IDENTIFICATION AND FUNCTIONAL ASPECTS

OF

PROTEIN KINASE ACTIVITY

IN

LEISHMANIA AETHIOPICA

A THESIS PRESENTED TO THE SCHOOL OF GRADUATE STUDIES

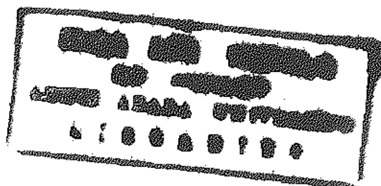
OF

ADDIS ABABA UNIVERSITY

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF

MASTER OF SCIENCE IN BIOCHEMISTRY



BY

DANIEL ASSEFA

AUGUST, 1993

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DEDICATION

This thesis is dedicated to the memory of

my father.

ACKNOWLEDGMENT

First of all I would like to extend my gratitude to my advisors, Dr. Göran Skoglund and Dr. Yesehak Worku who guided and educated me through out my work and tolerated my novice. I am indebted to the late Dr. Getachew Boledia for his inspiration of and continued interest in the work. I also offer my thanks to Dr. Hanna Akuffo for provision of the strains and for introducing me to cell culture. I thank the ministry of health for sponsoring me and the staff of the department of Biochemistry for the continued support that they showed me in all aspects of my research endeavour. I appreciate the help and friendliness that all the people at the Berzellius laboratory at the Karolinska Institute extended. I also would like to express my deep gratitude to friends and colleagues in the BRTP especially Dr. Yosef A. Mengesha the BRTP coordinatior for making the lab a habitable place and for the smooth operation of my project. I am grateful to Ato Alehegn Kasse of AAUMF and Ato Kassa Beimnet of AHRI for helping me with the photographs. Last but not least my deepest gratitude goes to my parents for making all this possible and to Selam for giving every thing a reason.

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ABBREVIATIONS

ADP .... Adenosine diphosphate

A-Kinase....... Cyclic AMP dependent protein kinase

AMP............. Adenosine monophosphate

ATP.............. Adenosine triphosphate

BSA.............. Bovine serum albumin

Ca-Kinase...... Calcium dependent protein kinase

cAMP........... Cyclic adenosine monophosphate

cGMP............Cyclic guanosine monophosphate

cIMP............ Cyclic inositol monophosphate

DNA .... Deoxyribonucleic acid

FLMP........... formyl-methionyl-leucyl-phenylalanine

GMP............ Guanosine monophosphate

LPG............. Lipophosphoglycan

MLC............ Muscle light chain

MLCK.......... Muscle light chain kinase

NADH.......... Reduced nicotinamide adenine dinucleotide

PDBu........... Phorbol-12, 13-dibutyrate

PKC............. Protein kinase C

PSP.............. Promastigote surface proteinase

PTK............. Protein tyrosine kinase

RNA............ Ribonucleic acid

ABSTRACT

Protein kinase activity was demonstrated on intact Leishmania aethiopica promastigotes but not on heat generated "amastigotes". Similar work conducted on lysed cells revealed enzyme activity in soluble and particulate fraction of flagellated parasites.

Enzyme on intact cell plasma membrane preferred Histone V-S over protamine as in vitro substrate whereas enzyme from soluble or particulate fraction of lysed cells displayed the reverse in vitro preference. On the other hand enzymes from both intact and lysed cells were inhibited by Staurosporine and Formycin ATP.

Incubation of intact stationary phase parasites with [^{32}P] ATP led to phosphorylation of eight protein bands. Similar experiment conducted using lysed cells revealed twenty bands. Incubation of the cells with [^{32}P] with the assumption of generating intracellular [^{32}P] ATP also led to a pattern of phosphorylation similar to that of lysed cells. It follows from the above that Leishmania aethiopica promastigotes possess both extra and intracellular endogenous substrates of protein kinases.

Measurement of soluble kinase activity revealed increased activity as cells went from log to stationary phase where intact cell activity remained constant. Exposure to elevated temperature (37°) induced transformation of the promastigotes to afflagellated organism "amastigotes". This transformation resulted in a potent down regulation of both the soluble and the ecto kinase activities.

In the promastigotes, Staurosporine caused morphological alterations. On the other hand Formycin ATP had a profound inhibitory effect on thymidine incorporation and cellular proliferation. These effects of Formycin ATP do not seem to be wholly accounted by its protein kinase inhibitory activity.

A classical protein kinase C like activity could not be detected in the promastigotes. However, evidence for the probable presence of a phorbol ester insensitive, calcium and phospholipid independent protein kinase C is presented. All the same these observations do not rule out the presence of more than one subclass of protein kinases.

This work suggests that major protein kinase activities may be involved in the regulation of proliferation, development and adaptation to "hostile" environment of Leishmania aethiopica promastigotes. It also argues that the continued study of key events in the biology of this organism is crucial in the development of better therapeuticstrategies.

\PTER ONE

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INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Leishmaniasis refers to a group of related diseases of man and animals caused by ›tozoan parasites belonging to the family Trypanomatidae and genus *Leishmania.* The ease has been known since ancient times. The Old world cutaneous form of the disease s recognized as early as the second millennium B.C in Iraq. Peruvian pottery paintings m the first millennium A.D. show nasal lesions compatible with New World shmaniasis.

VIE

ıws

The parasites were first observed in macrophages by Cunningham in 1885. In 1900, ishman reported seeing these microorganisms in the autopsied spleen of a private who l died of "Dumdum" fever acquired in Dum Dum, India and described them as the cause :he disease. A similar observation was reported from Sudan by Donovan in 1903 (Peters, 38).

It is estimated that 12 million people in the world are affected by the disease with),000 new cases appearing annually and a further 350 million at risk (TDR/WHO 'ision of Control of Tropical Diseases, 1990). Because of the considerable mortality and rbidity it causes especially in tropical and sub tropical areas, it is now one of the six jor diseases that the World Health Organization (WHO) has included for study in it's cial program for research and training in tropical disease (WHO, 1984). Based on the rall clinical significance, among the protozoal diseases of humans, leishmaniasis is ırded second to malaria.

.1 Classification

Leishmania are classified on the basis of, pathological, serological, biochemical and graphical differences. Though the classification of the *Leishmania* parasite into different

1.1.2 Life cycle and Morphology

The parasite is a dimorphic unicellular protozoa. In culture systems and insects, it is present as an extracellular long slender spindle promastigote with anteriorly located single flagelum. It has a diameter of 1.5-3.5 µm and is 15-20 µm in length with a 15-28 µm flagella. The small non flagellated amastigotes are found in the mammalian host. They measure 2-3 µm (Sanyae and Sen Gupta, 1967; Shaw and Lainson, 1976; Veress *et al.*, 1980). While these parasites are intracellular, during heavy infection they are spotted free floating on splenic and bone marrow smears. Wright or Geimsa staining of the parasites show a large nucleus, a kinetoplast, basal body and a blue cytoplasm. Beneath the plasma membrane is found a pellicular lining. The flagellum pocket serves as a modified reservoir of nutrients into which the lysosomal products are released (Fig. 1). The flagellum in promastigotes is used for movement in the blood and culture medium and for attachment in the gut of invertebrate host. The nucleus is surrounded by a double layered nuclear membrane and division is by binary fission.

Fig. 1.1 Schematic Diagram of Promastigotes and Amastigotes of *Leishmania*.

Promastigote

Amastigote

The parasite is transmitted by members belonging to three genera of sand flies: *Phlebotomes*, *Lutzomyia* and *Psychodopygus*, but some times by inoculation, blood transfusion and congenital transmission (Low and Cook, 1926). The first description of sand flies as the vector of leishmaniasis has been attributed to Bueno, who stated in 1764 that "Uta" sand fly was the vector of what is still called "Uta", peruvian cutaneous leishmaniasis.

Amastigotes are ingested with the first blood meal of the female sand fly and are transformed immediately or after one binary division into promastigotes. If the sand fly is the appropriate host of a given species, infection is established. If not, the parasites are either destroyed or excreted with faeces. Various species have their own restricted development area in the alimentary tract. Later on, the parasites move further up spreading the infection to oesophagus and pharynx. This migration is to be accompanied by changes in forms of the *Leishmania* parasites from elongate promastigotes to round or oval paramastigotes. This may not apply to all *Leishmania* species. Finally infection of the buccal cavity (cibarium) occurs by simple overflow, partially obstructing the digestive tract which results in the regurgitation of and injection of parasite during feeding (Walters *et al.*, 1989). Generally development in the sand fly varies from 5-10 days.

On inoculation of mammals, the parasite is phagocitized. The process is facilitated by a receptor ligand interaction. A number of molecules that are involved in host-parasite interaction were observed on the surface of macrophages and promastigotes. On the parasite surface a 63-65 kd glycoprotein (gp 63) which is the major promastigote surface protease has been described in this relation in many species (Chang, 1979; Russel, 1986, 1987). Other molecules which may contribute to the receptor mediated attachment include fructose and mannose containing glycoconjugates (Platink, 1989), and a complex lipophosphoglycan (LPG) (Handman and Goding, 1985; Puens *et al.*, 1988). Likewise,

macrophage receptors involved in the interaction include complement receptors (CR1, CR3) (Russel, 1986, 1987; Russel and Wright, 1989), fibronectin receptor (Rizioi *et al.*, 1988), receptors that recognize sugars like mannose (Wilson and Pearson, 1988), receptors for advanced glycosylation products (Mosser *et al.*, 1987) and, CD18 family of integrins (Lymphocyte function antigen-1, Mac-1 and P150/P95) (Mosser *et al.*, 1992). This interaction between the two cells seem to be regulated by Ca^{2+} (Mistra *et al.*, 1990).

1.1.3 Leishmaniasis

Leishmaniasis may be subdivided into three types. These are, cutaneous, mucocutaneous and visceral leishmaniasis. The clinical spectrum varies from a self healing localized ulcer to a widely disseminated, progressive debilitating disease which can be fatal. With the exception of visceral leishmaniasis in India, the disease is a zoonosis. The geographical distribution of the disease is determined by a complex interaction of the host-parasite system. The disease has been reported in over 100 countries ranging from tropical to sub-tropical countries of Africa, Asia, Europe, Central and South America.

1.1.3.1 Cutaneous Leishmaniasis

Cutaneous Leishmaniasis is commonly divided as the old and new world cutaneous leishmaniasis. Old world cutaneous leishmaniasis is caused by three species. *L. major*, whose carrier hosts are primarily gerbils, is found in desert areas of Central Asia, North Africa and Middle East. *L. tropica* whose carrier hosts are humans and dogs and is mainly found in the Middle East, and Mediterranean area. Finally *L. aethiopica* is found in Kenya South West Africa and Ethiopia and the primary reservoirs are Rock and Tree hyrax and other small mammals (Ashford *et al.*, 1977). In Ethiopia the disease was described as early

as 1919 by Martoglio and 1948 by Congolesi (cited by Ashford and Smith 1985). It is widely distributed at altitudes of 1500-2000 meters (Ashford, 1973). The main vectors in this area are *P. longipes* and *P. pedifer*. Occasionally *L. donovani* infection may be present as a simple cutaneous leishmaniasis.

The incubation period of these infections can vary from two weeks to several months even years (Smith, 1955). The disease can be present as a simple localized cutaneous leishmaniasis with an ulcer at the inoculation site or a diffuse lepromatous lesions. The lesions remain confined to the skin except in some *L. tropica* cases where localized lymph nodes may be affected. The name Leishmania recidivan is given to a relapsing tuberculoid form of the disease caused by *L. tropica* which may involve the mucous membranes and run a chronic course extending from twenty to forty years. Spontaneous cure is achieved in *L. major* quickly (six months) while lesions of *L. tropica* infection tend to develop and heal slowly (one year or more). *L. aethiopica* lesion heals in six months to one years time but occasionally it may take longer.

New world cutaneous leishmaniasis is caused by *L. braziliensis* and *L. mexicana* subspecies. Occasionally, *L. chagasi* causes simple cutaneous lesions. The disease is wide spread over the whole of the Americas except Uruguay, Chile and Canada. Normally, it is found in small forest rodents and larger mammals as inapparent infection or a small cutaneous lesion. It is transmitted by sand flies belonging to the *Lutzomyia* and *Psychodopogus* genera. Direct transmission by contact has also been reported (Marsden *et al.*, 1985). The pathology of the disease caused by *L. mexicana* complexes resemble old world leishmaniasis particularly *L. major* and in some cases *L. aethiopica.* But *L. braziliensis* has a different pathology. This form usually affects mucous membranes, cartilages and bones resulting in marked destruction and severe disfigurement. This form

is called mucocutaneous leishmaniasis. Unlike the simple cutaneous forms no spontaneous healing has been observed in the mucocutaneous forms.

Diffuse cutaneous leishmaniasis (DCL) was first reported from Ethiopia by Price and Herbert in 1965. It is caused predominantly by *L. aethiopica* and the *L. mexicana* subspecies. It represents a disseminated lepromatous form with nodular lesions that never ulcerate and are filled with parasites. There is no visceralization but parasites have been recovered from bone marrow and peripheral blood and treatment is usually difficult. The lesions may persist up to 20 years.

1.1.3.2 Visceral Leishmaniasis

Visceral leishmaniasis is known commonly as Kala-Azar (meaning black fever in Hindi). Other names used frequently are, Dum-Dum fever, Assam fever and infantile splenomegaly. The disease syndrome ranges from an asymptomatic self healing infection to a progressive condition which may end fatally. In advanced cases it is manifested by fever, hepatosplenomegaly, anaemia, cachexia, malaise, and superinfection. In East Africa, visceral leishmaniasis is caused by *L donovani* and is found to be widely distributed in Ethiopia, Kenya, Somalia, Sudan, and Uganda. Occasional reports have come from Chad, Upper Volta, Zaire and Zambia. *Phlebotomes orientalis* is thought to be the most important vector in the area.

1.2 METABOLISM IN *LEISHMANIA*

1.2.1 Glucose

The parasites are capable of both glycolysis and gluconeogensis and the glycolytic enzymes are contained in glycosomes. The pentose phosphate pathway and tricarboxilic acid cycle are also present.

Experiments conducted under hypoxic atmosphere (95%N_2: 5% CO_2) in *L. braziliensis panamensis* promastigotes indicated that glucose degradation leads to production of glycerol, pyruvate, lactate and alanine (Darling *et al.*, 1987). Similar experiments on amastigotes of *L. mexicana pifanoi* showed accumulation of acetate and alanine (Rainey and MacKenzie, 1991). It follows from the above that both forms of leishmania are endowed with glycolysis and transamination.

Unlike most eukaryotic cells *Leishmania* cells are unable to use glucose in the absence of CO_2. Glycolysis is observed when cells are exposed to an atmosphere that is composed of 5% CO_2 (Darling *et al.*, 1989). However, the enzyme of leishmania glycolytic pathway whose activity is influenced by CO_2 is not well known.

Glucose metabolism is also influenced by oxygen concentration. When *L. major* promastigotes were incubated in the presence of 5% CO_2, the utilization of glucose and relative amounts of products changed as a function of pO_2. As pO_2 was decreased from 95% to 6% in a CO_2:O_2: N_2 atmosphere, glucose utilization increased along with the production of glycerol and lactate. The level of other glycolytic intermediates also showed a PO_2 dependent fluctuation (Keegan and Blum, 1990, 1991).

Leishmania can convert Phosphoenolpyruvate to oxaloacetate by Phosphoenolpyruvate carboxykinase (Mottram and Coombs, 1985). Isotope labelling studies by Chatterjee and Datta (1973) and Darling *et al.* (1989) had showed that the resulting oxaloacetate can be converted to succinate by reverse Kreb's cycle thereby regenerating NAD^+ from NADH.

1.2.2 Lipids

Chloroform-Methanol (2:1) extract of promastigotes accounts for 2-15% of the dry

weight of a number of species of *Leishmania*. Phospholipids constitute about 70% of the promastigotes membrane lipid while the rest is accounted for by neutral lipids. The total lipid content and fatty acid composition of promastigotes is influenced by temperature. Cell culture in lipid free medium have demonstrated that the organism is capable of synthesising its lipid requirements (Wassef *et al.*, 1985).

In contrast to glucose and amino acids promastigotes do not oxidize fatty acids freely. On the other hand, amastigotes do have a well developed ability to oxidize fatty acids by β-oxidation (Hart and Coombs, 1982). A similar observation has been made for exogenous triglycerides in *L. mexicana* and to a lesser extent in heat shocked *L. b. panamenesis* (Blum, 1987; Darling and Blum, 1987).

1.2.3 Proteins and amino acids

The total protein content of *Leishmania* is higher in promastigotes than in amastigotes. For instance in *L. mexicana*, the quantity per cell is 5.3 pg for promastigotes and 1.3 pg for amastigotes (Chang and Bray, 1985).

