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A cyclic nucleotide-independent protein kinase in *Leishmania donovani*

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1. *Leishmania donovani* promastigotes labelled for 2 h with $^{32}P_i$ incorporated radioactivity into at least 21 different proteins, as determined by SDS/polyacrylamide-gel electrophoresis. Pulse–chase studies with $^{32}P_i$ demonstrated that the labelled proteins were in a dynamic state: some radiolabelled proteins rapidly disappeared and others appeared after the chase. The possibility of an ectokinase on the parasite was examined; incubation of intact parasites for 10 min at 25 °C in an osmotically buffered medium containing [γ-^{32}P]ATP, but not [α-^{32}P]ATP, resulted in the labelling of 10 different protozoal proteins, presumably localized to the surface of the organism's plasma membrane. Intact promastigotes also catalysed the transfer of ^{32}P from [γ-^{32}P]ATP to histones. 2. The histone-dependent kinase was solubilized by repeated freezing and thawing, and sonication, and purified 118-fold by chromatographing the high-speed (200000 *g*, 1 h) supernatant fraction on QAE-Sephadex, Sephadex G-150 and hydroxyapatite columns. The kinase eluted as a single activity peak from all three columns. 3. The partially purified histone-dependent kinase had the following properties: (i) pH optimum, 7.0; (ii) optimum temperature, 37 °C; (iii) K_m for mixed calf thymus histone, 0.15 mM; K_m for ATP, 0.8 mM; (iv) preferred fractionated histone acceptors, $H_{2b} > H_4 > H_{2a} > H_3$ (H_1 does not serve as an acceptor); (v) optimum activity required 10–20 mM-Mg^{2+}; (vi) inhibited 50–80% by 0.01 mM- and 1 mM-Ca^{2+}; (vii) activity was not stimulated by calmodulin, cyclic AMP (1 mM) or cyclic GMP (1 mM) nor inhibited by a cyclic AMP-dependent protein kinase inhibitor (50 μg/assay); (viii) apparent M_r 75000, as determined by Sephadex G-150 gel filtration chromatography; (ix) phosphorylated exclusively serine residues. 4. Protein kinase activity was low in the early exponential phase of the growth curve and increased 6-fold upon entry into the stationary phase.

INTRODUCTION

Leishmania donovani, the aetiological agent of kala-azar or visceral leishmaniasis, has a dimorphic life cycle: in the alimentary tract of its insect vector, the sandfly, it occurs extracellularly as the motile flagellated promastigote form, whereas within the phagolysosomal system of mammalian macrophages, it exists in non-motile non-flagellated amastigote form.

The organism is of interest for two reasons. First, it is responsible for considerable mortality and morbidity, especially in tropical and subtropical areas of the world (Mahmoud & Warren, 1977). Second, the host–parasite interaction represents an interesting biological phenomenon, providing a fertile area for studies aimed at enhancing our understanding of the biochemistry and cell biology of this interaction.

Recently, a number of reports have appeared describing tartrate-resistant acid phosphatase activity in certain intracellular parasites, namely *Leishmania donovani* and *Legionella micdadei* (Remaley *et al.*, 1985; Saha *et al.*, 1985). The phosphatase activity is localized to the outer surface of the organisms, and has the capacity to disarm phagocytic cells by blocking their ability to produce reactive oxygen intermediates (e.g. O_2^-, H_2O_2) (Remaley *et al.*, 1984). Although considerable effort has been directed at the leishmanial phosphohydrolases [e.g. nucleotidases (Gottlieb & Dwyer, 1981*b*) and acid phosphatases (Glew *et al.*, 1982)], little is known about the converse reaction, namely protein phosphorylation.

Protein kinases are a class of enzymes which catalyse the transfer of the γ-phosphate from nucleoside triphosphates (usually ATP) to protein acceptors. In addition to numerous reports describing protein kinases in animals, there are reports of protein kinase activity in protozoa (Majumder *et al.*, 1975), fungi (Pall, 1981), plants (Keates, 1973) and bacteria (Rahmsdorf *et al.*, 1974). However, reports of protozoal parasite protein kinases are few in number. It has been shown that *Plasmodium berghei*, a malaria parasite, and *Babesia bovis*, a cattle parasite, contain protein kinase activity and that infection results in the phosphorylation of erythrocyte membrane proteins (Wiser *et al.*, 1983; Williadsen, 1984). *Trypanosoma brucei* contains at least two different protein kinases (Walter & Opperdoes, 1982), and its variant surface glycoproteins, which allow the parasite to escape the host's immune system, are rapidly phosphorylated (de Almeida & Turner, 1983).

In the present report we demonstrate that *Leishmania donovani* promastigotes contain a protein kinase that is localized to the outer surface of the parasite. The kinase has been solubilized and purified 118-fold, and some of its properties have been characterized.

Abbreviations used: TCA, trichloroacetic acid; PAGE, polyacrylamide-gel electrophoresis; BSA, bovine serum albumin; MUP, 4-methylumbelliferyl phosphate; HBS, Hepes buffer in 0.9% NaCl soln.
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MATERIALS AND METHODS

Materials

[^{32}P]Orthophosphate (2 mCi/ml), [α-^{32}P]ATP and [γ-^{32}P]ATP (5–10 Ci/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A. 4-Methylumbelliferyl phosphate, phosphorylase kinase, 1,2-dioleoyl-rac.-glycerol (diolein), casein, protamine, phosvitin, kemptide, calmodulin, trypsin, calf thymus histones and protein kinase inhibitor from rabbit muscle were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. QAE-Sephadex and Sephadex G-150 were purchased from Pharmacia Chemicals, Piscataway, NJ, U.S.A. Hydroxyapatite was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. Purified pyruvate kinase was a gift from Dr. James Blair, West Virginia University, Morgantown, WV, U.S.A. Myosin from rabbit muscle was a gift from Dr. Jimmy Collins, University of Pittsburgh, Pittsburgh, PA, U.S.A. Histone fractions were a gift from Dr. Sidney Morris, University of Pittsburgh.

