carboxy group of an aspartate residue contained within a highly conserved region of the P-type ATPase. This phosphorylation domain is absolutely conserved in a range of organisms from bacteria to higher eukaryotes. The fact that the phosphorylated intermediate of the precipitated *Leishmania* protein is stable at acid pH and labile at alkaline pH suggested the existence of an aspartyl phosphate intermediate. The degeneration of the phosphoprotein upon incubation with hydroxylamine indicated that the phosphate was linked to the *Leishmania* ATPase via an acyl linkage. The identification of [^{3}H]homoserine upon reduction of the phosphoprotein with sodium boro[^{3}H]hydride indicates that the phosphorylated residue is an aspartate as in other P-type ATPases.

Together, these data indicate that *L. donovani* has a 105 kDa P-type ATPase on its plasma membrane. This enzyme forms a characteristic phosphoryl intermediate. The phosphate is transiently bound to an aspartate residue, forming a β-aspartyl phosphoryl intermediate. It appears that the *Leishmania* plasma membrane P-type ATPase forms a catalytic intermediate that is similar to enzymes found in higher eukaryotic organisms such as the Na^+/K^+-ATPase, and the Ca^{2+}-ATPase of the sarcoplasmic reticulum.

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