A number of amino acids are extracted from the culture medium and utilized by the parasites (Hart and Coomb, 1982). For instance L-proline intake by mid-log phase *L. donovani* promastigotes is carrier mediated and the energy is provided by a proton gradient (Zilberstein and Dwyer, 1985). After deamination of amino acids the resulting α-Keto acid is channelled in to the Krebs cycle (Glew *et al.*, 1988). The rate of catabolism is higher in promastigotes than that of amastigotes.

An interesting feature regarding amino acid utilization in *Leishmania* is the use of alanine as an osmolite. The parasites contain a very large intra cellular pool of alanine. Like some eukaryotic cells (Chamberlain and Strange, 1989) this pool seems to be linked

with response to osmotic stress. In *L. major*, exposure to hypo osmolar environment resulted in release of alanine and other NPS, (ninhydrin-positive substances) while in hyperosmotic conditions in the presence of glucose, the intracellular pool increases to about 1-8 folds (Darling and Blum, 1990; Darling *et al.*, 1990). The regulation of alanine metabolism and its permeability appears to play key a role in determining the ability of *L. major* promastigotes to cope with osmotic stress in the insect host.

1.2.4 Nucleic acids

The DNA is organized in the nucleus, kinetoplast and virus like particles in the cytoplasm. The average amount per cell is 91 X 10^6 and 242 X 10^6 base pairs for the promastigotes of *L. donovani* and *L. braziliensis*, respectively (Chang *et al*, 1985). Molecular karyotypes of promastigotes and amastigotes are indistinguishable. Furthermore transformation between developmental stages is not accompanied by detectable chromosomal rearrangement. There is also lack of detectable chromosomal condensation during cell cycle (Lighthall and Gianni, 1992).

Leishmania are incapable of a *de novo* synthesis of purines and therefore require exogenous purine. Studies conducted on *L. mexicana mexicana* demonstrated that purine salvage enzymes are compartmentalized. Species and stage specific differences in the salvage pathway are exhibited (Hassan *et al.*, 1985; Hart and Coombs, 1982). For instance amastigote posses 50 fold more adenosine kinase than promastigotes (Datta, 1987).

Leishmania posses the enzymes for *de novo* synthesis of pyrimidines. Unlike most eukaryotes the first three enzymes of the *de novo* pyrimidine pathway are not organized into a multi-enzyme complex. Further more it has a unique thymidylate synthetase which catalyses reductive methylation of deoxyuridine monophosphate to produce deoxythymidine

monophosphate.

1.3 SURFACE ENZYMES

1.3.1 Phosphatases

L. donovani contains at least three membrane bound acid phosphatases (Glew *et al.*, 1982; Kreutzer *et al.*, 1980). Likewise *L. mexicana amazonensis* amastigotes contain large amount of acid phosphatase activity some of which is contributed by the plasma membrane of the flagellar pocket. While both forms of *Leishmania* possess surface phosphatase, staining studies revealed more on promastigotes (Antoine *et al.*, 1987).

Recently a number of functions have been postulated for these enzymes. Dephosphorylation of target cell molecules may interfere with the microbicidal response of the host cell. This has been partly substantiated by the finding that pre-incubation of human neutrophils with a pure preparation of leishmanial acid phosphatases (ACP-P_1) resulted in a marked reduction of superoxide anion production by neutrophils stimulated with the bacterial chemotactic peptide FMLP (Remaley *et al.*, 1984, 1985). Whether this is an important feature of the parasites defence mechanism is open to question. In fact the expression of the enzyme is low in metacyclic and amastigotes contrary to might be expected if it is to provide significant protection. So far no particularly good substrate for these enzymes on the macrophage surface has been identified (Glew *et al.*, 1988).

Leishmanial phosphatase block calcium mobilization from intracellular stores in a dose dependent manner. This effect is similar to its effect on the respiratory burst (Remaley *et al.*, 1984, 1985). It follows from the above that phosphatase may block production of reduced oxygen metabolites in activated neutrophils. This information is intriguing in the light of the findings that phosphatases hydrolyse inositol phsopholipids including IP_3 (Das

et al., 1986) and that the respiratory burst in macrophages is mediated by the IP_3 derived diacylglycerol/protein kinase C pathway which involves Ca^{2+} release.

Large amount of phosphatase activity from *L. donovani* is accumulated in the extracellular growth medium as a result of secretion by actively growing cells. This is a relatively acidic protein able to hydrolyse sugar phosphates, ATP, 5'-AMP and 3'AMP. Lovelace and Gottlieb (1986) examined 40 isolates of *Leishmania* species representing all of the major infectious species to man for secretion of an extracellular acid phosphatase. All but *L. major* and *L. tantolae* secrete acid phosphatase. In some cases the amount seems to be strain dependent. The extracellular acid phosphatase produced by various strains vary in terms of their electrophoretic mobility and sensitivity to L-tartrate inhibition.

1.3.2 Neuclotidases

L. donovani possess both 3'and 5' nucleotide monophosphatases (Dwyer *et al.*, 1984). These enzymes seems to play a nutritional role since *Leishmania* cannot synthesise purine nucleotide *de novo*. They have pH optimum of 8-9 and 6.5 - 7 respectively (Glew *et al.*, 1988).

1.3.3 Proteinases

1.3.3.1 Promastigote surface proteinase (PSP)

Promastigot surface proteinase is a membrane bound enzyme. It is a dimer with Zn^{++} at its active site. This enzyme is detected in all species of *Leishmania* promastigotes that have been examined so far (Bouvier *et al.*, 1987). It is said to be expressed in reduced quantities by amastigotes of *L. amazonensis*, *L. mexicana*, and *L. major* (Etges and Bouvier 1991). However failure of monoclonal antibodies directed against promastigote PSP to

recognise the amastigote counterpart may indicate differential processing of amastigotes PSP (Fong and Chang, 1982). The enzyme is conserved in non infective laboratory strains suggesting an important metabolic role.

Leishmania require an exogenous source of preformed haem for growth (Chang and Chang, 1985). In this context proteolytic degradation of haemoglobin which is readily available in the midgut of the haematophagous vector would provide an abundant nutrient to promastigotes (Grim *et al.*, 1982).

1.3.3.2 Cystine proteinases

Some *Leishmania* species particularly *L. mexicana* exhibit a high cystine proteinase activity. In amastigotes it accounts for over 90% of the total proteinase activity and is located in lysosome like organelles that has acidic pH (Pupkin *et al.*, 1986). Gelatine SDS-PAGE electrophoresis revealed four distinct bands of cystine proteinase activities from stationary-phase population of *L. mexicana mexicana* (Robertson and Coombs, 1992) that are distinct from all amastigote enzymes.

Multiplicative promastigotes show relative deficiency when compared to stationary phase ones (Lockwood *et al.*, 1987). The differential expression suggests that it is part of a necessary adaptation to a "hostile" environment. In support of this is the fact that inhibition of the proteinase activity does have anti-leishmania activity (Pupkin and Coombs, 1984). At the present time no particular role has been identified for these enzymes although a number of hypotheses have been proposed (Coombs *et al.*, 1991).

1.4 PROTEIN KINASES

Protein phosphorylation constitute a significant part of cellular regulatory system.

Protein kinases which belong to this system transfer mostly the γ phosphate group of ATP to protein there by either activating or deactivating an enzyme or a hormone.

The first clue of a regulatory protein phosphorylation came from the work of the Coris and Green in 1943 and 1945 (cited in Krebs, 1985). They postulated that glycogenolysis is regulated by phosphorylation and dephosphorylation of an enzyme called glycogen phosphorylase. The first kinase was identified by Krebs and co-workers in 1959 (Krebs *et al.*, 1959). The continued study of glycogen metabolism resulted in the discovery of the cAMP-dependent protein kinase (Walsh, 1968), providing an explanation for the control of intracellular protein function through covalent modification induced by extracellular stimuli. In the 1960s a number workers showed that processes other than glycogenolysis may be regulated by phosphorylation/dephosphorylation. For instance the regulation of pyruvate dehydrogenase by phosphorylation/ dephosphorylation events was discovered (cited in Krebs, 1986).

Protein kinases in general show a marked homology among themselves. Based on sequence similarities it appears that most if not all eukaryotic protein serine/threonine and protein tyrosine kinases arose from a single archetypal gene (Krebs, 1985; Hunter, 1987).

The enzyme kinase usually catalyses the phosphorylation of a number of proteins. Likewise a single substance can serve as substrate for more than one kinase. But there are also examples where only a single substrate have been identified. eg β-adrenergic receptor kinase and rhodopsin kinase.

Phosphorylation and dephosphorylation are coupled with conformational change. This in turn has influence on activity of the covalently modified protein. One evidence of the phenomenon is X-ray crystallographic data of glycogen phosphorylase. It was seen that the conversion from B form to A form is induced by addition of phosphate to ser-14. This is followed by formation of 3 new salt bridges one of which is between phosphate group

and Arg-69 of the same subunit while the other two are inter-subunit bridges. Flitterich and Madsen have calculated the binding energy of these salt bridges that is translated into a highly significant decrease in the allosteric constant for phosphorylase A as compared to phosphorylase B (Sprang *et al.*, 1988). The three dimensional structural analysis has also shown that phosphorylation can affect protein activity by electrostatic repulsive effects (Hurly *et al.*, 1990).

In general protein kinases occur in inactive state. The enzymes rarely show more than 20 fold difference in activity between activated and basal states. But a marked amplification can occur by a cascade like arrangements resulting in amplification at each step.

In a phosphorylation-dephosphorylation system regulation can be exerted both at the kinase and the phosphatase level. This is emphasised by the finding of at least two phosphatase inhibitors which are active only when phosphorylated (Cohen, 1985). The presence of regulating protein phosphatases will add to the flexibility and sensitivity of the regulatory system.

Protein kinases show cell or tissue specificity. For example, many cells of the haematopoietic linage seem to express their own *src* gene family members. Further more protein phosphorylation-dephosphorylation is much more abundant in eukaryotic cells than in prokaryotic cells, suggesting that the process evolved to meet the needs of more complex organisms. Edwin Krebs has postulated that the protein phosphorylation dephosphorylation may be largely concerned with the handling of external signals.

4.1 Classification

Protein kinases can be classified based on two ways; on phoshorylated amino acid and activating ligand.

1.4.1.1 Classification based on amino acid specificity

Though about nine amino acids are known to serve as substrate for protein kinases, based on their amino acid preference protein kinases are grouped in to two.

1. Serine/threonine kinases EC 2.7.10. eg.Protein kinase C, cAMP dependent kinases.
2. Tyrosine kinases EC.2.7.11. eg. Insulin receptor, EGF receptor.

Recently new evidence is accumulating that indicate the presence of dual specificity kinases. So far at least 11 such enzymes have been identified eg ERK1 and ERK2. (Lindberg *et al.*, 1992). But the elucidation of structural motifs that allows the prediction of the amino acid specificity has not yet been made.

1.4.1.2 Classification based on activating ligand

1. Cyclic AMP dependent protein kinase (A-kinase)
2. Cyclic GMP dependent protein kinase (cGK)
3. Protein kinase C (PKC
4. Ca^{2+}/Calmodulin dependent protein kinase (Ca-kinase)
5. Protein Tyrosine kinase (PTK)

A protein kinase taxonomy consisting of a series of families can also be generated based on the different degrees of sequence relationships among protein kinase genes eg. *src* gene family, *abl* gene family EGF receptors family, insulin receptor family etc.

1.4.2 Characteristics of major protein kinase groups

1.4.2.1 Cyclic AMP dependent kinases (A-Kinases)

In eukaryotic cells the cAMP-dependent kinases are the only receptors for cAMP. These enzymes have a phylogenetic distribution that extends from mammals to yeast. But

so far there is no compelling evidence for the existence of a cAMP-dependent protein kinase in prokaryotes.

Cyclic AMP dependent kinases are composed of catalytic and regulatory subunits forming a tetramer,R_2C_2. The binding of cAMP to the regulatory unit results in the dissociation and the release of the catalytic subunit from the inhibitory action of the regulatory subunit

(Edelman *et al.*, 1987). Regulatory subunit isolated from the bovine enzyme contains four domains, two cAMP binding, a dimerization and a C interaction (inhibitory) domains. There are two types of regulatory subunits designated R_I and R_{II}. Each in turn has an α and β isoforms (Hubbard and Cohen, 1993). Each subunit of A-Kinase is known to be phosphorylated at multiple sites. The significance of this phosphorylation is not well understood except in the case of Ser 95 phosphorylation site in RII which reduces the rate of reassociation with the C subunit. RI phosphorylation at ser-99 by cGMP-dependent kinase *in vitro* results in loss of inhibitory activity toward C and loss of one cAMP binding site (Edelman *et al.*, 1987).

The cellular level of cAMP is regulated by ligands such as glucagon, epinephrine and prostaglandin E_2 which bind to their respective receptors. The interaction of these receptors with Gs proteins leads to the activation of a membrane bound adenylate cyclase increasing the formation of cAMP. The signal is terminated when the GTP attached to the α subunit of the Gs protein is hydrolysed. On the other hand receptors for other hormones like somatostatin by interacting with a different sets of subunits results in the inhibition of adenylate cyclase. The concentration of cAMP may also be regulated by the action of nucleotide phosphodiesterase (Edelman *et al.*, 1987).

There are two well known types of mammalian A-Kinases; type I and type II that are distinguished on the basis of DEAE-chromatography, cAMP analog specificity, ability

to undergo autophosphorylation and tissue specificity. Most of the structural and functional differences are supplied by the two RI, RII sub units.

1.4.2.2 Cyclic GMP dependent protein kinases (CGK)

Cyclic GMP dependent protein was first reported in 1970 by Kuo. Its physiological function is not well understood. It is widely distributed in muscle, lung, heart, cerebellum and other tissues. The enzyme appears to be primarily cytosolic but a distinct membrane specific CGMP-dependent kinase (CGK) had been described (Edelman *et al.*, 1987).

The enzyme consists of two subunits which are identical and arranged in antiparallel manner with the regulatory domain of one subunit inhibiting the catalytic domain of the other. There are two intrasubunit cGMP binding sites. The dimerization domain is not homologous to the corresponding part in cAMP dependent kinases.

Present evidence indicate that cGMP has a narrower specificity than A-kinases. It is regulated by nanomolar levels of cGMP; but cAMP and cIMP in micromolar concentrations also activate the enzymes. Unlike A-kinases, the binding of cGMP does not cause dissociation of subunits. The enzyme is phosphorylated at multiple sites. But this has little or no effect on cGMP interaction with the enzyme. If autophosphorylation takes place *in vivo*, it may allow the enzyme to be activated by cAMP as well as cGMP.

Recently a distinct non nucleotide dependent protein kinase which contains both the catalytic and the regulatory domains in the same chain and with similar affinity to both cAMP an cGMP has been purified from a developing insect (Edelman *et al.*, 1987).

1.4.2.3 Protein kinase C (PKC)

Agonist induced generation of diacylglycerol from phosphatidylinositol-bis-phosphate (PIP2) has been widely described. The receptor mediated turnover of PIP2 is

coupled through a G-protein-dependent activation of phospholipase C in a mechanism analogous to that established for trans-membrane signalling linked to cyclic AMP formation (Berridge and Irvine, 1984).

Protein kinase C is diacylglycerol (DAG) activated, phospholipid and Ca^{2+} dependent serine/threonine specific enzyme. DAG increases the affinity of protein kinase C for Ca^{2+} and for phophatidylserine thereby activating the enzyme at intracellular Ca^{2+} levels. It interacts with protein kinase C at the same site as phorbol esters (Bell, 1986). The ability of phorbol esters to bind and activate protein kinase C is shared by a number of other tumour promoters including mezerin, aplysiatoxin, debromoaplysiatoxin and teleocdidin. Though this activation requires the presence of acidic phospholipids, it does not require the membrane. Studies in cultured cells and under defined conditions *in vitro* suggest that translocation of protein kinase C from the cytosol to the membrane occurs on activation and that translocation may be an important event in signal transduction (Bell, 1986).

At least ten subspecies of PKC have been identified. These subspecies have a common structure closely relate to, but clearly distinct from each other. The carboxy-terminal half of each enzyme is the catalytic domain and this domain of the bovine PKC shares substantial homology with other serine, threonine, and tyrosine protein kinases. In addition they have a conserved sequence which is similar to the consensus sequence of a cysteine-zinc -DNA-binding proteins that are related to transcriptional regulation, but there is no evidence that PKC binds to DNA (Parker *et al.*, 1986).