Organism and growth conditions

A cloned strain of *L. donovani* (sudan-1) promastigotes was cultivated and harvested as described by Gottlieb & Dwyer (1981*a*), except for the addition of streptomycin (50 μg/ml) and penicillin (50 μg/ml) to the growth medium.

Labelling of parasite membranes with $^{32}P_i$

Promastigote cells from 7-day-old cultures (1×10^6 cells) were harvested and washed twice with 25 mM-Hepes buffer in 0.9% NaCl soln. (HBS), pH 7.2, supplemented with 5 mM-glucose, and then suspended in 0.95 ml of the same medium. [^{32}P]Orthophosphate (0.1 mCi) was added to the cell suspension, which was then incubated for 2 h at 25 °C. The phosphate chase was effected by rapidly washing the cells twice and then resuspending them in fresh medium for 1–15 h at 25 °C. Portions (0.2 ml) were removed at various times and rapidly frozen at −70 °C until the time of analysis. To prepare a crude membrane fraction, the ^{32}P-labelled promastigotes were thawed and subjected to three freeze–thaw cycles and then centrifuged (10000 *g*, 10 min). The crude membrane fraction was extracted three times with ethanol/acetone (3:1, v/v) to remove lipids. The delipidated membrane fractions were then subjected to SDS/PAGE and autoradiography to identify radiolabelled proteins. The X-ray film was developed and scanned with a densitometer (Helena Laboratories, Beaumont, TX, U.S.A.).

Purification of histone kinase

All procedures, unless otherwise specified, were carried out at 4 °C in 10 mM-sodium citrate buffer, pH 6.5, containing proteinase inhibitors (50 μM-leupeptin and 50 μM-aprotinin). A twice-washed pellet composed of 2×10^{11} cells, harvested 1 day after they had entered the stationary phase of growth, was suspended in HBS containing protease inhibitors and subjected to three freeze–thaw cycles. The cell suspension was sonicated three times for 30 s. Finally, the sonicated material was homogenized in a Potter–Elvehjem homogenizer (10 strokes) followed by stirring for 15 min. The crude homogenate was centrifuged at 200000 *g* for 30 min. The resulting supernatant, containing 70–80% of the total protein kinase activity, was applied to a QAE-Sephadex A-50 column (2 cm × 10 cm), equilibrated with 10 mM-sodium citrate buffer, pH 6.5. Approximately 10% of the kinase activity appeared in the breakthrough fraction; the remaining activity, accounting for more than 80% of the activity applied to the column, was eluted with a 0–0.5 M-NaCl gradient in the same buffer.

The eluate from the QAE-Sephadex column was concentrated and chromatographed on a Sephadex G-150 column (1.2 cm × 100 cm) equilibrated in the same sodium citrate buffer. The single peak of kinase activity was pooled and loaded directly on to a hydroxyapatite column (1 cm × 10 cm) equilibrated with 10 mM-sodium citrate buffer, pH 6.5. All of the kinase activity that was recovered from the hydroxyapatite column (about 65% of that applied to the column) appeared in the breakthrough fraction. The hydroxyapatite column breakthrough was concentrated by dialysis against 50% glycerol in 10 mM-sodium citrate buffer, pH 6.5, containing protease inhibitors.

The protein kinase assay

Protein kinase activity was routinely determined with mixed histones used as the phosphate acceptor; the incubation medium contained the following additions, in a final volume of 0.1 ml: 10 mM-$MgCl_2$, 50 mM-NaF, 0.3 mM-histone, 50 mM-Tris/HCl buffer (pH 7.0), 1 mM-[γ-^{32}P]ATP, 50 mM-NaCl and 5–20 μl of enzyme solution (0.1–1.0 μg of protein). The reaction was initiated by the addition of ATP and continued for 10 min at 37 °C. The reaction was terminated by addition of ice-cold 10% (w/v) trichloroacetic acid (TCA) followed by 10 μl of bovine serum albumin solution (20 mg/ml). The pellet obtained by centrifugation (5000 *g*, 10 min) was dissolved in water and reprecipitated three times with 10% TCA. Finally, the precipitate was dissolved in water and a portion was counted for radioactivity as described by Corbin & Reimann (1974). The reaction was linear for 10 min at 37 °C and within the range of quantities of kinase assayed in the present study (results not shown). To determine which particular histone species were radiolabelled, the reaction product was subjected to SDS/PAGE and analysed by autoradiography.

When the synthetic peptide, kemptide, was tested as substrate, portions of the reaction mixtures were applied to discs of Whatman P81 paper and washed with 30% acetic acid (four times for 20 min each). The paper discs were dried and radioactivity was determined with a scintillation counter (Maller *et al.*, 1978).

Phosphorylation of histones using [γ-^{32}P]ATP and intact parasites: demonstration of ectokinase activity

Histones were also phosphorylated using intact promastigotes as the source of protein kinase. In this case, 1×10^6 promastigotes (7-day culture) were washed three times and suspended in 25 mM-HBS, pH 7.0, supplemented with 5 mM-glucose, 0.3 mM-histone, 10 mM-NaF, 10 mM-$MgCl_2$ and 1 mM-[γ-^{32}P]ATP or -[α-^{32}P]ATP. The cells were incubated for 10 min at 25 °C and incorporation of ^{32}P into TCA-insoluble products was determined as described above. At the end of the 10 min incubation period 100% of the cells were still motile and apparently intact. In order to determine whether the ectokinase could phosphorylate promastigote cell surface proteins, the experiment was repeated, except that the histones were omitted from the incubation medium. Phosphorylated proteins were

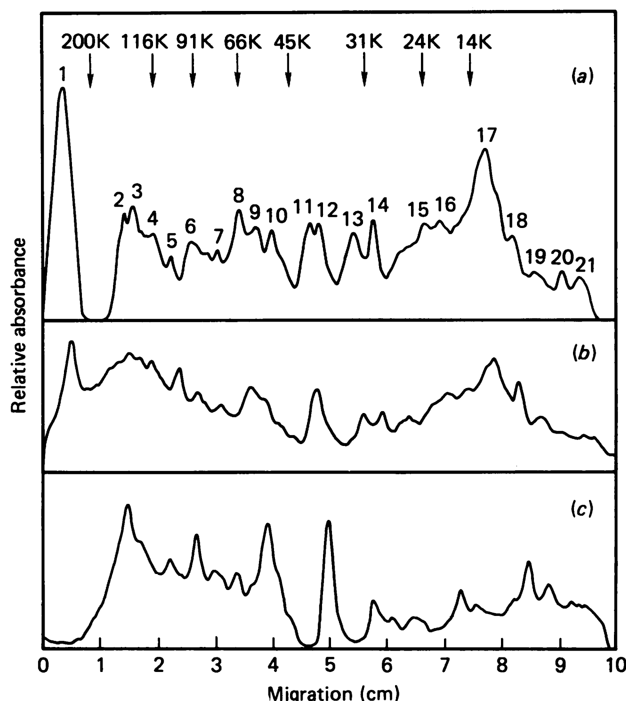

Fig. 1. Analysis of phosphoproteins on SDS/PAGE after phosphorylation with [^{32}P]orthophosphate

SDS/5–20%-PAGE of $^{32}P_i$-labelled proteins from *L. donovani*. Promastigotes were grown in liquid medium and labelled with [^{32}P]orthophosphate as described in the Materials and methods section. After pulse-labelling for 2 h, the radioactivity was chased for 0 h (*a*), 5 h (*b*) and 15 h (*c*). A 10 μg portion of protein from each sample was subjected to SDS/PAGE and then autoradiographed as described in the Materials and methods section. Bands are numbered 1 to 21. The film was scanned with the aid of a densitometric scanner.

quantified by the technique of Corbin & Reimann (1974) and analysed by SDS/PAGE as described below.

The acid phosphatase assay

Acid phosphatase activity was determined fluorimetrically at 37 °C with MUP as the substrate, as described by Glew *et al.* (1982).

Protein determination

Protein concentration was estimated by the method of Bradford (1976) with bovine serum albumin used as standard.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

^{32}P-labelled proteins were resolved by SDS/PAGE as described by Laemmli (1970) using 5–20% polyacrylamide gels. Proteins were stained with Coomassie Brilliant Blue (G-250) and press-dried on to Whatman 3MM paper. Radiolabelled proteins were visualized by autoradiography with the aid of a Dupont Lightning-plus Screen. The autoradiogram film and wet gel (before drying) were scanned with a densitometric scanner.

Phospho amino acid analysis

Radiolabelled proteins were resolved by gel electrophoresis, excised from the dried gel with a razor blade and digested in 6 M-HCl at 110 °C for 2 h. Authentic internal standards were added and phosphorylated amino acids were separated on Eastman cellulose thin-layer plates by electrophoresis for 2 h in the first dimension (formic acid/acetic acid/water, 8:2:90, by vol.), followed by chromatography in the second dimension for 3 h with a solvent system consisting of butanol/pyridine/acetic acid/water (50:33:10:40, by vol.).

RESULTS

Labelling of *L. donovani* membranes with $^{32}P_i$

To estimate the number and kinds of phosphorylated membrane proteins in *L. donovani* promastigotes, we incubated 7-day-old promastigotes with inorganic ^{32}P. Because we were interested primarily in the plasma membranes, after first breaking the cells by freeze–thawing we prepared a crude membrane fraction by centrifuging the homogenate at 10000 ***g*** for 10 min. Phospholipids were removed by extraction with ethanol/acetone. SDS/PAGE resolved 21 ^{32}P-labelled proteins, which ranged in size from about 10000 to 365000 daltons (Fig. 1).

Not only were many proteins phosphorylated, but most were metabolically unstable as well (Figs. 1*a*–1*c*). Some ^{32}P-labelled bands (e.g. Fig. 1, bands 1 and 13–17) lost their label during the chase period (e.g. Fig. 1, bands

Table 1. Summary of the purification of protein kinase from *L. donovani*

Purification step	Total activity (units)*†	Total protein (mg)	Specific activity (units/mg)	Yield‡ (%)	Purification (-fold)
1. Crude homogenate	40300	1210	33.2	—	—
2. High-speed supernatant	33800	774	43.6	100	1
3. QAE-Sephadex	27000	84	321	80	7.37
4. Sephadex G-150	23700	11.9	1990	70	45.6
5. Hydroxyapatite	15500	3.0	5160	46	118

* One unit of kinase activity equals 1 pmol of ^{32}P incorporated/min.
† Activities were measured with mixed histone (type II-S, calf thymus) as substrate.
‡ Calculated by setting the activity in step 2 at 100%.