Northern blot analysis with specific oligonucleotide probes and *in situ* messenger RNA hybridization has suggested that some PKC subspecies are expressed specifically in certain tissues. For instance in the rat, the γ subspecies appears to be expressed solely in the brain and spinal cord, and is particularly concentrated in the hippocampus, cerebral

cortex and amygdaloid complex. Many tissues including liver, kidney, spleen, lung, heart and testis contain the β subspecies (particularly β_{II}) in variable ratios but the α -subspecies of PKC is the most widely distributed. Protein kinase C-L, is abundant in lung tissue, (Bacher *et al.*, 1991). Most cell types contain more than one subspecies (Nishzuka, 1988).

There are numerous evidence that indicate that the activity of the different isozymes differ with regard to their kinetic behaviour and activating substances hence have distinct roles. β_I and β_{II} for instance show substantial activity in the absence of Ca^{2+} while ζ is not activated by DAG/Phosphatidylserine. Otte and Moon (1992) have shown that the isoenzymes α localized in the dorsal ectoderm of Xenopus and β which is uniformly distributed have different effects on neural induction and competence.

Protein kinse C is involved in many cellular activities. The enzyme is involved in regulation of cell proliferation, differentiation and gene expression. For instance it is implicated in induction of the IL-2 receptor. It is also involved in transmitter release at the nerve terminal (Shapira *et al.*, 1987). The macrophage oxidative burst response is also dependent on PKC activation. The enzyme is also implicated in the activation of some proto-oncogene (Krebs, 1979). A number of reports implicate a role of PKC in oncogenesis (Castagna, 1987). Elevated levels of DAG have been observed in cells expressing *ras* and *sis* oncogenes. Evidently protein kinase C does not show homology to oncogenes that have been described to date. PKC also provides negative feed back control over various steps of the cell-signalling processes, operating both short-term and long-term responses of the cell.

The enzyme has been identified in lower organisms, eg. the nematode *C. elegans* (Sassa and Miwa, 1992), *S. mansoni* (Weist *et al.*, 1992), *E. histoltica* (De Meester *et al.*, 1990) trypanosomes (Keith *et al.*, 1990) and the yeast (Ogita *et al.*, 1990). Interestingly in

the yeast this enzyme did not respond significantly to phorbol esters and showed substrate specificity distinctly different from the mammalian one. Unlike the rat PKC this one phosphorylated threonyl residues rather than serine.

1.4.2.4 Calcium (Calmodulin)-dependent protein kinases (Ca-kinase)

The ability of cells to sequester calcium in special organelles like sarcoplasm, nucleus, mitochondria and the endoplasmic reticulum and release in response to various stimuli makes it a good signalling molecule. Cells maintain a steep concentration gradient of calcium between the extracellular environment and the cytosol by the active extrusion of Ca^{2+} through the plasma membrane and accumulation in the intracellular organelles. Many of the calcium mediated activities seem to be dependent on the binding of the divalent cation to calmodulin, a 17 kd protein originally described as an activator of cyclic nucleotide phophodiesterase. The Ca^{2+}/calmodulin complex regulates a number of enzymes including several kinases (Means, 1980; Edelman *et al.*, 1987).

Among the well characterized members is the myosin light chain kinase (MLCK). The enzyme has been isolated from cardiac and smooth muscles as well as other non muscle tissues with varying sub-cellular distribution (Edelman *et al.*, 1987). Most MLCK require the one to one binding of calmodulin on which all the four calcium binding sites are occupied for activity. This binding results in a conformational change which can be detected by changes in its fluorescence characteristics. The effect of the phosphorylation of the myosin light chain (MLC) differs in different tissues. In the smooth muscle it is to initiate contraction while in the striated muscle it serves to modulate the degree of tension produced during contraction. In non-muscle tissue, though not well understood, it may be related to the regulation of motile process such as capping phagocytosis, movement, secretion etc. MLCK isolated from different sources show difference with regard to their

molecular weight, structure and arrangement of domains. Some MLCK have been shown to undergo autophosphorylation but the significance is not well understood (Edelman *et al.*, 1987).

Other well described Ca^{2+}/calmodulin dependent kinase are the muscle and liver phosphorylase kinases. Phosphorylase kinase has four different subunits, $\alpha4,\beta4,\gamma4$ and $\delta4$. The γ subunit is the catalytic sub unit and the MW is the same as catalytic subunits of A-kinases. The α and β sub units are involved in activation of the enzyme by phosphorylation/ dephosphorylation. The δ sub unit is calmodulin and the enzyme can be activated by the binding of Ca^{2+} to endogenous calmodulin or by the binding of calmodulin/ Ca^{2+} complex. During electrical stimulation of muscle, Ca^{2+} is released from the sarcoplasmic reticulum, initiates muscle contraction and also activates phosphorylase kinase which phosphorylates phosphorylase b and thereby activates glycogenolysis (Cohen, 1982). No dissociation of subunits occurs when the enzyme is activated.

1.4.2.5 Protein tyrosine kinases (PTK)

Phosphotyrosine residues were not identified until 1979. They were detected in immuno-precipitates of viral transforming protein hydrolysate incubated with [^{32}P] ATP. The first protein tyrosine kinases (PTK) activity to be detected were associated with viral transforming proteins, particularly those of acutely oncogenic retroviruses (Erikson *et al.*, 1979). These showed homology in catalytic domain with the serine-specific cyclic AMP-dependent protein kinase. Since then many PTKs have been detected including those in insects and simpler eukaryotic species (Simon *et al.*, 1983). Protein phosphatases specific for phosphotyrosines have also been reported fulfilling a critical requirement for a regulatory system based on tyrosine phosphorylation.

Hunter and Cooper (1985) recognized two broad groups with significant overlaps.

These are;

1. The retroviral protein tyrosine kinases and their cellular homologes of which pp60$^{v\text{-}src}$ is well characterized member.
2. The growth factor receptor-associated protein-tyrosine kinases which includes the epidermal growth factor receptor (EGF) (Cohen *et al.*, 1982) and are usually transmembrane.

Except for the insulin and Insulin like growth factor-1 (IGR-I) receptors the PTKs all have a single polypeptide chain which is active in the monomeric state. PTKs can be integral part of the plasma membrane as in the case of growth factor receptors and P120$^{gag\text{-}abl}$ or be peripherally associated with cytoplasmic membranes eg PP60 $^{v\text{-}src}$, P140$^{gag\text{-}fps}$ and p56^{lstra}. Still others appear to be soluble like p75 and the c-fps protein (Hunter and Cooper, 1985).

Unlike the other kinases many PTKs show an apparent preference for Mn^{2+} over Mg^{2+} for phosphorylation of both protein and peptide substrates. Though ATP is the preferred substrate GTP can also be used in some instances.

The knowledge regarding the tyrosine kinase has expanded tremendously by the study of oncogens many of which are themselves tyrosine kinases (Cantley *et al.*, 1991). Several hormone and growth factor receptors including those mentioned above are kinases with specificity for tyrosine in their substrate proteins. All are trans-membrane proteins with the tyrosine kinases domain situated in the cytoplasmic portion of the protein. The binding of the appropriate ligand to the extracellular domain results in increased catalytic activity of the tyrosine kinases both towards the receptor itself and other substrates (Yarden, 1988).

The viral PTKs autophosphorylate at a homologous tyrosine in the heart of the catalytic domain. In EGF receptor the majority of autophosphorylation site lie outside the

catalytic region. The activity of many PTKs including the $pp60^{v\text{-}src}$ is increased by the phosphorylation of tyrosine residues outside the catalytic region. All of the PTKs are also modified by at least one serine kinase (Hunter and Cooper, 1985).

Information on endogenous substrates is limited. A major substrate for the EGF-receptor tyrosine kinase appears to be lipocortin, a cytoskeletal component that is also a potent inhibitor of phospholipase A_2 *in vitro* (Pepinsky, 1986). Activation of the insulin receptor tyrosine kinase *in vivo* results in the phosphorylation of a 180 kd protein (White 1985) as well as calmodulin. However the importance of this phosphorylation in mediating the action of the growth factors has not been defined.

One group of protein substrates are related by the presence of sequence motifs termed *src* homology or SH^2 domains (Koch, 1990). Two of these are enzymes that modulate the metabolism of phosphoinositides: phospholipase C-γ, and phosphatidylinositol 3-kinase. A third is rasGAP, a molecule that modulates the GTPase activity of *ras*. Others substrates recently identified include phophotyrosine phosphatase, cytoskeletal protein and analogous to *c-crk*, lipocortin/calpatin family of proteins etc.

Other kinases that could not fall in groups but for some of which affecting ligands have been identified include, Casein kinases, Heam regulated kinase, Double stranded RNA--dependent kinases, etc. Apart from these commonly identified ones their are also bifunctional protein kinase/phosphatases (Edleman *et al.*, 1987).

1.5 PROTEIN KINASES IN PROTOZOAN

Protein kinase activity had been demonstrated in a number of protozoan parasites. Das *et al.* (1986) demonstrated the presence of a cyclic-nucleotide-independent protein kinase in *Leishmania donovani*. The incubation of the parasite with ^{32}P for two hours

resulted in the labelling of at least 21 proteins that are in a dynamic state. The enzyme purified 118-fold was not activated by cAMP or cGMP and had an apparent MW of 75 kd. This enzyme showed a six fold increase in activity in promastigotes at the stationary phase as compared to the early exponential phase of growth. A protein kinase activity has also been partially purified from *L. donovani* and *L. mexicana* (Berman, 1988).

A soluble 85 kd protein kinase has been isolated from the promastigote form of *L. donovani.* This enzyme preferentially utilized protamine as exogenous phosphate acceptor. A number of common stimulators such as cAMP, cGMP, Ca^{2+}/calmodulin, Ca^{2+}/phospholipid did not stimulate enzyme activity and it used mixed histones less efficiently (Banerjee and Sarkar, 1990).

Lester *et al.* (1990) in their study of extracellular phosphorylation on *L. major*, have found eleven phosphoproteins of which ten are membrane-associated ranging in molecular weight from 48-140 kd. This phosphorylation pattern was not affected by heparin and spermine. Of the total enzyme, 70-80% of the activity was membrane associated. Serine-threonine kinase substrates like histone I, histone F2b, and kemptide, casein or phosvitin were phosphphorylated and protamine was found to be a better substrate for both cytosolic and membrane fractions in comparison with mixed histones. Regarding phosphorylation by intact parasites (ectokinase activity) histone phosphorylation was 2.5 times greater than endogenous phosphorylation and protamine phosphorylation was significantly less than endogenous phosphorylation (35%). Three specific phosphoproteins were released in to the medium. Analysis of the 40 and 55 kd proteins that are released revealed phospho-O-serine indicating that serine residue is the site of phosphorylation.

A histone-tubulin protein kinase of *L. donovani* has been shown to be inhibited by heparin. It is not known if this has a physiological implication (Mukhopadhyay *et al.*,

1989). Berman has studied the effects of antileishmanial purine nucleoside analogues on protein kinase of *L. mexicana amazonensis* promastigotes and amastigotes and found them to be inhibitory (Berman, 1988).

Recently the ability of the purified leishmanial protein kinase-1 isolated from *L. major* to phosphorylate the α-chain of complement factors C3 and C5 was demonstrated. It was found that the P-C3 was more resistant to cleavage by trypsin than the non-phosphorylated one. The physiological effects of such phosphorylation has not been studied but it can be speculated that it may interfere with the action of C3 convertase, and the reactivity of C5 with the C5 convertase (Hermoso, 1991).

These leishmanial kinases which phosphorylate only the broad spectrum exogenous substrates like protamine and histone does not fit into any of the major classes of protein serine-threonine kinases.

Protein kinase C like activities in bloodstream and procyclic forms of *Trypanosoma brucei* have been partially purified and characterized (Keith *et al.*, 1990). One of these enzyme was found to contain an epitope recognizable by anti-body raised against mammalian PKC. *Trypanosome cruzi* has PKC that can be activated by phorbol myristate acetate and diacylglycerol and is inhibitable by inhibitors of mammalian PKC like staurosporine and polymyxin B. Optimal concentrations for the activators and inhibitors are almost identical to those reported for the mammalian protein kinase C (Gomez *et al.*, 1989). Tumor promoters of the phorbol ester family which are well known activators of PKC induce morphological change in these organisms.

T.brucei contains a number of phosphotyrosines that seem to be differentially regulated during the life cycle suggesting that tyrosine kinases may play an important role in trypanosome biology (Parsons *et al.*, 1990). In addition cAMP dependent protein kinase

has been described in *T.cruzi* and *T.gambiense* (Walter, 1976; cited in Das *et al.*, 1986) and some of the *in vitro* properties determined.

A calcium/phospholipid dependent protein kinase C has been identified in *Entamoeba histolytica* by (De Meester *et al.*, 1990) whose cytolytic activity is activated by phorbolesters indicating the role of the PKC. They also found a 68 kd substrate in the organism which may be analogous to the 68 kd substrate found in macrophages. Protein kinase has also been detected in *Plasmodium berghei* (Wiser, 1976).

4.6 HOST PARASITE INTERACTION

4.6.1 Adaptation of the parasite

Mononuclear phagocytes function both as host cells required for survival of the infecting agent and as the effector arm of immune response. The survival of the parasite depends on its capacity to transform and stay intracellular. This transformation to amastigote stage is accompanied by profound morphologic and metabolic changes. Among these, respiration rate and glucose metabolism are markedly decreased as compared to promastigotes (Hart and Coombs, 1982) and non esterified fatty acids become a predominant source of energy. There occurs also increased resistance to lysis by human serum (Bandyopadhyay *et al.*, 1991) and change in enzyme profile. Development of pormastigotes *in vitro* is associated with changes in membrane carbohydrates.

Incubation at acidic pH (4.5) has resulted in change in morphology and the expression of amastigote specific proteins in *Leishmania major* and *Leishmania mexicana* promastigotes (Zilberstein *et al.*, 1990). In addition, heat shocking produces heat shock proteins preceding the morphological, metabolic and biological changes characteristic of the amastigote stage in a number of species (Lawrence and Roberto-Gerro, 1985; Smejkal

et al., 1988; Van der Ploeg *et al.*, 1985). These and other findings seem to indicate that pH may be important to trigger the parasites transformation into amastigotes in the old world leishmaniasis while in the new world species temperature may play the predominant role as suggested by Zilberstein (1990).

The nutritional requirements of the parasite seem to be fulfilled by facilitated uptake. Incorporation of glucose and amino acids is a carrier mediated process in several leishmania species (Bonay and Cohen, 1983; Scaefer and Mukada, 1976). In this context a leishmania glucose transporter molecule has been characterized (Zilberstein *et al.*, 1986). Energy for transportation is supplied by a proton gradient established by a proton-translocating ATPase (Zilberstein and Dwyer, 1988). This pump also contributes to lysosomal acidification and the maintenance of internal pH. Localization studies revealed that leishmanial H^+/ATPase is distributed on the plasma membrane, flagellar surface, golgi apparatus as well as membrane of intracellular organelles (Liveanu *et al.*, 1991). For this mechanism to be effective in wide range of pH, the membrane potential must compensate for the apparent decrease in pH gradient that may occur in the promastigotes (Zilberstein, 1991).

1.6.2 Host effector mechanisms

Apart from the classical O_2 dependent cytocidal mechanisms, a newly discovered non oxidative pathway which utilizes arginine derived nitrogen derivative (nitric oxides) appear to have a major role in the intracellular killing of *Leishmania*. In mice IFN γ and TNF α mediated induction of nitric oxide synthase catalysing the synthesis of nitric oxide has been shown. These substances mediate their actions through a protein kinase C dependent pathway. The pathway is important for BCG and INF gamma induced anti leishmanial activity in animal models (Green *et al.*, 1990; Liew *et al.*, 1990; Green *et al.*,

1991). But the extension of this to human cells has met with some difficulty (Locksley and Louis, 1992).

A mechanism not dependent on soluble mediators requiring intimate contact between lymphocyte and macrophages had been described (Wyler *et al.*, 1987). In addition, macrophage precursors devoid of phagocytic activity have been shown to kill *Leishmania* promastigotes and amastigotes extracellularly by a mechanism that remains to be explained (Baccarini *et al.*, 1988).

T-helper1 (Th1) and T-helper2 (Th2) cell reconstitution experiments in severe combined immunodeficient mice (SCID) has shown that these cells alone are capable of mediating the spectrum of diseases without the participation of B-cells or additional T-cells (Holaday *et al.*, 1991). Experimental infection in mice with *L. major* correlate the expansion of Th-1 cells with cure and those of Th-2 cells with progressive disease.