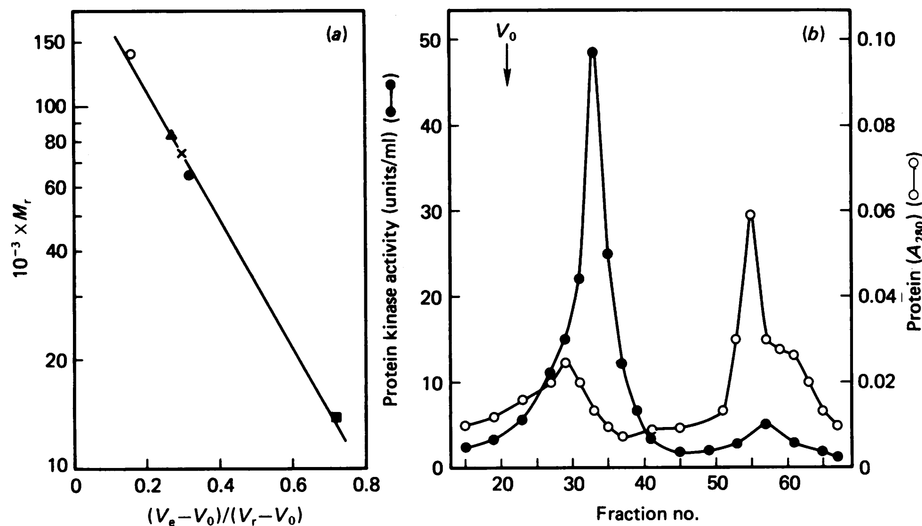

Fig. 2. Gel-filtration chromatography of protein kinase on Sephadex G-150

A portion (1.0 ml) of the protein kinase preparation after the QAE-Sephadex step (Table 1) was applied to a 1.2 cm × 100 cm Sephadex G-150 column equilibrated in 10 mM-citrate buffer, pH 6.5. Fractions (2 ml) were collected and analysed for kinase activity (●) and protein (○) (*b*). The column was calibrated by chromatographing a solution of standard proteins on the same columns containing lactate dehydrogenase (○), bacterial alkaline phosphatase (▲), bovine serum albumin (●) and lysozyme (■). The void volume (V_0) was determined by using Blue Dextran, and the retention volume (V_r) with 0.5 M-NaCl. The column was calibrated for M_r by plotting the equilibrium distribution coefficients, $K_D = (V_e-V_0)/(V_r-V_0)$, of each of the standard proteins versus log M_r (*a*). The M_r of protein kinase (×) was estimated to be 75000.

2, 5, 11 and 12). Some of the bands labelled during the 2 h incubation with $^{32}P_i$ did not lose radioactivity during the chase period (e.g. Fig. 1, bands 4 and 8). The apparent loss of radioactivity from a particular protein band could be the result of dephosphorylation or due to turnover of the entire protein. Likewise, the appearance of a new ^{32}P-labelled band during the chase period, or an increase in the specific radioactivity of a particular band, might result from proteolysis of a larger radiolabelled protein.

Purification of histone kinase

The partial purification of the leishmanial protein kinase was monitored with histone as the exogenous phosphate acceptor in the kinase assay. Because the parasites were grown on a relatively small scale and stored frozen (−70 °C) until we had accumulated a quantity of cells sufficient to support the isolation of protein kinase, we began the purification using freeze–thawed promastigotes. Preliminary studies showed that greater than 80% of the total protein kinase activity was rendered soluble by this procedure (Table 1). The high-speed supernatant (200000 *g*, 30 min) containing most of the histone-specific protein kinase activity was chromatographed sequentially on columns of QAE-Sephadex (results not shown), Sephadex G-150 (Fig. 2) and hydroxyapatite. With the ion-exchange and gel filtration columns, protein kinase activity was eluted as a single relatively sharp and symmetrical peak, indicating that we are probably dealing with a single species of protein kinase. From these three steps, we recovered nearly half of the soluble protein kinase activity contained in the original high-speed supernatant; the overall purification achieved was 118-fold (Table 1). At this point we decided to characterize some of the physicochemical and kinetic properties of the enzyme.

Properties of the partially purified leishmanial protein kinase

Size. In terms of size, when chromatographed on a calibrated Sephadex G-150 column (Fig. 2) the protein kinase was eluted between bacterial alkaline phosphatase and bovine serum albumin; the apparent M_r of the native protein kinase was 75000.

Acceptor specificity. A variety of potential phosphate acceptors were compared in the standard protein kinase assay (Table 2), namely mixed histones, protamine, rat liver pyruvate kinase, phosphorylase kinase, casein, myosin, phosvitin and kemptide; only the mixed histones and protamine supported detectable activity. When the mixture of ^{32}P-phosphorylated histones was subjected to analysis by SDS/PAGE, we found that the H_{2b} and H_4 components had each taken up about half of the total ^{32}P that was incorporated into the histone mixture (results not shown). In an effort to define better the specificity of the kinase, we compared five subfractions of histone for their ability to serve as phosphate acceptors in the standard protein kinase assay. As shown in Table 2, the histone fractions are not equally effective acceptors; H_{2b} was the best protein kinase substrate, followed in order by H_4, H_{2a} and H_3. The H_1 fraction did not serve as a phosphate acceptor in the protein kinase assay. Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) was not a substrate for protein kinase.

When the radiolabelled histone product was subjected to acid hydrolysis and the phospho amino acids were separated on cellulose thin-layer plates, we found that all

Table 2. Phosphorylation of different proteins by partially purified protein kinase from *L. donovani*

Protein acceptor*	Amount per incubation (μg)	Kinase activity† (units/ml)
Mixed histones	300	220
Histone subfractions		
H_1	300	< 2
H_3	300	8.2
H_{2b}	300	56.6
H_{2a}	300	14.7
H_4	300	33.9
Protamine	300	54.2
Pyruvate kinase	10	< 2
Phosphorylase kinase	40	< 2
Casein	500	< 2
Myosin	20	< 2
Phosvitin	200	< 2
Leu-Arg-Arg-Ala-Ser-Leu-Gly (kemptide)	100	< 2

* The protein kinase assay was carried out with the specified amount of acceptor in a 0.1 ml assay mixture. The reaction was carried out for 10 min at 37 °C and terminated by TCA precipitation as described in the Materials and methods section.

† One unit of kinase activity equals 1 pmol of ^{32}P incorporated/min.

Table 3. Effect of various additions to and deletions from the assay medium on protein kinase activity

Addition (+) or omission (−)*	Protein kinase activity† (units/ml)
Control	37.5
−50 mM-NaCl	5.2
−10 mM-Mg^{2+}	8.6
−10 mM-NaF	24.3
−histone (0.3 mM)	5.62
+1 mM-Mn^{2+}	22.5
+0.01 mM-Ca^{2+}	18.4
+1 mM-Ca^{2+}	6.56
+0.01 mM-cyclic AMP	35.6
+1 mM-cyclic AMP	24.3
+0.01 mM-cyclic GMP	28.1
+1 mM-cyclic GMP	25.1
+rabbit muscle protein kinase inhibitor (50 μg/assay)	32.9
+calmodulin (3.5 μg)	13.5
+10 μM-Ca^{2+} and calmodulin (3.5 μg)	32.2
+10 μM-Ca^{2+} and phosphatidylserine (10 μg)	33.4
+10 μM-Ca^{2+} and phosphatidylinositol (10 μg)	27.4
+15 μM-diolein	37.0
+10 μM-Ca^{2+}, phosphatidylinositol (10 μg) and 15 μM-diolein	18.4

* The phosphorylation reaction was carried out with 10 μl of partially purified protein kinase (after the hydroxyapatite step). The 'control' contained all the components of the standard reaction mixture and the reaction was carried out for 10 min at 37 °C as described in the Materials and methods section.

† One unit of protein kinase activity is equal to 1 pmol of ^{32}P incorporated/min.

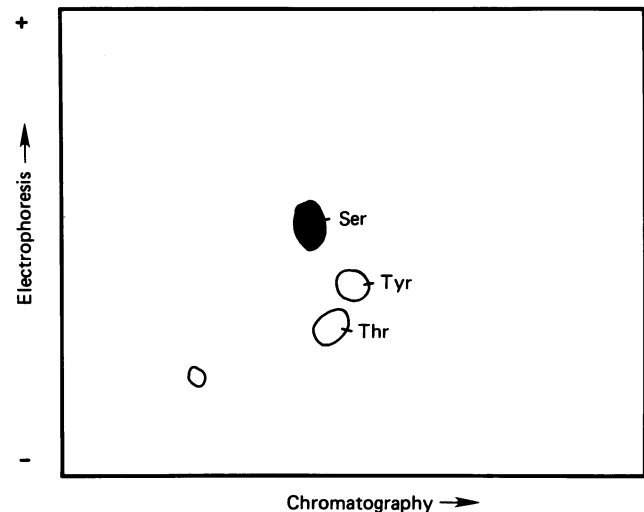

Fig. 3. Phospho amino acid analysis of radiophosphorylated histone

Histones were phosphorylated with the partially purified protein kinase from *L. donovani*. The phosphorylation reaction was carried out for 10 min at 37 °C. The phosphorylated histones were separated by SDS/PAGE. After elution from the gel and hydrolysis in 6 mM-HCl at 110 °C for 2 h the sample was freeze-dried and authentic standards (e.g. phosphoserine, phosphothreonine and phosphotyrosine) were added. The phospho amino acids were separated on a cellulose thin-layer plate as described in the Materials and methods section.

(> 99%) of the ^{32}P ran with the phosphoserine standard (Fig. 3). We conclude that the leishmanial protein kinase transfers the γ-phosphate of ATP to serine residues in the histones.

Metal ion requirements. Omission of NaCl from the standard protein kinase assay reduced protein kinase activity by about 80% (Table 3). Likewise, omitting $MgCl_2$ from the incubation medium decreased protein kinase activity by about 75%. The elimination of NaF [which was included in the reaction mixture to inhibit phosphatase (Remaley *et al.*, 1985)] from the assay mixture reduced activity by about one-third; we therefore included fluoride ions in the standard protein kinase assay. Optimum histone-dependent protein kinase activity required 10–20 mM-$MgCl_2$ (results not shown). Inclusion of 0.01 mM- or 1.0 mM-Ca^{2+} in the standard protein kinase assay inhibited activity markedly (Table 3). Neither calmodulin nor a Ca^{2+} (10 μM)/calmodulin mixture had any stimulatory effect on the protein kinase. Mixtures of Ca^{2+} (10 μM) and the acidic phospholipids, phosphatidylserine and phosphatidylinositol also had only a slight inhibitory effect on protein kinase activity. Neither diolein (15 μM) nor a phosphatidylinositol (10 μg)/Ca^{2+} (10 μM)/diolein (15 μM) mixture had any effect on the protein kinase.