Certain lymphokines appear to be important for immune recovery. Included are IFN-γ produced by T-cells $CD4^+$ and GM/CSF (granulocyte monocyte colony stimulation factor) (Weiser *et al.*, 1987), macrophage inhibitory factor and a membrane associated TNFα on activated $CD4^+$ lymphocyte (Sypek and Wyler, 1991). Others like IL-3,IL-4 and IL-10 counter act IFN-γ (Liew, 1989) and are disease promoters and the production of IL-4 and IL-10 by $CD4^+$ cells is associated with progressive disease (Heinzel *et al.*, 1991). Exceptions to this pattern are present (Stenger *et al.*, 1991; Kay *et al.*, 1991; Titus *et al.*, 1991; Locksley and Louis, 1992).

Leishmania live within phagolysosomal compartments that have been demonstrated to contain class II MHC antigens. The sensitization of $CD4^+$ cells, which recognize antigenic peptides in association with class II MHC molecules on the surface of antigen-presenting cells (APCs), during infection is consistent with this intracellular localization

of the parasite. However, recently there has been a number of evidences that indicate that the $CD8^+$ has a role in the immunity to the parasite particularly in the production of INfγ and TNF (Hill, 1991; Smith *et al.*, 1991).

1.6.3 Evading strategies of the parasite

Leishmania parasite has evolved various mechanisms of inactivating or evading the hosts immune system. Amastigotes contain large amount of scavenging enzymes like superoxide dismutase and catalase whose targets are the reactive oxygen species (ROS) and lead to a weaker respiratory burst than for promastigote (Channon and Blackwell, 1985). Furthermore *Leishmania* has 'trypanothione,'a reducing agent which may detoxify oxygen metabolites especially H_2O_2. It is covalently linked with glutathione and act as a co-factor for a parasite specific trypanothion peroxidase. Lipophosphoglycans (LPG) of *L. donovani* cell surface are also very effective scavengers of both hydroxyl and superoxide radicals (Chan *et al.*, 1989; Eze, 1991).

Lipophosphoglycan by favouring parasite-macrophage interaction using CR3 increases the chance of intracellular survival because this receptor causes a relatively small degree of respiratory burst activity (Wright and Silverstein, 1983). In addition, LPG and its truncated derivatives like GIPL-6 inhibit protein kinase C (PKC), the major modulator of macrophage oxidative metabolism (McNeely and Turco, 1987; Descoteaux *et al.*, 1991). Recent results provide evidence for the defective regulation of intracellular Ca^{2+} in *L. donovani* infected human monocytes. This correlated with a decreased accumulation of inositol 1,4,5, trisphosphate and a decreased superoxide and H_20_2 production on FMLP stimulation (Oliver *et al.*, 1991). The importance of LPG in this respect is shown by the fact that abnormal species of LPG contribute to the avirulence of some laboratory *L. major*

strain (LRC L119) (McCowlle and Homan, 1992).

As mentioned earlier parasite acid phosphatase is also reported to block the production of superoxide anion in neutrophil stimulated with the chemoattractant peptide Met-Leu-Phe (Remaley *et al.*, 1985).

The production of cytokines and response to mitogenic stimulus has been found to be depressed in *Leishmania* infected cells (Reiner, 1987; Ridel *et al.*, 1987; Murray *et al.*, 1986). Furthermore the expression of both class I and class II MHC gene products as induced by INFγ, was suppressed *in vitro* infection of macrophages from BALB\C mice with *L. donovani* infection (Reiner *et al.*, 1987). In addition, *L. donovani* infected cells show impairment of macrophage *d-fos* and TNF-α gene expression in selective manner (Descoteaux and Matalashewski, 1989). The "*Lsh*" gene has been found to be associated with host susceptibility in mice infections (Bradley, 1977).

1.6.4 Interaction with host serum factors

The trypanosomatids have been shown to interact with mammalian serum factors in a way that favours their survival. Heparin with its multivalent glycosaminoglycan may act as a bridge between specific receptors on the viable leishmania and macrophage surface facilitating attachment and entry of *L. donovani* in mouse macrophage (Butcher *et al.*, 1992). In addition heparin has been found to interfere with the histone phosphorylation by the ecto-kinase *in vitro* (Mukkopadhyay *et al.*, 1989). Basic fibroblast growth factor-like proteins (bFGF) that may have a role in cellular attachment growth and differentiation has also been identified both in leishmania and trypanosomes (Kardami *et al.*, 1992). GM-CSF is a growth factor for the *L. mexicana amazonensis* parasite (Charlab *et al.*, 1990). Its infectivity is increased by GM-CSF treatment possibly by being protected from heat

induced death (Barcinski *et al.*, 1992). These findings point to the general trend that the parasites are well equipped to capitalize on the hosts resource in different ways.

Though much is known about leishmania biology, there are many unanswered questions. These include; Why is there preferential tissue tropism among the different species ?, What are the basic molecular mechanisms of transformation that allow parasitazation?, Why is leishmaniasis reactivated after apparent resolution like in Post kala-azar dermal leishmaniasis and Leishmanids?

The non-uniformity in response to treatment between patients from different geographic areas (Berman, 1988), the inconvenience of parenteral adminstration of drugs, the presence of unwanted side effect (Davis, 1969), relapses (Wyers, 1971) and the emergence of considerable resistance to commonly used antimony drugs in patients with leishmaniasis, justifies the continued study of the biology and biochemistry of the species in an attempt to define better therapeutic strategies (Grogel *et al.*, 1992). As a part of the biochemistry of the species the study of signal transduction mechanisms with the aim of finding differences with analogues systems within the genus and with mammalian systems may help in identifying vulnerable spots in the parasites biology (Benson *et al.*, 1992; Henderson *et al.*, 1992; Croft and Hogg, 1988). Furthermore the biological and clinical differences exhibited between the different species of *Leishmania* makes the study of each species as a separate entity mandatory.

Survey of the available literature demonstrates that little work is done on *L. aethiopica* so far when compared to other *Leishmania* species. Because of the above and the relevance of this species in our geographic area the authors have chosen to study protein kinase activity of *Leishmania aethiopica* .

CHAPTER TWO

AIMS OF THE STUDY

Leishmaniasis is a major tropical disease whose prevention and therapy has met with considerable difficulty. The understanding of the basic molecular processes that regulate the proliferation, development and infectivity of this protozoa, will help in development of better therapeutic methods than are currently available to this and other diseases caused by intracellular protozoan parasites. In this context, the specific objectives of this study are;

1) Identification of protein kinase activity in *Leishmania aethiopica* and partial characterization to enable comparison with protein kinases of other *Leishmania* species.

2) Determination of subcellular localization and substrate specificity of protein kinases in *Leishmania aethiopica.*

3) Analysis of protein kinase activation or inhibition on survival, developmental transformation and proliferation of *Leishmania aethiopica.*

4) To investigate particularly the presence of protein kinase C in *Leishmania aethiopica.*

CHAPTER THREE

METHODOLOGY

3.1 Materials

Cell culture media and constituents were from GIBCO (Paisley, Scotland). Staurosporine and Kinase substrate (Histone III-S, Protamine, Myelin basic protein and ATP) were from SIGMA. [^{32}P] ATP, (300 Ci/mol),^{32}P and MC5 monoclonal antibody was from Amersham, UK. Formycin ATP was from Calbiochem,USA. Other antibodies were from GIBCO BRL USA. Phorbol-12, 13-dibutyrate was from Du PONT. Molecular weight markers and all reagents for electrophoresis were from Biorad.

3.2 Cell source and cell culture

Leishmania aethiopica strain P40^2, isolated from a case of cutaneous leishmaniasis in Ethiopia, was kindly provided by Dr. Hannah Akuffo, Karoliniska Institutet, Stockholm, Sweden. The parasites were maintained at room temperature (23°C) in RPMI (Rothwell Park Memorial Institute) 1640 medium supplemented with 15% heat inactivated foetal calf serum, 2mM L-glutamine and antibiotics (200 U/ml penicillin and 200 μg/ml streptomycin). Cultures were passaged every seven days by diluting 10 times in fresh medium.

3.3 Temperature-dependent transformation

Temperature-dependent transformation (heat-shock) was achieved by resuspending late log phase promastigotes in fresh medium and continuing the cultivation at 37°C for 72 hours.

3.4 Viability testing of heat transformed parasites

3.4.1 Dye exclusion

Hundred microlitre parasite culture suspension was taken and mixed with equal volume of 0.4% trypan blue in Phosphate buffered saline (PBS). After ten minutes at room temperature samples were taken and viewed under a microscope (400X). Two hundred cells were counted to check for dye exclusion. Inclusion of the dye was taken as evidence of cell death.

3.4.2 Retransformation

One microlitre culture suspension of heat shocked cells was collected by centrifuging at 3700 rpm in a WIFUG studie-M table centrifuge (England) for 10 min. The pelleted cells were resuspended at 10^6 cells/ml in a fresh medium at 23°C. Parasite proliferation was monitored at intervals of 1-2 days by counting using a haemocytometer.

3.5 Total protein determination

Total cellular protein was determined by Bradford method (Bradford, 1976) using Bio-Rad Protein Assay kit. Parasites were collected by centrifugation (3700 rpm in WIFUG studie-M table centrifuge for 10 min), washed twice in the same way using ice-cold PBS and resuspended (10^8 cells/ml) in Tris buffer, pH 7 (20 mM TRIS-HCl (pH 7), 2 mM EDTA, 0.5 mM EGTA and 0.1% Triton X-100). The solution was sonicated for 10 sec at setting 10 using Soniprep 150 sonifier. The volume was adjusted to 100µl with sample buffer. To this 4 ml of a solution containing 11% phosphoric acid, 3% methanol and Coomassie Brilliant Blue G-250 was added. After 10 min at 25°C, absorbance was read at 595 nm. Bovine serum albumine was used to construct a standard.

3.6 Cell extraction and subcellular fractionation

3.6.1 Ultracentrifugal fractionation

Parasites were collected by centrifugation (3700 rpm in WIFUG studie-M table centrifuge for 10 min), washed twice with ice-cold phosphate buffered saline (PBS) lacking Mg^{2+} and Ca^{2+} and were resuspended (2 x 10^7 cells/ml) in buffer "A" pH 7.5 (20 mM TRIS-HCl, 2mM EDTA, 0.5 mM EGTA, 50 µM leupeptin, 1 mM PMSF). Following sonication for 3x10 seconds in the Soniprep 150 sonifier at setting 10 and after microscopic confirmation of cellular disruption at 400X, the preparation was spun at 100,000 x g (4°C) for 1 hour. The supernatant was used as a cytosolic fraction and the pellet was resuspended with mild sonication (setting 6 for 2 x 6 seconds) and used as a solubilized particulate fraction.

3.6.2 Triton extraction

Cells were collected and suspended in the "A" buffer with 0.1 % Triton X-100. After extraction for 30 minutes on ice, non-solubilized material was removed by centrifugation (5700 rpm in WIFUG studie-M table-centrifuge for 10 min) and the resulting supernatant was assayed for kinase activity. In some experiments, the non-solubilized material was resuspended in the same buffer by sonication at setting 6 for 2 x 6 seconds on a soniprep 150 sonifier and used as a solubilized particulate fraction.

3.7 Protein kinase assay

3.7.1 Ectokinase activity

The following paragraph describes protocol used to measure ectokinase activity both on exogenous as well as endogenous substrates. Live intact promastigotes were used as a source of enzyme activity.

In the first case parasites were washed and suspended in HBS pH 7.4 (20 mM Hepes, 150 mM NaCl and 5 mM glucose). One million cells in 100µl of incubation buffer (10 mM $MgCl_2$, 100 uM ATP, ^{32}P-ATP 2-3 x 10^6 cpm and 100µg of substrate) were incubated at room temperature for 10 minutes. Different concentrations of staurosporine and formycine ATP were also included. After taking samples for viability test, the reactions were stopped by the addition of 1 ml of 10 % TCA and 40 µl of 0.63 % BSA. The sedimentable fraction was pelleted down by centrifugation at 3000 x g for 20 minutes. The pellet was redissolved in 50µl of 0.2 M NaOH and reprecipitated with 1ml of 10% TCA. After three cycles of washing the final precipitate was dissolved in 50 µl of 0.2 M NaOH solution and 450 µl. of H_2O. The whole sample was mixed with 4 ml of Readysafe scintillation fluid and radioactivity was counted.

Secondly samples used for the detection of endogenous substrates by electrophoresis/autoradiography had 4 x 10^6 cells/200µl of incubation buffer with no exogenous protein substrate. The reaction was stopped by ice cold PBS. After washing twice with the same buffer they were extracted with 1% Triton in buffer "A" 10% TCA was used to precipitate proteins and this was washed with ice cold acetone and then solubilized in solubilization buffer (3.10.2) and electrophoresed as described in 3.10.3.

3.7.2 Kinase assay in cellular fractions

The different cellular fractions derived from triton X-100 solubilized cell preparations (3.6.1) and detergent free preparations (3.6.2) were used as enzyme sources. As the case was with ectokinase, exogenous as well as endogenous protein phosphorylating activity was measured. To this end, exogenous protein phosphorylation activity was measured in a final assay mixture of 250 µl (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 100 µM ATP 2-3 x 10^6 cpm and 250 µg of histones or protamine or 100 µg myelin basic

protein. However, this solution was supplemented with 0.8 mM Calcium and 20 μg/ml phosphatidylserine and in some incubation 200 nM Phorbol-12,13-dibutyrate (PDBu) whenever protein kinase C activity was measured. To the assay the assay mixture was added 50 μl of leishmania extract.

Incubation to measure protein kinase activity was carried out in two stages. First 200 μls of concentrated assay mixture containing all but [^{32}P] ATP and substrate (preceding paragraph) was preincubated at 30°C for 2 minutes. Secondly, the reaction was started by adding 50 μl of a mixture containing [^{32}P] ATP and substrate. After 3 min., the incubations were terminated by the addition of 1 ml of 25 % TCA followed by 2 ml of 5 % TCA and 100 μl 0.63 % BSA. The precipitated protein was pelleted by centrifugation at 3000 x g for 20 min. The residue was dissolved in 100 μl of 0.2 M NaOH and reprecipitated with 4 ml of 5 % TCA. However, protamine-containing samples were dissolved in 0.6 M NaOH and reprecipitated with 10% TCA. In experiments where subcellular distribution of the enzyme was assayed all samples were processed in the same way as the protamine containing samples. After three cycles of washing as in 3.7.1, the final pellet was dissolved in 100 μl of NaOH-solution and diluted to 1 ml with H_2O. After mixing, 0.5 ml of this solution was mixed with 4 ml scintillation fluid and radioactivity was counted.

For measurement of protein kinase activity towards endogenous substrates cells were extracted with 0.1% Triton as in 3.6.2 and 3 x 10^6 cells were used in each incubation in the absence of exogenous protein substrates. The mixture was incubated for 10 minutes at 30°C. The reaction was stopped with 1 ml of 10% TCA and washing was done with ice cold acetone. The resulting sample was then analyzed by electrophoresis/autoradiography.

3.8 Thymidine incorporation

Stationary phase parasites were collected as in 3.5 and resuspended (10^6 cells/ml) in a fresh medium. Onto 200 µl of the suspension, 25 µl (1 µCi) of [^{3}H]thymidine and desired amount of inhibitor (staurosporine or formycin ATP) or phorbol-12,13-dibutryate was added. Appropriate controls were also prepared. After incubation at 23^{0}C for three days in a 96 well culture plates, cells were harvested using Skatron semiautomatic cell harvester. Filters were dried and mixed with 2 ml of Readysafe scintillation cocktail (Beckman Instruments) and radio activity of triplicates were measured.

3.9 Phorbol ester binding

Parasites were collected and washed as in 3.7.1 and resuspended (10^7 cells) in 0.45 ml of HBS. To this 50µl containing 2 µCi [^{3}H]PDBu in the same buffer was added to control and experimental tubes. Final concentration of [^{3}H]PDBu was 20 nM. Any binding of radiolabelled PDBu in the control was displaced by adding 2 µl unlabelled PDBu. Incubation with [^{3}H]PDBu was conducted at room temperature for 30 minutes.

The binding was stopped by adding 1ml of ice cold HBS. Excess PDBu was washed as in 3.5. The final pellet was dissolved in 0.3 ml of 0.2M NaOH and 1% SDS for 20 min. Then the samples were mixed with 6 ml of scintillation fluid and measured in duplicates.

3.10 Protein Phosphorylation

3.10.1 Labelling of endogenous ATP with ^{32}P

Late log or stationary phase cells were taken and washed as in 3.7.1 and resuspended in HBS ($2x10^7$ cells/ml). The cells were incubated with 25 µCi of ^{32}P/ml for 1hr at 25^{0}C in order to label the endogenous ATP.