Histone requirement. Omission of histones from the assay medium markedly reduced the rate of incorporation of [γ-^{32}P]ATP into proteins (Table 3); the residual incorporation observed in the absence of histone acceptor suggests that the partially purified kinase preparation contained some endogenous phosphate

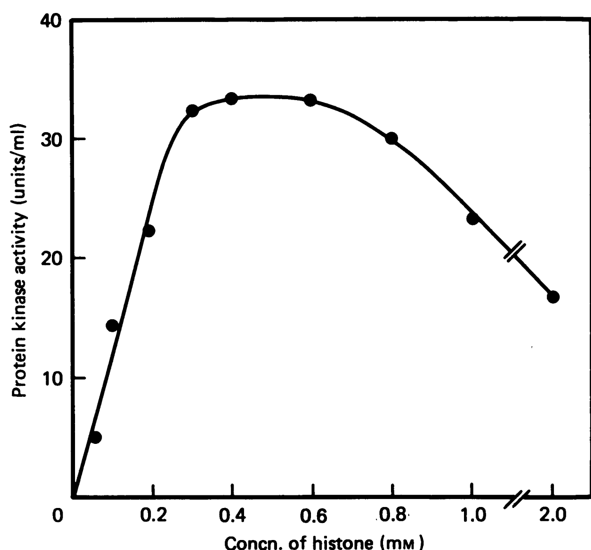

Fig. 4. Effect of histone concentrations on kinase activity

Protein kinase activity was measured at pH 7.0 at different concentrations of mixed histones ranging from 0.05 to 2.0 mM. Reactions were carried out for 10 min at 37 °C. Histones were precipitated with 10% (w/v) TCA and the TCA-precipitable radioactivity was determined as described in the Materials and methods section.

acceptors (which have not been characterized). It is unlikely that the incorporation of ^{32}P into TCA-insoluble material obtained in the absence of histone is the result of autophosphorylation. When the histone concentration of the assay medium was varied, we observed a bell-shaped curve, with optimum kinase activity being achieved with 0.3–0.6 mM-histone (Fig. 4).

Effect of cyclic nucleotides. The activity of the protein kinase was not stimulated by 0.01 mM- or 1 mM-cyclic AMP or -cyclic GMP; in fact, activity was moderately inhibited by these cyclic nucleotides (Table 3). The cyclic AMP-dependent protein kinase inhibitor of rabbit muscle (50 μg/assay) had only a slight inhibitory effect on the activity of the leishmanial protein kinase.

Since some protein kinases are activated by partial proteolysis (Inoue *et al.*, 1977) we attempted to activate the leishmanial protein kinase by exposure to trypsin. However, treatment with even small amounts of trypsin for only a few minutes resulted in extensive inactivation of the kinase (data not shown).

Effect of temperature. Between 20 °C and 37 °C, protein kinase activity increased linearly with temperature; the highest specific activity was obtained when the incubation was carried out at 37 °C (results not shown). Above 40 °C, the enzyme lost activity rapidly.

Demonstration of ectokinase activity using intact promastigotes

The first indication that a significant portion of the protein kinase activity of *L. donovani* promastigotes was located on the external surface of the organism came from an experiment in which we incubated intact osmotically buffered promastigotes (10^6 cells) in 0.1 ml of fresh growth medium supplemented with 0.3 mM-histone, 1 mM-[γ-^{32}P]ATP, 50 mM-NaCl, 10 mM-Mg^{2+} and 10 mM-NaF for 10 min at 25 °C and compared the extent of incorporation of ^{32}P into proteins with that catalysed by exactly the same number of cells that had been first broken by freeze–thawing. The intact cells incorporated ^{32}P into TCA-precipitable product at 50% of the rate at which the broken cells expressed protein kinase activity (results not shown). Because essentially all of the osmotically buffered parasites were still motile and apparently intact at the end of the 10 min period of incubation with [γ-^{32}P]ATP and histone, and assuming that neither the histones nor the ATP entered the cells, it appears that the outer surface of *L. donovani* promastigotes contains a protein kinase that is capable of phosphorylating histones.

We were next interested in determining whether intact promastigotes would phosphorylate their own proteins. We therefore repeated the experiment described in the preceding paragraph, except that we omitted histones from the incubation medium. Despite the omission of histones from the medium, the rate of incorporation of [γ-^{32}P]ATP into protein was still substantial, being about 40% of that achieved when histones were present (results not shown).

When we analysed the ^{32}P-labelled proteins, labelled by intact parasites in the absence of histones, by