3.10.2 Preparation of samples for electrophoresis

Cells prelablled as in 3.10.1 were incubated for another 20 minutes in the absence or presence of 200nm PDBu. At the end of the 20 minutes they were washed using ice cold PBS. The final pellet was solubilized in 1ml of 1%triton in "A" buffer (3.6.1) for 20 min at 4°C. This was centrifuged for 20 min at 5700 rpm (WIFUG studie-M table centrifuge) and supernatant was collected. It was precipitated with 1 ml of 10% TCA and was allowed to stand for 10 minutes. Again the samples were washed as above with 2 ml of ice cold acetone. From the final pellet, samples were solubilized in 30 µl solubilization buffer (50mM Tris.HCl pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol and 5% 2-mercaptoethanol). After boiling for 3 minutes the samples were subjected to SDS-PAGE on a 10% gel (Solubilized material/lane = 3 x 10^6 cells).

3.10.3 Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A discontinuous polyacrylamide gel was prepared using a mini Protean II dual slab cell (Biorad) as described below.

To cast a 10% separation gel, 2.65ml Distilled, deionized water, 3.4ml Tris-HCl pH 8.8, 0.67ml 2% SDS and 5.95 ml of 22% w/v acrylamide 0.6% Bis was mixed and deaerated under water jet suction for 10 minutes. Then 6 µl TEMED and 0.6 ml w/v 1% ammonium persulfate was added and the mixture was poured into prepared gel-plates. The gel was overlayed with 10% ethanol in distilled water and allowed to polymerize for 45 minutes. The stacking gel was made by dissolving 0.5g sucrose in 2.5 ml Tris-HCl buffer pH 6.8, 0.7 ml Bis-acrylamide and 0.5 ml of 2% SDS solution. The solution was deaerated as above and after 5 µl of TEMED and 0.4ml of 1% ammonium persulfate were added, it was poured on to the separating gel which has been rinsed with distilled water with the well combs in position. The gel was allowed to stand for 45 minutes. Then the combs were

removed and the wells were rinsed with distilled water. Finally samples were loaded and the gels were run under electrophoresis running glycine buffer pH 8.3 (250mM Glycine 25mM Tris and 0.1% SDS) at 200 volts for 45 minutes. Molecular weight markers (Biorad) were run in one lane and on each gel.

3.10.4 Gel Staining and Drying

When the electrophoretic run was completed, gels were put in 200 ml of filtered staining solution containing 0.25g of R250 Coomassie brilliant blue and water, methanol, glacial acetic acid in a 9:9:2 v/v ratio. After 4 hours at room temperature, the gels were destained by soaking in a 7.5% glacial acetic acid and 5% ethanol in water solution for 12 hours. Finally they were immersed in distilled water, wrapped with saran wrap immediately and dried in a Biorad 543 gel dryer for 1.5 hours at 80°C.

3.10.5 Autoradiography

The dried gel was put on a Kodak X-OMAT radiography film and the film was exposed in an intensifying screens cassette at -70^{0}C for an appropriate period of time.

3.11 Western Blot

3.11.1 Sample preparation

Cells were collected and washed with cold PBS as in 3.6.1 and solubilized with solubilization buffer (10^6 cells/100μl). Samples were boiled for 3 minutes and sonicated for 10 sec at setting 10 using soniprep 150 sonifier. SDS-PAGE was performed as above (solubilized material/lane = 3 x 10^6 cells).

3.11.2 Transfer to Nitrocellulose Membrane

Gel proteins were transferred to nitrocellulose paper in a Mini protean II dual slab cell using the method of Towbin *et al.* (1979) as follows. The gels and cut nitrocellulose membranes were equilibrated in the transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, 0.1% SDS, pH 8.3) for 10 and 15 minutes respectively. Filter papers of the same size as the gels and fibre pads were also saturated with the same buffer. Then a fibre pad, filter paper, gel, membrane, filter paper and fibre pad sandwich was assembled in the gel holders with the membrane side towards the anode. The gel holder was then put in a transfer tank filled with prechilled transfer buffer. Transfer was accomplished at 100 volts in 1 hour.

3.11.3 Staining of the Nitrocellulose Membrane

The side of the membrane that has the molecular weight markers was cut and placed in Amido-swartz stain for 3 hours. It was then washed with water till the bands could be clearly visualized. This was stored at 4°C in a desicator.

3.11.4 Immune Detection of Protein kinase C

All detection reagents were from Amersham Enhanced Chemiluminecsense (ECL) kit. Blots from 3.11.1 were taken and blocked in a 5% dried milk in TBS-T washing buffer (20mM Tris base, 137 mM NaCl, pH adjusted to 7.6 with 1M HCl and 0.1% tween-20) at room temperature for 1 hour. Then membrane are washed with the washing buffer by rinsing two times and washing for 15 minutes twice and 5 minutes once on a rocking plate. The membrane were then incubated at room temperature with the following primary antibodies diluted in TBS-T (Table 3.1).

Table 3.1 Incubation of primary antibodies.

Nº	Antibody	Source	dilution	Inc. time
1	Anti-PKC α,β,γ	Rabbit	1/1000	1 hour
2	Anti-PKC ε	Rabbit	1/1000	1 hour
3	Anti-PKC $\alpha,\beta_I,\beta_{II}$	Mouse (cloneMC5)	1/500	4 hours

Presence of PKC-Anti PKC complex was detected by anti IgG antibody tagged horse raddish peroxidase. Incubation was conducted at 1:4000 dilution of the secondary antibody. After washing, the presence of the latter was detected in solutions that contained H_2O_2 and luminol (a cyclic diacylhydrazide). The excess fluid was drained off and membranes were wrapped in saran wrap. Following this light emission intensity of oxidized luminol was detected by exposing a Kodak X-OMAT radiography film from 3-15 seconds at room temperature. Films were then developed.

CHAPTER FOUR

RESULTS

4.1 Extra- and intracellular protein kinase activities in *L. aethiopica*

When live promastigotes of *L. aethiopica* were exposed to exogenous phosphate acceptor proteins, in the presence of Mg^{2+} and ^{32}P-ATP, ^{32}P was incorporated in the TCA-precipitable proteins, suggesting the presence of an ectokinase activity on *L. aethiopica*. Incorporation on endogenous proteins in the absence of exogenous substrate was only minor (Table 4.1). The ectokinase activity strongly preferred histone V-S as a substrate over protamine, myelin basic protein and histone III-S, under incubation conditions that did not affect cell viability as judged by trypan blue exclusion.

When *L. aethiopica* parasites were extracted in a hypotonic buffer containing chelating agents (EDTA and EGTA), protease inhibitors (PMSF, leupeptin) and 0.1 % Triton X-100 (section 3.6.2), a kinase activity was released into the resulting supernatant. The incorporation of ^{32}P into extracted endogenous proteins constituted only a minor fraction of the incorporation observed in the presence of exogenous substrate (Table 4.1). The extractable kinase preferred exogenous substrate in the order protamine > Histone V-S > myelin basic protein > histone III-S (Table 4.1), thus differing in substrate preference from the ectokinase activity measured on live promastigotes. Using histone V-S as a substrate, the extracts showed 8-9 times higher protein kinase activity compared to the ectokinase.

Table 4.1 Substrate specificity of extracted kinase and of ectokinase from *L. aethiopica.**

Substrate	Ectokinase activity	Kinase activity in extract
Endogenous proteins	0.1 ± 0.1	2.9 ± 0.8
Histone III-S	0.3 ± 0.1	3.4 ± 0.6
Histone V-S	9.3 ± 3.1	81.5 ± 6.5
Protamine	0.9 ± 0.2	135.4 ± 19.6
Myelin basic protein	0.5 ± 0.1	15.2 ± 1.0

* Activity is expressed as pmol of ^{32}P transferred/min/10^6 cells. Values represent mean ± SEM (n = 3-4)

4.2 Subcellular Distribution of Protein Kinase Activities

L.aethiopica were disrupted by sonication and fractionated by ultracentrifugation at 100,000 x g for 60 min. Protein kinase activity was highest in the resulting high-speed supernatant, compared to the particulate fraction, using either histone or protamine a substrate. Histone phosphorylation in the particulate fraction was barely detectable, whereas in the supernatant it amounted to about 50% of the protamine phosphorylation (Table 4.2).

The distribution of kinase activities was also studied using the triton extraction technique. Triton extracts and triton-insoluble sediments were prepared and the latter was resuspended in sonication buffer and sonicated. The kinase activity towards histone V-S and protamine was then measured in the fractions. As shown in Table 4.2, the soluble fraction prefers protamine over histone V-S. In the sonicated triton-insoluble fraction no activity towards histone V-S was detected, but with protamine as substrate, an activity

amounting to about half of that seen in the soluble fraction was measured.

Table 4.2 Subcellular distribution of kinase activities in *L. aethiopica**

Fractionation procedure	**Substrate**	**Soluble fraction**	**Particulate fraction**
Ultracentri-fugation	histone	28.1 ± 4.4	1.7 ± 1.2
	protamine	62.2 ± 8.8	33.2 ± 6.4
Triton solu-bilzation	histone	42.1 ± 2.5	0
	protamine	110.5 ± 4.5	42.2 ± 8.9

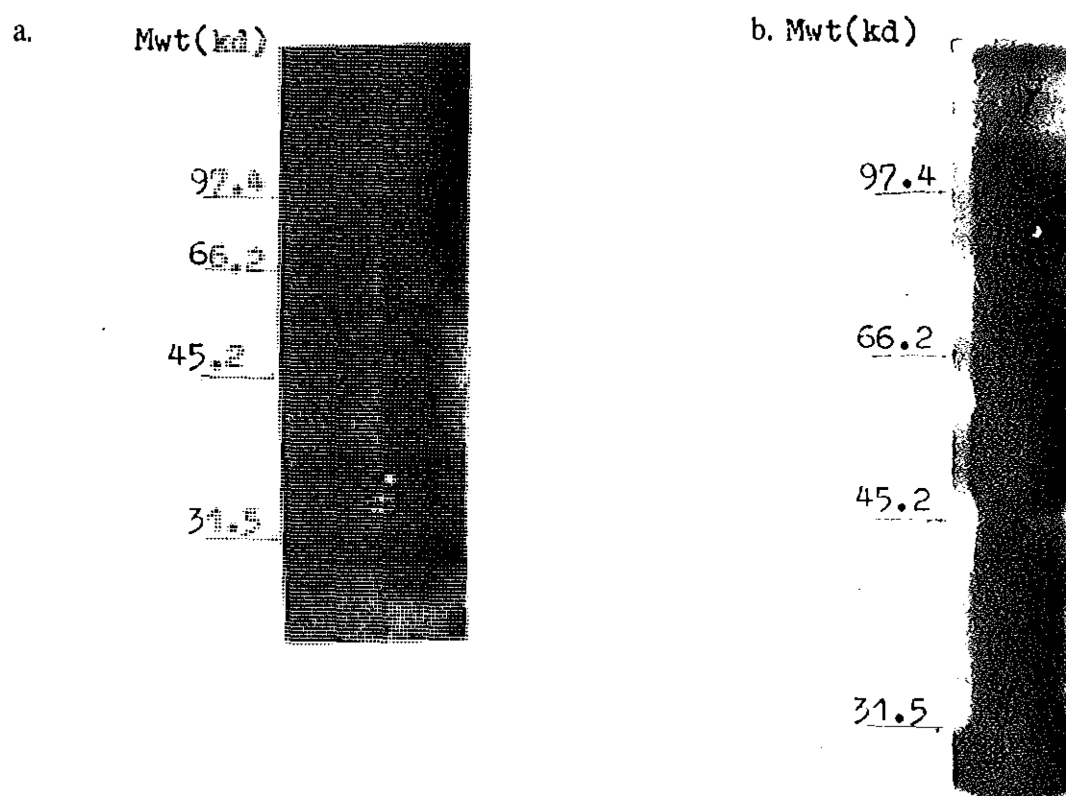
* Activities are expressed as pmol of ^{32}P transferred/min/10^6 cells (promstigotes) and values represent mean ± SEM (n = 3). The total protein content = 1.2 mg/ 10^6 promastigotes. The protein content of detergent free particulate and soluble fractions were comparable.

4.3 Phosphorylation of Endogenous Substrates

The presence of endogenous substrates that can be phosphorylated by exogenous ATP was investigated using a larger number of parasites than used for the kinase assays. The ectokinase incubations, without exogenous substrates, were fractionated on a 10% SDS gel and autoradiographed. The film revealed the presence of at least 8 bands of phosphorylated proteins in the incubation conditions specified. Likewise the triton extract when incubated with ^{32}P-ATP in the absence of exogenous substrates and followed by SDS-gel electrophoresis and autoradiography, revealed the presence in the parasite of at least 20 phosphorylated protein bands distributed over a relatively wide range of molecular weight

(Figs. 4.1a & b). The autoradiographic patterns as well as the relative intensities of the different bands differed markedly between the two incubation conditions (Fig. 4.1a & 4.1b).

Fig. 4.1 Endogenous substrates for ectokinase and triton extracted kinase activities of *Leishmania aethiopica.*



(a) Ectokinase activity and (b) is extracted kinase activity on endogenous substrates.

4.4 Effect of Cell Growth and Transformation on Protein Kinase Activities

In order to investigate eventual changes in kinase activity in relation to growth and differentiation, the histone V-S phosphorylating activity of the triton-solubilized kinase and the ectokinase were chosen for study. Kinase activities expressed by cells from cultures growing at low densities (early log phase) were compared with activities in cells from cultures growing at higher densities (late log/stationary phase). It was found that the

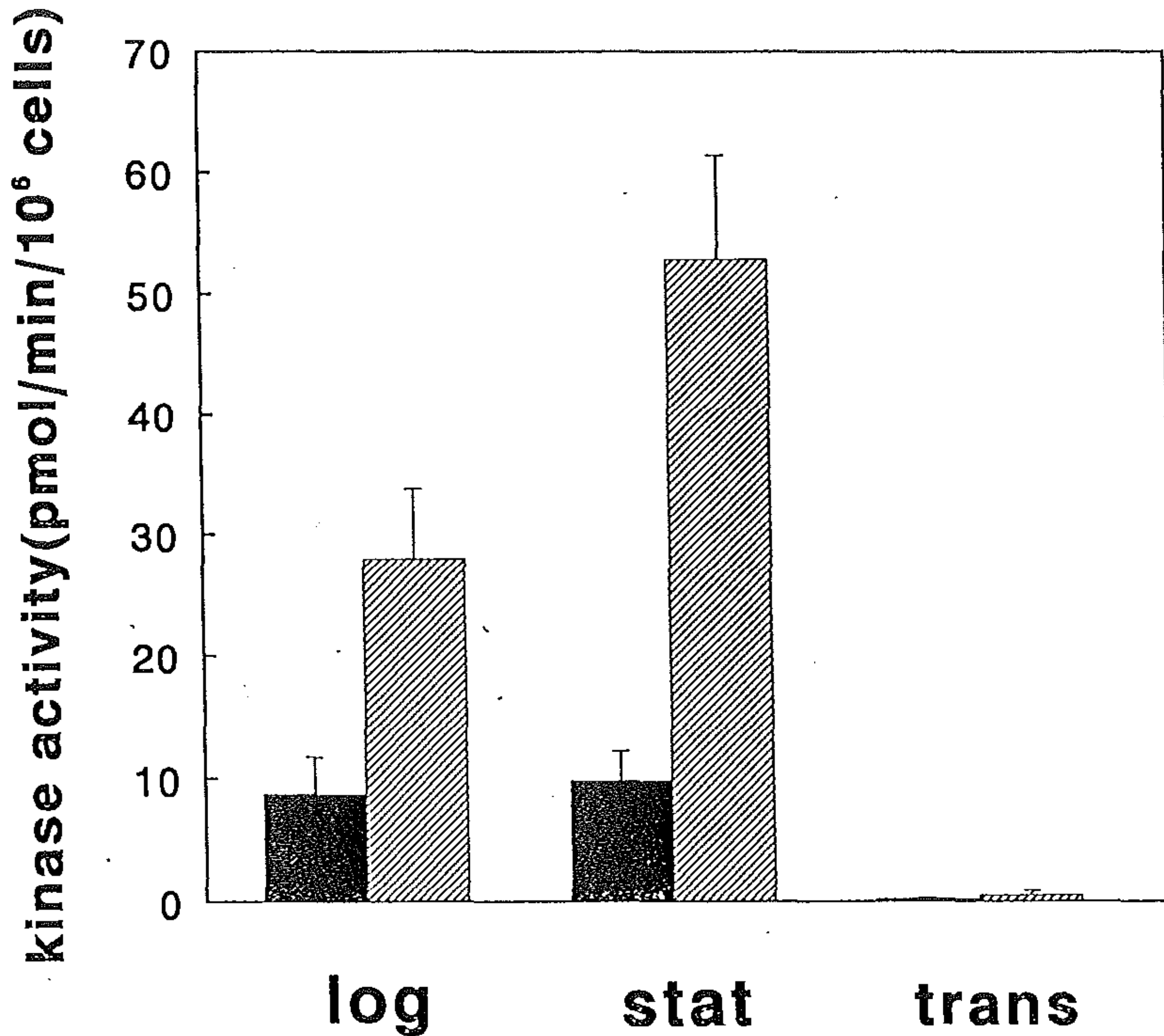
activity solubilized by 0.1 % triton was about twice as high in the high density cultures as compared to the less dense cultures (Fig. 4.2). On the other hand the ectokinase activity did not exhibit a similar trend in this respect.

Heat-shock treatment has been shown to transform many species of *Leishmania* from the promastigote to an amastigote-like state (Hunter, 1982; Smejkal et al. 1984). This information led to the investigation of kinase activities in cells incubated at 37°C for 72h. The data presented in Fig. 4.2 shows that heat-shock treatment reduced the triton-solubilized as well as the ectokinase activities to almost undetectable levels (less than 2 % of the controls). Similar results were obtained using protamine.

The heat-treated cells were small, compared to the promastigotes, rounded and non-motile without prominent flagellum. The viability of the heat-treated cells, assessed by trypan blue exclusion, exceeded 90% after 72 hours. When the heat-transformed parasites were reverted to the normal culture temperature (23°C) they retransformed during 2-3 days to elongated, flagellated and motile cells. Following this lag period, the retransformed cells started to grow at the same rate as their non-transformed counterpart (Fig. 4.3).

In another set of experiments cells were transformed at pH 4.75 (Zeiberstein *et al.*, 1991). Again, cells appeared small, rounded, non-motile and again with out prominent flagellum. In cells transformed this way, the ectokinase activity and triton-soluble kinase activity also was below the detection limit. In fact, a ten hour exposure to pH 4.8 at room temperature reduced the ectokinase activity by about 60%. When this was done at 34°C the reduction approached 96% (Data not shown).

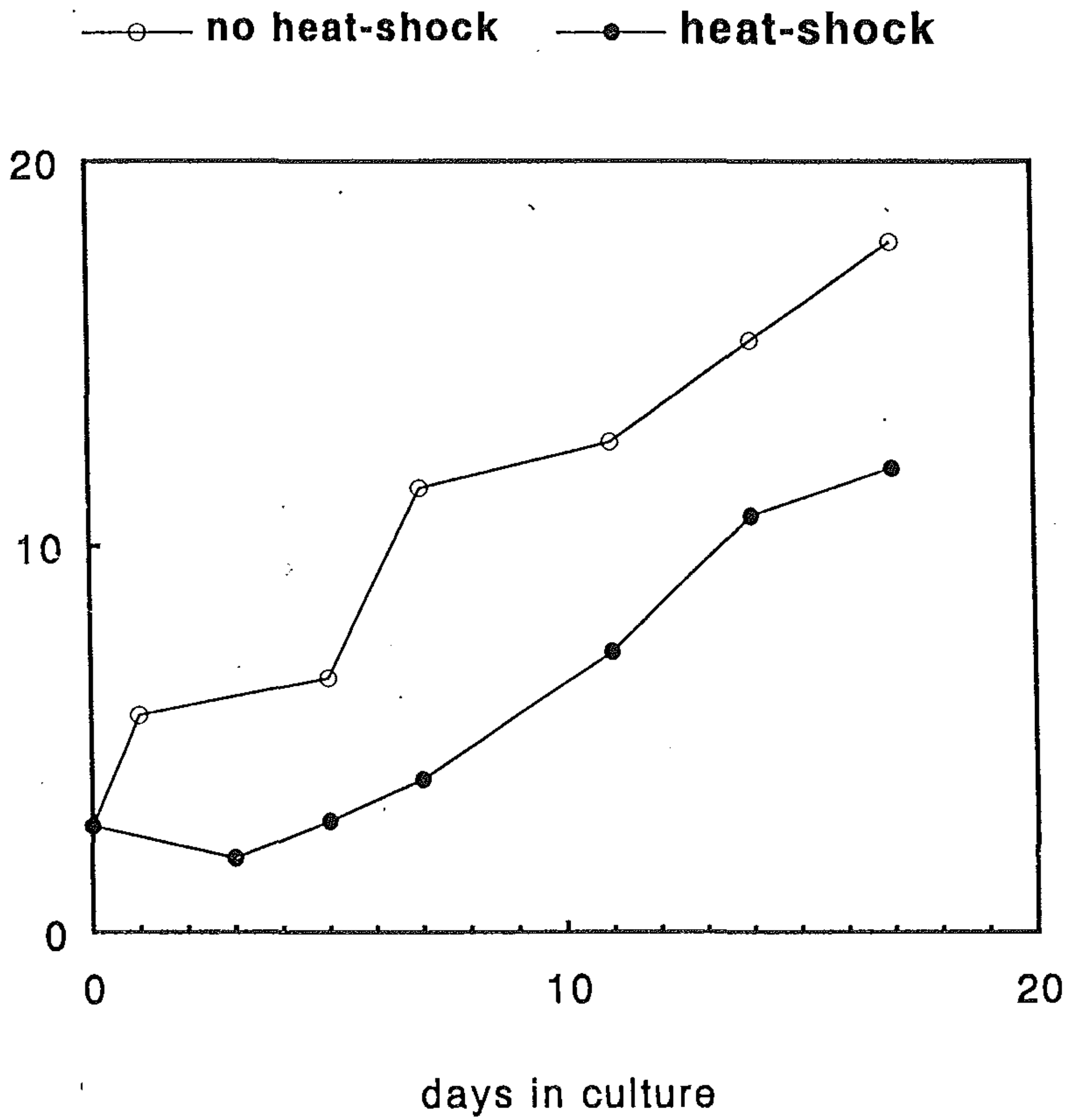
Fig. 4.2 Protein kinase activities during growth and transformation of *L. aethiopica.*



Hatched bars represent kinase activity in 0.1% tritone estract and filled bars represent the ectokinase activity. Histone V-S was the substrate.

log = dilute cultures (4 x 10^6/ml), *stat* = late log or stationary phases of growth (4 x 10^6/ml). *trans* = cells transformed by heat-treatment. Results represent the mean ± SEM of 4-7 experiments assayed in duplicate.

Fig. 4.3 Retransformation of heat shocked cells. The figure shows a representative experiment done in duplicate.

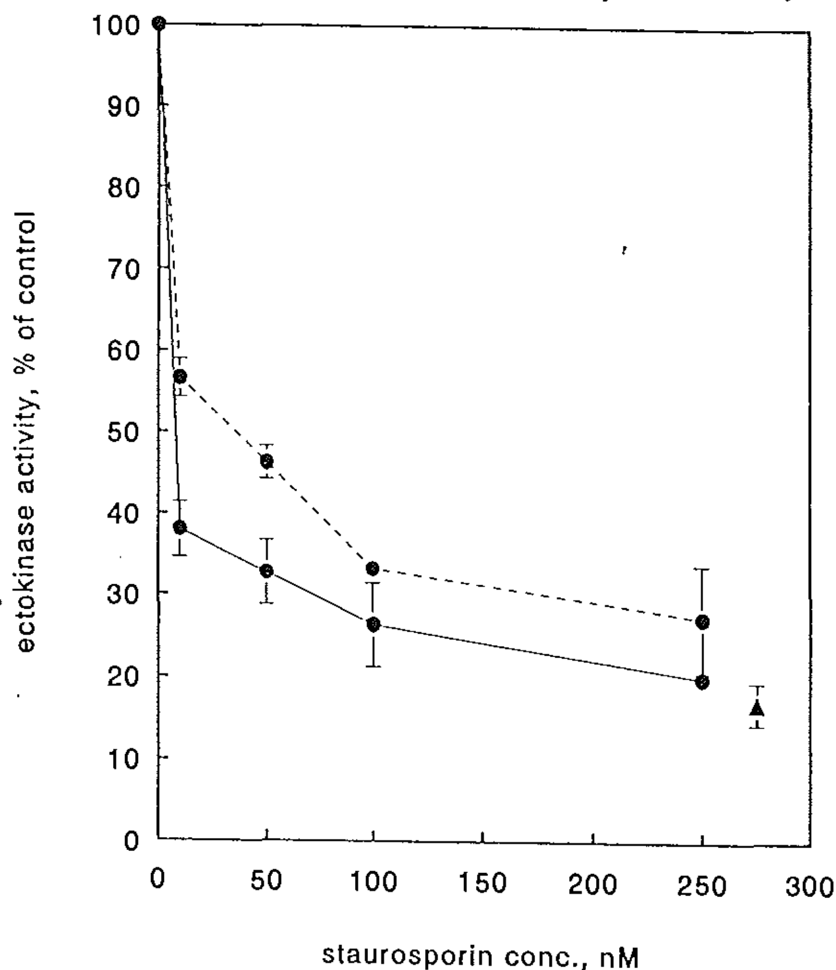


4.5 Effect of Kinase Inhibitors

4.5.1 Effect of kinase inhibitors on protein kinase activities

The ability of protein kinase inhibitors to lower or suppress *L.aethiopica* kinase activities was studied by using different cellular fractions. 60% of ectokinase histone phosphorylating activity can be inhibited using 10 nM staurosporine. At 100 nM the inhibition rose to 75%. A slightly lower degree of inhibition was observed when protamine was used as a substrate (Fig. 4.4). Formycin ATP at 250 μM inhibited the ectokinase by approximetly 82% .

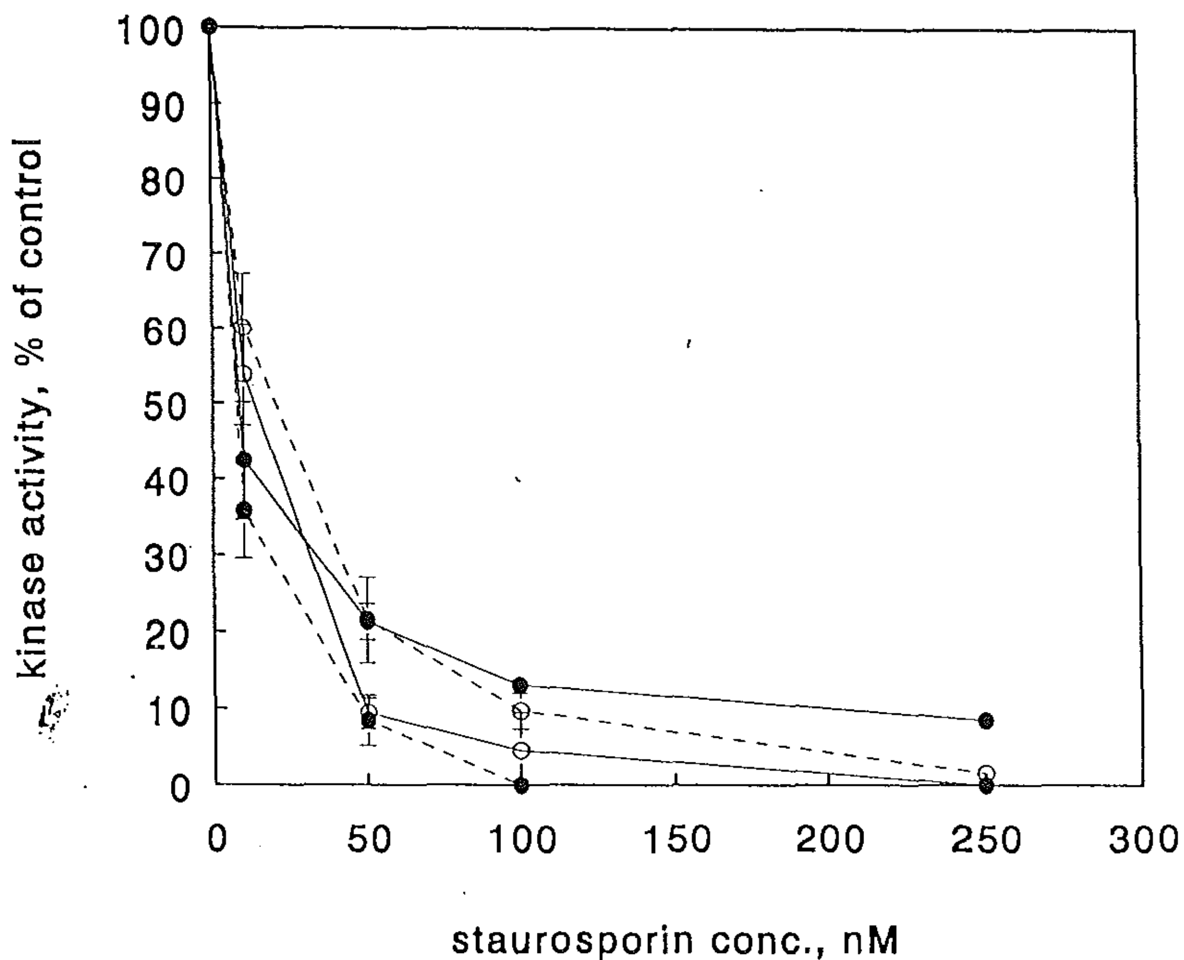
Fig. 4.4 Effect of staurosporine on ectokinase activity of *L. Aethiopica.*



Solid line (—●—) represent histone substrate (n=4) and broken line (- - ● -) protamine. Values represent mean ± SEM.; ▲ represent inhibition by 250μM Formycin ATP where histone was the substrate. Value is mean ± SEM (n=2). All were assayed in duplicate.

In the subcellulr fractions, investigations were focused on the protamine kinase activity which is readily detectable in all fractions used. Staurosporine inhibited the protamine kinase activity of all studied fractions (triton released supernatant, triton resistant sediment, high-speed supernatant and high-speed pellet) with comparable efficiency. At 10 nM of staurosporine, the degree of inhibition ranged between 40 - 65% in the different fractions (Fig. 4.5). At 100 nM of staurosporine, inhibition was between 90 - 100% (Fig. 4.5).

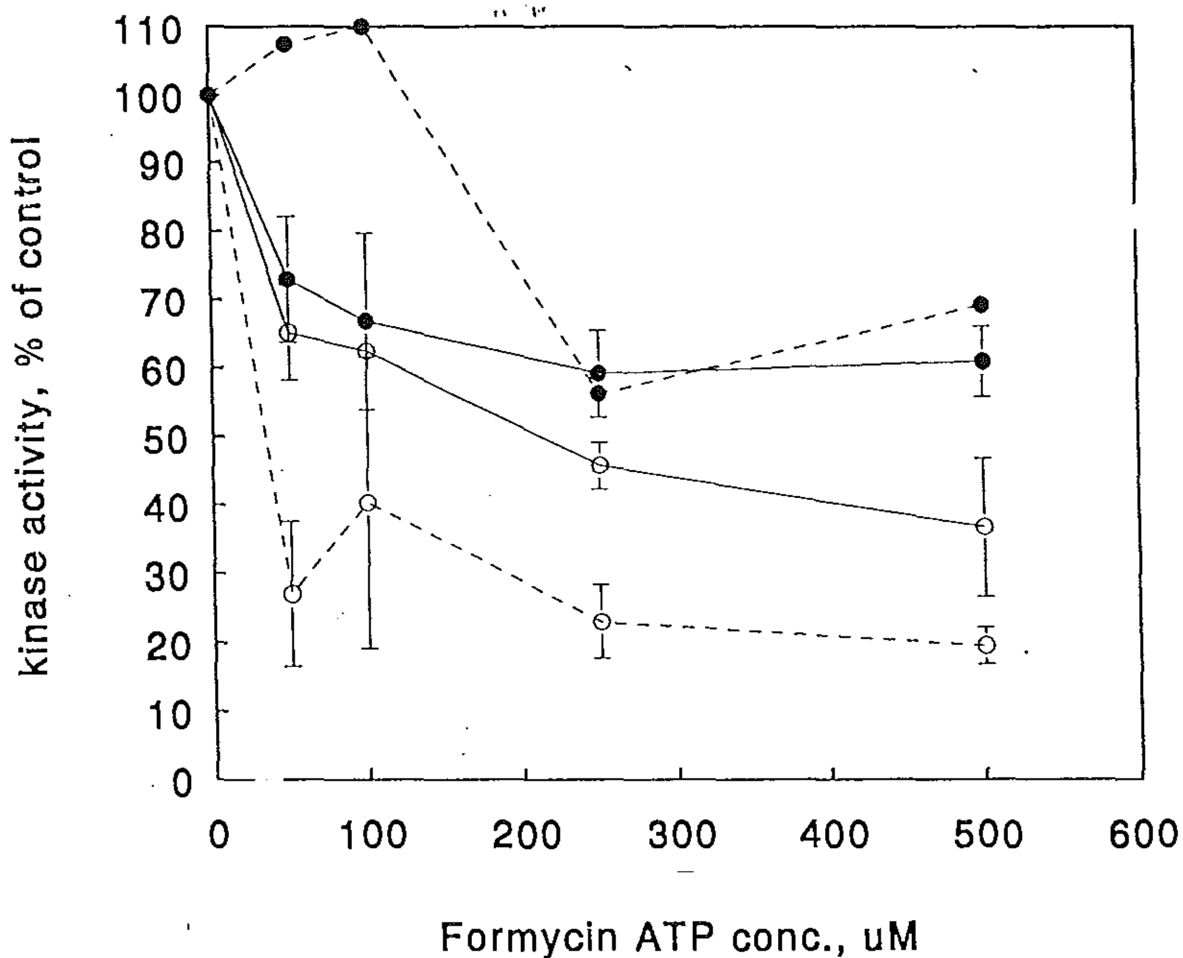
Fig. 4.5 Effect of staurosporine on protamine kinase activity in subcellular fractions.