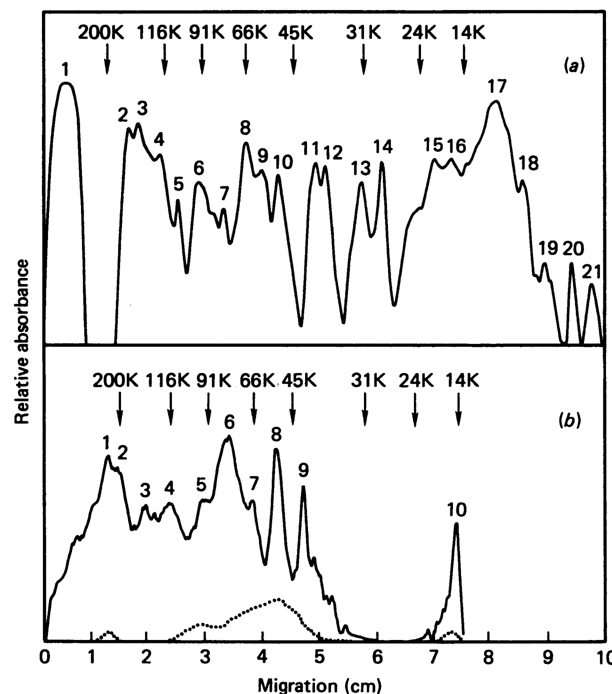

Fig. 5. Surface protein phosphorylation of *L. donovani* promastigote

Osmotically buffered intact promastigotes (10^6 cells) were incubated with 1 mM-[α-^{32}P]ATP or -[γ-^{32}P]ATP, 50 mM-NaCl, 10 mM-Mg^{2+} and 10 mM-NaF for 10 min at 25 °C. Phosphoproteins were then analysed by SDS/PAGE and autoradiography. The X-ray film was scanned with the aid of a densitometric scanner. (*a*) Phosphorylation of *L. donovani* cells with [^{32}P]orthophosphate; (*b*) the phosphorylation obtained with [γ-^{32}P]ATP (——) and [α-^{32}P]ATP (· · · ·).

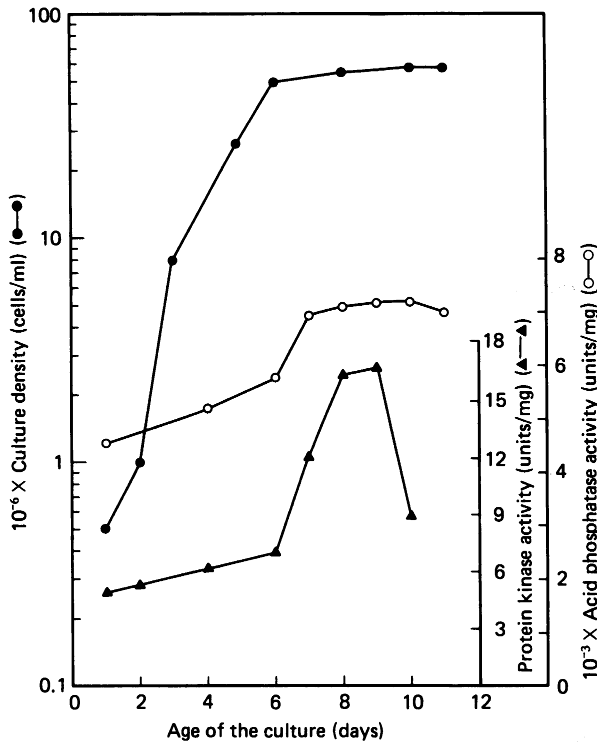

Fig. 6. Protein kinase activity during the growth of *L. donovani*

A flask containing 200 ml of medium 199 and 20% fetal calf serum was inoculated with 0.5×10^6 cells. Portions were removed at the indicated times, and cell density (●) was determined by haemocytometry. Cells were harvested and washed, and the crude homogenates were prepared as described in the Materials and methods section and assayed for histone kinase activity (▲). Acid phosphatase activity (○) was determined as described in the Materials and methods section.

SDS/PAGE we observed that more than 10 protein bands were radiolabelled (Fig. 5*b*). About half of the radioactive proteins had the same electrophoretic mobilities as proteins that were labelled when intact promastigotes were incubated with $^{32}P_i$; specifically, bands 3 and 5–8 that were radiolabelled by the ectokinase and [γ-^{32}P]ATP were also labelled when $^{32}P_i$ was added to a culture of exponential-phase parasites (Fig. 5*a*).

In view of the intense acid phosphatase and nucleotidase activities (Gottlieb & Dwyer, 1981*b*) that have been established as being localized to the outer surface of the plasma membrane of *L. donovani* promastigotes, it occurred to us that the observation in the preceding paragraph could be accounted for by the dephosphorylation of ATP, followed by the transport of radiolabelled $^{32}P_i$ into the cells and incorporation of the radioisotope into intracellular phosphates. In order to test this possibility, we utilized a strategy, employed by Schieven *et al.* (1985), of comparing the incorporation of ^{32}P from [α-^{32}P]ATP and [γ-^{32}P]ATP into parasite proteins when the isotopes were incubated for 10 min with intact promastigotes. Fig. 5 demonstrates extensive phosphorylation of promastigote proteins by [γ-^{32}P]ATP and very little labelling with [α-^{32}P]ATP under the same conditions. Furthermore, when we took the extracellular medium from the experiment in which we had incubated [γ-^{32}P]ATP and promastigotes and chromatographed it on a charcoal column, to which the ATP would bind, we found that less than 5% of the radiolabelled ATP had been broken down. Thus these results indicate that labelling of the parasite proteins is apparently due to phosphorylation by authentic ectokinase.

Histone-specific protein kinase activity during the growth of *L. donovani*

The goal of our last experiment was to estimate protein kinase activity as a function of the phase of cell growth. As shown in Fig. 6, kinase activity per cell was relatively low and increased only slightly during the exponential phase of growth; however, as the cells entered the stationary phase, protein kinase activity per cell increased about 6-fold. Late in the stationary phase (day 10), there was a precipitous decline in protein kinase activity.

DISCUSSION

The major accomplishment of this study of the enzymology of *L. donovani* was the discovery and partial purification of a cyclic nucleotide-independent protein kinase in promastigotes that prefers histone as substrate and which phosphorylates serine residues. The kinase requires high concentrations of NaCl (approx. 50 mM) and $MgCl_2$ (approx. 10 mM) for optimum activity, and functions best at pH 7.0.