Solid lines represent triton x-100 fractionated material and dotted line ultracentrifugal fractionation. The open symbols are soluble fraction while the closed are particulate fractions. Values represent mean ± SEM (n=4) assayed in duplicate.

Formycin ATP also inhibited the protamine kinase activity in all the subcellular fractions, albeit not completely. At 250 μM of Formycin ATP, inhibition ranged between 40 - 70%. Even at the highest concentration used (500μM), between 20 - 70% of the kinase activity remained (Fig. 4.6).

Fig. 4.6 Effect of Formycin ATP on protamine kinase activity in subcellular fractions.



Solid lines represent triton x-100 fractionated material and dotted line ultracentrifugal fractionation. The open symbols are soluble fraction while the closed are particulate fractions. Values represent mean ± SEM (n=3) assayed in duplicate.

4.5.2 Effect of kinase inhibitors on cell growth and morphology

The effect of kinase inhibitors on leishmania proliferation was monitored by studying the influence of staurosporine and formycin ATP on cell count and [^{3}H]thymidine incorporation into parasite DNA. In this context, 10 nM staurosporine was without effect on cell growth, whereas at 250 nM inhibition became apparent (Table 4.3). Under the same conditions, 250 μM of Formycin ATP not only blocked cell growth but also reduced the number of living cells.

Table 4.3 Effect of inhibitors on *L. aethiopica* proliferation.

	Day 0	Day 3	day 5
Controls	1.0	5.3 ± 0.8	7.78 ± 0.04
Staurosporine			
10 nM	1.0	6.4 ± 0.08	7.27 ± 0.9
50 nM	1.0	2.4 ± 0.01	4.35 ± 0.02
100 nM	1.0	1.97 ± 0.01	4.55 ± 0.4
250 nM	1.0	1.55 ± 0.04	1.85 ± 0.01
Formycin ATP			
250 μM	1.0	0.44 ± 0.03	0.33 ± 0.04

Cell number is expressed as 10^6 cells /ml. Values are mean ± SEM (n=4)

The incorporation of [^{3}H]thymidine into parasite DNA was not significantly reduced below 100 nM of staurosporine. Higher concentrations (250 nM) of the inhibitor resulted in a 39% inhibition (Table 4.4). On the other hand Formycin ATP obliterated [^{3}H]thymidine incorporation at a concentration of 50 μM.

Table 4.4 Effect of inhibitors on [^{3}H]thymidine incorporation by *L. aethiopica.*

Staurosporine	Percentage with regard to control	Formycin ATP	percentage with regard to control
Control	100	Control	100
10 nM	94 ± 3.09	50 μM	3.0 ± 1.22
50 nM	99.2 ± 9.38	100 μM	2.33 ± 1.05
100 nM	83.5 ± 8.07	250 μM	2.8 ± 0.45
250 nM	61.0 ± 2.28	500 μM	2.87 ± 2.05

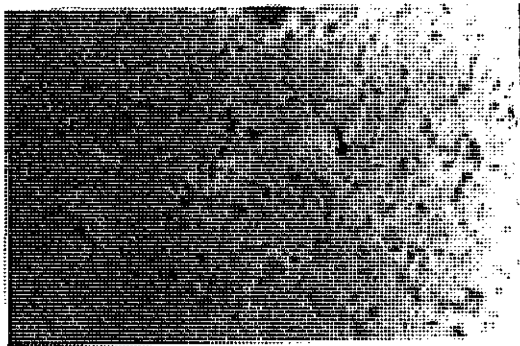
Thymidine incorporation of experimentals were expressed as percentage cpm of controls. The results are mean ± SD. (n=3)

Contrary to its effect on [^{3}H]thymidine incorporation, staurosporine has a profound effect on morphology at concentrations of 50 μM or more. Microscopic observation has revealed the cells to be very much enlarged (at least twice or thrice in diameter) with clear cytoplasm. These "giant" parasites retained the general ovoid shape (Fig. 4.7 a&b) Along with these, other very small atypical looking flagellated parasites could be identified. In addition numerous rounded enclosed cell ghost like structures were seen. These structures

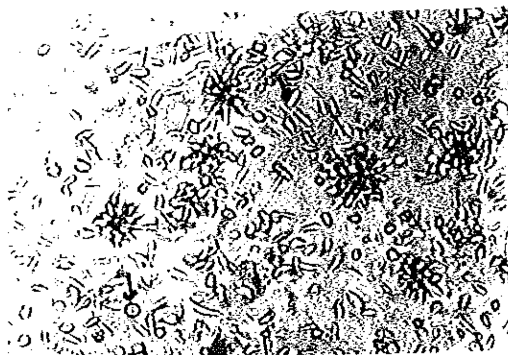
were not similar to the disintegrating cellular material that could be seen in old but normal cultures leishmania. The controls did not exhibit any of the above. In addition it was noticed that these large cells seem to have slightly sluggish motility.

Fig. 4.7 Morphological alterations in *Leishmania aethiopica* produced by staurosporine.

a. Control



b. Staurosporine treated cells



The Figures above show some of the size differences exhibited between controls and promastigotes exposed to 200 nM staurosporine. Ordinary light microscope at 400X magnification was used. Arrows indicate typical cells.

4.6 Investigation of Protein Kinase C Expression

The protein kinase C family of enzymes is involved in numerous regulatory functions concerning the vital aspects of cell biology such as differentiation and growth control. Protein kinase C is also found in some protozoa (see "introduction"). In this context a series of experiments were conducted to elucidate whether *L. aethiopica* does express members of this class of protein kinases.

4.6.1 Assay of protein kinase C activity

The presence of a Ca^{2+}/Phospholipid dependent protein kinase activity in the triton solubilized cells was investigated. For this purpose histone III-S was used as exogenous substrate. Ca^{2+}, phosphatidylserine and phorbol dibutyrate were included in the incubation mixtures as indicated in figure (4.7). The addition of 0.8 mM calcium and 20µ/ml phosphatidylserine did not show a significant difference in kinase activity when compared to controls. However, the further addition of 200 nM of Phorbol dibutyrate decreased the kinase activity by 30%, contrary to what might normally be expected if there was a phorbol ester sensitive protein kinase C like activity in the incubation mixture.

Fig. 4.8 Protein kinase C activity assay of *Leishmania aethiopica* promastigotes.

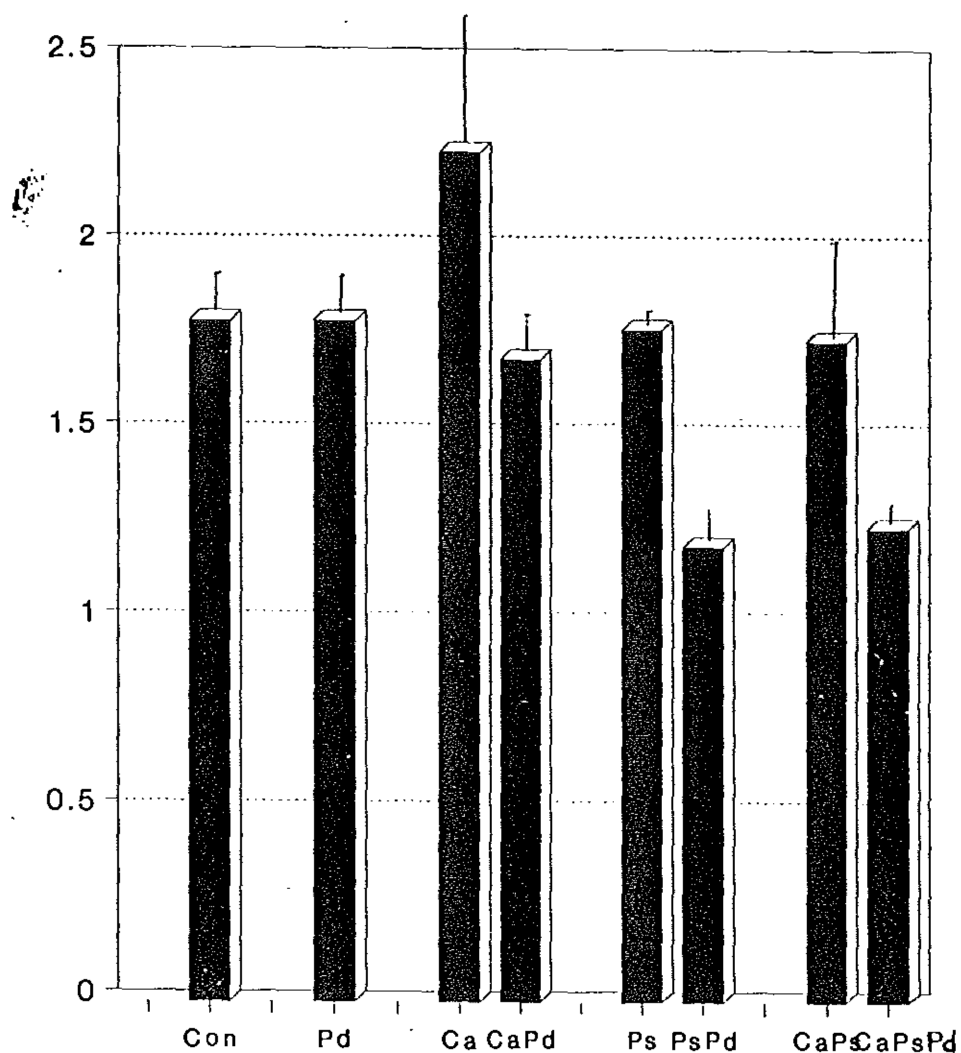


Fig. 4.8 represents mean activity in pmol ^{32}P transferred/min /10^6 cells ± SD. The kinase activity in the presence and absence of known protein kinase C activators is shown. Con= control, PD= phorbol dibutyrate, Ca= Ca^{+2}, Ps= phosphatidylserine. (n=2).

4.6.2 Effect of phorbol ester on cell growth and [^{3}H]thymidine incorporation

The effect of phorbol dibutyrate on cell growth and thymidine incorporation by growing cells was investigated. In the first case, cells were incubated with two different concentrations of PDBu. As can be seen from Table 4.5 no significant effect of PDBu was detected in terms of cell number when compared to controls. Furthermore ≤ 400 nM PDBu did not produce any morphological or motility change as seen under a microscope at magnification of 400X.

In the second case cells were incubated with [^{3}H]thymidine and 200 nM of phorbol dibutyrate as described in the methods section. When the thymidine incorporation in CPM of the experimental group was compared with that of controls a 100.6% ± 4.5% incorporation was detected (n=7).

Table 4.5 Effect of Phorbol ester on proliferation of *L. aethiopica.*

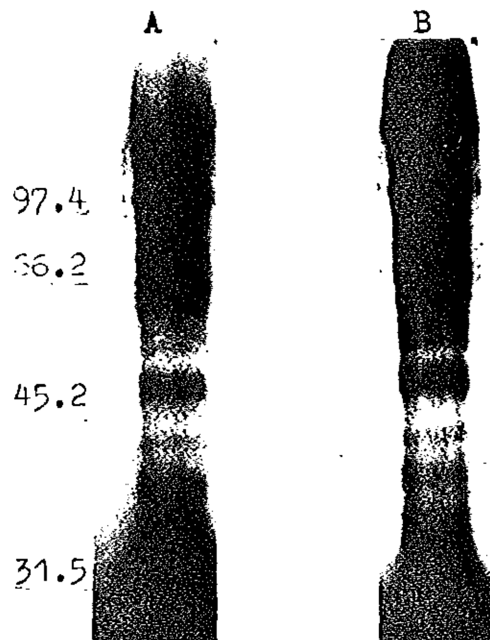
	Day 1	Day 3½	Day 5
Control	1	4.2 ± 0.1	5.9 ± 0.4
200 nM PDBu	1	4.1 ± 0.1	5.6 ± 0.2
400 nM PDBu	1	4.0 ± 0.2	6.0 ± 0.3

Cell number is given as mean ± SEM million cells/ml (n=4).

4.6.3 Effect of Phorbol ester on endogenous protein phosphorylation

The effect of phorbol ester on phosphorylation of endogenous proteins was investigated using cells in which we pre-labelled the endogenous pool of ATP with ^{32}P. When such cells were exposed to 200 mM PDBu and then analyzed by electrophoresing on a 10% SDS gel followed by autoradiography, the pattern of the phosphorylated protein bands was exactly the same as that of the controls. In addition no significant difference in intensity of the bands could be detected visually (Fig. 4.9).

Fig. 4.9 Effect of phorbol ester on phosphorylation of endogenous proteins by *L. aethiopica.*



"A" = control ; "B" = Experimental with 200nm PDBu.

4.6.4 Phorbol Ester Binding Activity

Phorbol ester binding activity of intact live parasites was examined as an indirect method of studying the presence of a protein kinase C capable of interacting with phorbol esters. This was done in two sets of experiments. In one set only PDBu and [^{3}H]PDBu were used. In the other set Ca^{2+} (1 mM) and Ca^{2+} ionophore (A 23187) (200 μM) were included in order to facilitate any possible binding. As shown in Table 4.6, no specific binding could be detected in any of the experimental conditions (Table 4.6).

Table 4.6 Phorbol dibutyrate binding by live promastigotes.

Sample	Control	A	B
% activity	100	95.5 ± 8.9	95 ± 9.7

Values are expressed in % ± SEM of the controls (CPM).

"A" represents experiments where only PDBu and [^{3}H]PDBu were used (n=7)

"B" represents experiments where Ca^{2+} and Ca^{2+} ionophore (A 23187) were included (n=4).

4.6.5 Western blot analysis of protein kinase C expression

The immune reactivity of *L. aethiopica* promastigotes with different anti-PKC antibodies was investigated by western blot analysis. Three groups of antibodies were used. The first was an anti-PKC α, β, γ (Rabbit anti-peptide antibody). This antibody was generated using a peptide sequence corresponding to amino acids 381-394 of PKC- γ. The antibody is also able to recognize the α and β isoforms. The second antibody was specific for PKC-ε where as the third antibody was a monoclonal anti-PKC (clone MC5) that recognizes residues 312-323 of bovine PKC-α. It was also able to recognize $\beta_I\beta_{II}$ isoform.

CHAPTER FIVE

DISCUSSION

Leishmania aethiopica promastigotes possess a measurable activity of protein kinase (Table 4.1 and 4.2). Localization studies were carried out on intact parasites and cell lysates using exogenous substrates. In this context, the phosphorylation of substrates which are cell impermeable such as histones and protamine (Table 4.1, Fig. 4.2) by live parasites demonstrates the existence of an ectokinase. Another set of experiments conducted using high speed supernatants (Table 4.2) indicates the existence of cytosolic protein kinase(s).

It is common knowledge that enzymes of biological membranes can be extracted using detergents (Helenius and Simons, 1975). While most membrane protein are soluble in triton, proteins of glycosomes or those that are firmly attached to cytoskeleton are triton resistant. The triton resistant cellular fraction of *L. aethiopica* has significant protein kinase activity (Table 4.2).It is therefore possible to draw the following conclusion from the preceding paragraphs:

1. *Leishmania aethiopica* promastigotes possess measurable protein kinase activity.
2. Promastigote kinases occur in at least two or probably three sites:
    a. On the outer aspect of the plasma membrane (ectokinase).
    b. In the cytoplasm.
    c. On Triton resistant intracellular structure.