The leishmanial protein kinase is similar in some respects to a protein kinase that has been described in *Plasmodium berghei* and *Plasmodium chabaudi* (Wiser *et al.*, 1983); these organisms also contain a protein kinase that is cyclic AMP-dependent and which phosphorylates serine residues. However, whereas the *L. donovani* protein kinase prefers histones, the plasmodial kinases are specific for casein (Wiser *et al.*, 1983). Another point of comparison between the various parasite protein kinases is in the bivalent cation requirement: the *L. donovani* protein kinase is strongly dependent on Mg^{2+}, whereas Mn^{2+} and Ca^{2+} are inhibitory (Table 3). This finding is in accordance with the report of Wiser *et al.* (1983), who demonstrated a similar bivalent cation requirement for the plasmodial protein kinases.

It is not unprecedented for a protozoal parasite to possess a protein kinase that is unresponsive to cyclic AMP. In fact, for the Trypanosomatidae, although they possess adenylate cyclase and their growth and differentiation are affected by cyclic AMP, our efforts so far to demonstrate stimulation of protein kinases by cyclic AMP have been unsuccessful [see Opperdoes (1985) for review]. At this moment it is difficult to explain the reason for the presence of cyclic AMP-independent protein kinase in *L. donovani* and in other Trypanosomatidae.

Although we have not yet carried out a comprehensive subcellular fractionation study to confirm the location of the protein kinase in *L. donovani* promastigotes, our results suggest that the enzyme, or at least a considerable fraction of the total enzyme activity, is localized to the outer surface of the plasma membrane; in other words, it appears to be an ectokinase. Several pieces of evidence support this conclusion. First, the enzyme is particulate in crude homogenates of fresh cells; solubilization requires subjecting the promastigotes to freezing and thawing or extraction with bile salts (e.g. sodium

cholate) (results not shown). Second, we found that live, intact, motile parasites incubated for brief periods of time (5–10 min) with impermeant radiolabelled [γ-^{32}P]ATP and histones were capable of phosphorylating histones at more than half the rate expressed by the same number of broken cells. Third, whereas we observe extensive phosphorylation of cellular proteins when we incubate [γ-^{32}P]ATP with intact promastigotes, much less labelling is seen when we incubate the cells with [α-^{32}P]ATP. Nevertheless, we have not examined the possibility that *L. donovani* promastigotes possess a transport system for internalizing ATP; noteworthy is the demonstration of Sanchez *et al.* (1976) that African trypanosomes possess a nucleotide transport system in their plasma membrane. Confirmation of the proposed attachment of their histone-dependent kinase to the outer surface of the parasite's plasma membrane will require subcellular fractionation, marker analysis and electron microscopy–histology studies.

The orientation of the active site of the cell surface protein kinase and the ability of the enzyme to phosphorylate extracellular acceptors raise a question regarding the nature of the natural acceptor. There are at least two possibilities: first, the parasite could use its own ectokinase to phosphorylate proteins on the surface of host cells, in this case phagocytes (e.g. neutrophils, macrophages). There is a precedent for this kind of kinase-mediated host–parasite interaction; malaria parasites and *B. bovis* (Wiser *et al.*, 1983; Willadsen, 1984) possess an ectokinase that is capable of phosphorylating erythrocyte membrane proteins.

Alternatively, the leishmanial ectokinase could utilize proteins on the parasite's own surface as phosphate acceptors. Under this consideration, there are two possibilities: first, the kinase on the surface of one promastigote might utilize some exposed protein on the surface of a second promastigote as the phosphate acceptor in the kinase reaction or, second, the ectokinase on the same cell might be situated in close proximity to a protein that is capable of serving as the phosphate acceptor in the kinase reaction. Evidence contained in this report indicates that intact promastigotes do, in fact, possess at least 10 proteins on their surface (Fig. 5*b*) that can serve as substrates in the kinase reaction; we observed that live motile promastigotes incorporate significant amounts of ^{32}P from [γ-^{32}P]ATP into membrane proteins. Furthermore, about five proteins that are radiolabelled by [γ-^{32}P]ATP and the ectokinase have the same apparent M_r values on SDS/PAGE gels as those that are labelled when live promastigotes are incubated with $^{32}P_i$ (Fig. 5).

Collectively, the demonstration of ectokinase activity in *L. donovani* promastigotes in this study, together with previous reports documenting the presence of intense acid phosphatase activity on the outer surface of the organism, indicate that the parasite possesses the capability of regulating the properties and function of not only its own cell surface macromolecules, but those of host cells as well by phosphorylation and dephosphorylation of key proteins. We have already shown (Remaley *et al.*, 1984) that the predominant tartrate-resistant leishmanial acid phosphatase has the effect of blocking the oxygen burst in phagocytic host cells. It is conceivable that the ectokinase of *L. donovani* plays some role in disarming host cells when the surface membranes of the parasite and the host cell first come in contact with each other. Noteworthy is the observation of Sacks & Perkins (1984) that *Leishmania* promastigotes taken from the stationary phase of growth are much more infectious than exponential-phase promastigotes. Since protein kinase activity increased 6-fold during the stationary phase, this enzyme may also play some pathophysiological role inside the host cell; worth considering in this regard is the possibility that lysosomal proteins in the host cell are inactivated by phosphorylation brought about by the parasite's ectokinase.

There are additional specific questions that are worthy of study. Does the parasite retain the histone-dependent kinase when it undergoes transformation, inside host cells, from the promastigote into the amastigote form? Do other *Leishmania* species possess this protein kinase? Do host cells contain proteins that can function as phosphate acceptors for the ectokinase? Does incubation of the solubilized purified histone-specific kinase with phagocytes have any effect on the metabolism of the latter? These and other questions will be addressed once the *L. donovani* ectokinase has been isolated in pure form; hopefully, a histone affinity column will facilitate this task.

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