Substrate preference of the three kinase fractions listed above were studied using histone, protamine and myelin-basic protein (Table 4.1). The ectokinase showed a ten fold more preference of mixed histones than protamine which was the next best substrate. On the other hand the solubilized and particulate fractions preferred protamine. However while the non-particulate fractions have significant histone kinase activity the particulate fraction has very little activity (Table 4.2). The histone kinase activity in the triton solubilized

fraction cannot be fully accounted for by a detergent released histone phosphorylating ectokinase activity, since the non-particulate detergent free fraction also has a substantial histone kinase activity.

The data from subcellular localization experiments show that significantly higher kinase activity is found in soluble fractions whether histone or protamine is used as substrate. While the ectokinase activity strongly preferred histone, no substantial histone V-S phosphorylating activity was found in the high speed sediment (Table 4.2). This suggests that the membrane located ectokinase either loses activity or changes substrate specificity when the membrane integrity is disturbed.

It is clear from the data presented that in *L. aethiopica* the particulate fraction accounts for 5-35% of the total kinase activity depending on the substrates used (Table 4.2). This is different from the work done by Lester *et al.* (1990) on *L. major* that showed the high speed sediment enzyme accounts for 75.8% of the total kinase activity. Work done by Das *et al.* (1986) on *L. donovani* revealed that about 20% of the histone kinase activity of the organism was accounted for by the particulate fraction. On the basis of the above it appears that protein kinase activities found in *Leishmania aethiopica* may have a subcellular distribution which is different from that of other leishmania species.

Both ectokinase and the triton solubilized enzymes were capable of phosphorylating endogenous substrates (Fig. 1.1). The number of bands obtained on SDS PAGE/autoradiography plate were eight for the ectokinase and twenty for the triton solubilized enzyme. The two sets are superimposable except for two bands (130, 140 kd) from the ectokinase incubations. This suggests that the superimposible bands may represent similar substrates. In [^{32}P] labelled parasites a similar pattern of phosphorylation was found to that of the triton solubilized ones (Fig. 4.9 control). Table 5.1 compares the number and molecular mass of the endogenous substrates of ectokinases from *L. aethiopica* and *L. major.*

Table 5.1 Comparison of endogenous substrate phoshphorylated by *L. aethiopica* and *L. major*. The figures are given in Kilo Dalton and for *L. aethiopica* range is given.

Species	Mol. Wt. of the different protein bands
L. aethiopica	139±1, 130±1, 113±1, 70±1, 63±1, 60± 2, 38±1, 36±1
L. major	140, 130, 116, 95, 63, 55, 48, 40, 38

Most of the endogenous substrates for the ectokinases of the two species are comparable in molecular weight but as can be seen from table 5.1 there are exceptions.

The endogenous protein phosphorylation by the ectokinase of *L. aethiopica* seems to be low. On the other hand the phosphorylation of endogenous proteins by the ectokinase activity of *L. donovani* (Das *et al.*, 1986) and *L. major* (Lester *et al.*, 1990) are 40% and 20% respectively, when compared to the phosphorylation of mixed histones. This could partly reflect the difference in assay conditions. But unlike in *L. aethiopica*, in *L. major* the protamine kinase activity of the ectokinase was half of that of endogenous proteins.

The triton-soluble histone V-S phosphorylating activity of *L. aethiopica* increased with increasing cell densities in the cultures without concomitant increase in ectokinase activity (Fig. 4.2). This data supports the conclusion reached earlier on the bases of subcellular study, i.e. the two kinase activities represent distinct enzymes. This finding suggests that there is a growth dependent regulation of at least one of the measured kinase activities. The stationary phase cells seem to have significantly more triton solubilized histone kinase activity than log phase cells. This finding is partly in agreement with the

results presented on the *L. donovani* kinase (Das *et al.*, 1986) where the total histone kinase activity (soluble and membrane bound) increased as the organisms approached stationary phase.

The survival of leishmania promastigotes in the vertebrate host is dependent on its ability to change into amastigotes. The exact biochemical events leading to this transformation are not clear. One possible trigger of the promastigote to amastigote transformation is the drastic temperature elevation that the parasite encounters when it is transferred from the invertebrate host to the mammalian host. In response to such stimulus a number of species including *Leishmania* produce a set of proteins collectively known as heat-shock proteins.

In this work it was demonstrated that *L. aethiopica* is able to undergo a similar change when exposed to 35°-37°C (section 4.4). Similar *in vitro* temperature-induced conversion of the promastigotes of various leishmania species to the round afflagelate extracellular form, resembling amastigotes has been accomplished (*L. mexicana*, Hunter, 1982; *L. braziliensis panamensis* Smejkal *et al.*, 1984). However such transformation is not typical of all species of *Leishmania*.For example, *L. major* (Shapira *et al.*, 1988) and *L. donovani* (Zilberstein, 1991) exhibit a relative unresponsiveness to heat shock. It has been suggested that pH rather than temperature may be important in the triggering of the development of amastigote like characteristic in old world *Leishmania* species (Zilberstein, 1991). Though a detailed molecular characterization regarding the development of amastigote like morphology was not done on *Leishmania aethiopica*, the finding discussed above invites the re-evaluation of the earlier assertion.

In *L. aethiopica*, in addition to the growth-dependent regulation of the extracted kinase, both the extractable kinase and the ectokinase appear to be subject to potent down

regulation following temperature-dependent transformation to an amastigote-like stage (Fig. 4.2). The transformation procedure did not affect the viability of the parasites as judged by dye exclusion and re-transformability (Section 4.4). In a preliminary experiment, transformation by growth at acidic pH also resulted in loss of both kinase activities. A ten hour exposure to pH 4.8 at room temperature reduced the ectokinase activity by about 60%. When this was done at 34°C, the reduction approached 96% (Section 4.4). In both cases the parasite viability was not affected markedly.

The finding of a transformation-dependent down regulation of protein kinases in *Leishmania aethiopica* could indicate that the enzymes are of no vital importance to the amastigote-like form of the parasite. This may suggest that the kinases serve their role in activities unique to the promastigotes in the insect host or immediately after inoculation in the mammalian host. As has been discussed in the preceding paragraphs, the protein kinase activity measurement in *L. aethiopica* during different growth stages, (early log phase, stationary phase and axenic amastigotes) demonstrates that protein kinase activity changes at least quantitatively during growth and transformation. This may be part of the necessary adaptive process that must take place in order to enable the parasite to survive in the vertebrate host. Such growth dependent regulation of the expression of enzymes and other molecules has been shown in a number of lower species including *Leishmania*. For instance developmental expression of PKC in *Schistosoma mansoni* (Wiest *et al.*, 1992) and proteinases in *Leishmania* (Robertson and Coombs, 1992) were reported.

In addition, in work done in other cellular systems elsewhere, a heat-shock protein associated down regulation of protein (Ashburner, 1982) and tubulin synthesis (Shapira *et al.*, 1988) in a manner similar to protein kinase down regulation in *L. aethiopica* has been demonstrated.

Protein kinase activities in all fractions from *L. aethiopica* are subject to marked inhibition by Staurosporine and Formycin ATP (Figs. 4.4, 4.5, 4.6). Staurosporine which is a kinase inhibitor which has some selectivity towards protein kinase C inhibits all fractions with comparable efficiency (Figs. 4.4, 4.5). The effect of staurosporine on other aspects of cellular processes was also studied. At concentration above >50 nM it showed a considerable inhibitory effect (55-71%) on cellular proliferation of the typical promastigotes (Table 4.3). On the other hand these cells were able to incorporate thymidine at a rate that is comparable or slightly lesser than that of the controls. In addition staurosporine produced a marked alteration of morphology and also affected motility (section 4.5.2). Moreover, a preliminary experiment on staurosporine treated promastigotes upon exposure to elevated temperature seems to indicate that they have a decreased transformation and survival ability.

The capability of staurosporine to inhibit the kinase activities of *L. aethiopica* at concentrations similar to those being effective on mammalian kinases suggests that they are similar, at least regarding the ATP-binding site, with which staurosporine interacts (Ruegg and Burgess, 1989). The poor effect of staurosporine on thymidine incorporation, suggest that the kinases are not centrally involved in control of DNA-synthesis. A more profound effect of staurosporine at similar concentrations was observed on cell growth. This indicates thymidine incorporation is not a reliable measure of cell proliferation in staurosporine treated *L. aethiopica* and also suggests that the measured kinase activities may serve some regulatory function regarding proliferation control.

The nucleoside triphosphate Formycin ATP has been suggested to exert part of it's cytocidal effects by inhibition of parasite protein kinases (Berman, 1988). Our results confirm that Formycin ATP is moderately inhibitory to leishmania kinases (Fig. 4.6). However, the effects on thymidine incorporation and cell growth occur at substantially

lower concentrations and are more profound, suggesting that the effect of the drug on parasite growth may not be totally related to the kinase inhibitory action.

Based on the available data one can speculate on the possible functions of the two main kinase activities as follows.

The ectokinase may regulate cellular activity by acting on one or more substrates on the surface of the same leishmania cell or an adjacent one. Examples of similar occurrence have been elucidated for ectokinases in other systems. The high affinity uptake of norepinephrine in synapses is regulated by a calcium-dependent phosphorylation of endogenous proteins of neural surface by ectokinase (Hendly, *et al.*, 1988). Phosphorylation/dephosphorylation events that involve ectoprotein kinases on platelets are also thought to regulate the initial events of platelet activation (Naik, 1990). In the same manner phosphorylation of *Leishmania* surface receptors, integrins, etc, may regulate crucial events in biology of leishmania. The fact that a potent ectokinase activity can be detected in cultures which have undergone extensive passage (>18 months), as we were able to do, indicate that indeed the ectoenzyme may be very essential.

Another way that ectoenzyme may benefit the cell may be by acting on substrates present in a hostile serum on macrophage surface membrane. This may enable the parasite to evade the immune system of the vertebrate host by inactivating some component of the system. Supporting this statement is the fact that a protein kinase of *L. major* origin was able to phosphorylate C3, C5, C9, complement components. In the case of C3, this was found to alter the kinetics of C3 cleavage (Hermoso *et al.*, 1991). But in this study ,the fact that the ectokinase in *L. aethiopica* does not show a growth dependent elevation of activity as the parasite approaches infective stage speaks against it being specific for complement components in the vertebrate host.

In *L. major* and *L. donovani* the development of the infective (Metacyclic)

promastigotes is accompanied by biochemical changes that distinguish it from the log phase promastigotes. These changes include alteration in protein/carbohydrate ratio, flagellar size, membrane exposed carbohydrates and enzymes, etc (Bandyopadhayay *et al.*, 1991). Given the versatility of protein kinases in the regulation of cellular functions at many stages including transcription (Hunter and Karin, 1992), it would be legitimate to assume that the observed rise of protein kinase in *L. aethiopica* may indicate its involvement in this developmental transformation.

It is a known fact that some protein kinases are involved in the regulation of organization of the cytoskeletal net work (Howard *et al.*, 1993). The data from the inhibition studies suggest that staurosporine sensitive protein kinase activity may be involved in the regulation of cytoskeletal function in *Leishmania aethiopica.* This observation in *L. aethiopica* is strengthened by the finding that staurosporine and its analog K252a, suppresses spontaneous and colchicine-induced front-tail polarity and locomotory activity of Walker carcinosarcoma cells. Furthermore it was also able to produce morphological changes in the same cells. ICGP412, a staurosporine derivative with a much higher specificity for protein kinase C and inhibitors preferring nucleotide sensitive kinases (KT5720 and KT 5882) did not produce similar effects (Zimmermann and Keller, 1992). It has been suggested that the rapid increase in cytoskeleton associated actin of neutrophils exposed to staurosporine is due to inhibition of an unknown staurosporine-sensitive enzyme not identical to PKC or one of the nucleotide dependent kinases (Niggli and Keller, 1991). In other cellular systems inhibitors of the staurosporine type were found to cause effects that are both agonistic and antagonistic to phorbol esters. For instance staurosporine induces the association of PKC with the neutrophil membrane and a dendritic shape in keratinocytes (Wolf and Baggiolini, 1988; Sako *et al.*, 1988).

Additional work need to be done in order to establish whether the development of atypical looking parasites in staurosporine treated *L. aethiopica* can be equated to loss of polarity. From the presented data it is difficult to define whether the effects observed are due to ectokinase inhibition or the inhibition of a intracellular kinase activity. However the fact that staurosporine is a cell permeable drug and that ATP was not included in the incubations (section 3.8) seem to point away from the ectokinase. Nevertheless the findings suggest that a protein kinase(s) may be involved in the regulation of cytoskeletal function in *Leishmania*.

In this respect it is believed that similar studies using more specific protein kinase inhibitors may help in determining the role that the different protein kinases of leishmania play in its biology.

Protein kinase C plays an important role in the regulation of cellular growth and differentiation. The presence of protein kinase C like activities in *L. aethiopica* was studied using different approaches. Specific binding of phorbol dibutyrate could not be demonstrated in *L. aethiopica* (Table 4.6). In line with this, the protein kinase activity in the triton soluble fraction could not be activated with phorbol ester or phorbol ester plus physiological activators namely phosphatidylserine and calcium (Fig.4.7). The same was true with a preliminary experiment where a DEAE sepharose purified extract eluted by buffer of differing ionic strength (50mM -1000mM NaCl) (results not shown). In fact phorbol ester addition to incubations caused a decrease of the total phosphorylation (Fig. 4.7). In addition to the above, phorbol dibutyrate did not produce a detectable change on the banding pattern of phosphoproteins as seen by a 10% SDS-PAGE (Fig. 4.8). Most of these findings agree with the earlier observation of Das *et al.* (1986), Berman (1988) and Banerjee and Sarkar (1990) regarding *L. donovani* and *L. mexicana.*

Though phorbol esters have been known to have biological effects notably modulation of terminal differentiation in various cell lines and primary cell cultures (Castagna, 1987), in *Leishmania aethiopica*, phorbol dibutyrate did not produce any change in thymidine incorporation (section 4.6.2), cellular proliferation (Table 4.9) and also on gross cellular morphology.

Taken altogether, the above observations would seem to indicate the absence of biological effect of Phorbol esters, and a phorbol ester receptor in *L. aethiopica.* This together with the data on PKC assay suggest that there might not be a phorbol ester sensitive protein kinase C in *L. aethiopica.* It is worth while to note that of the known PKC subfamilies ζ does not respond to phorbol esters.

However the immune reactivity of anti PKC (clone MC5) monoclonal antibody with the leishmanial proteins would seem to indicate the presence of a protein kinase C-like peptide sequence (Fig. 4.9). This sequence which different from the phorbol ester binding site, is conserved in PKC and is thought to be unique to it. The protein band detected by the antibody shows an approximate molecular weight that is similar to other protein kinases. This suggests the presence of protein kinase C similar to α, β_I or β_{II} type. The failure of the other antibodies which also includes antibodies against α, β and γ to react with leishmania protein could be explained by the fact that they recognize different epitopes. Therefore the presented data points towards the probable presence of a phorbol ester insensitive protein kinase C in *L. aethiopica*. Further work is required to clearly establish the identity of the peptide/protein. This is particularly important since Protein kinase C plays a pivotal role in the biology of cells.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, the findings from this work can be summarized as follows;

1. *Leishmania aethiopica* has at least two major protein kinase activities. The first one is a histone preferring ectokinase and the second a protamine preferring soluble kinase.
2. The two kinases show some difference in subcellular distribution when compared to protein kinases described in other *Leishmania* species.
3. The two kinases are differentially regulated along the growth of the parasite.
4. Both are down regulated in the axenic (temperature transformed) amastigotes.
5. Substantial amount of protein kinase activity can be detected in association with the triton resistant fraction implying a possible association with cytoskeletal components.
6. Both enzymes are susceptible to inhibition of formycine ATP and Staurosporine.
7. Formycine ATP has a cytocidal activity that is not wholly dependent on its protein kinase inhibiting property.
8. Staurosporine produces marked morphological alterations suggesting a possible role of internal protein kinases in cytoskeletal and/or general regulation of proliferation.
9. Classical protein kinase C like activity and phorbol ester binding could not be demonstrated in *L. aethiopica.*
10. *Leishmania aethiopica* has a 90kd protein/peptide recognizable by monoclonal anti-PKC (clone MC5) antibody.

With the above information at hand, this work can also be expanded along the following lines in the future.

1. Identify possible substrates of the ectokinase on the surface of Leishmania and also on the surface macrophages and determine their significance.
2. Look into the possible role of protein kinases in the promastigote-amastigote transformation.
3. Look into the presence and function of protein kinases in amastigotes with special attention to the different pH and temperature that the organism is exposed to.
4. To look into the characteristics of the 90kd protein band detected by the anti-PKC (clone MC5) monoclonal antibody.

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