Structural and Functional Investigations of *Leishmania* Oligosaccharides and the Predominant Surface Glycoprotein, Gp63

by

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We accept this dissertation as conforming
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ABSTRACT

Leishmania express a predominant surface glycoprotein, gp63, having endoprotease activity. Characterization of this activity precludes any discussion regarding its potential function during the different life-stages of the parasite. Using insulin B-chain as the substrate, gp63, isolated from *L. major* and *L. mexicana amazonensis* promastigotes, displayed distinct pH optima of 7.0 and 5.0, respectively. Gp63, from both species of *Leishmania* was capable of digesting large proteins at pH 5.0 provided the proteins had been denatured. Gp63 was found to have a broad substrate specificity, cleaving primarily on the amino side of hydrophobic and polar residues with no other apparent structural requirement. The Km of *L. major* gp63 was 63 μM when a 13 amino acid peptide was used as the substrate.

Further investigation of gp63 was concerned with the functional significance of its N-linked oligosaccharides. *Leishmania major* promastigotes, when grown in the presence of tunicamycin (TM), produced a plasma membrane bound, proteolytically active gp63 with a lower mol. wt. than the native glycoprotein. However, this lower mol. wt. form of gp63 continued to be recognized by concanavalin A (Con A), suggesting that inhibition of N-linked glycosylation was not complete. Metabolic labeling of gp63, using ^{35}S-methionine, demonstrated that in the range of 5-10 μg ml^{-1} TM, only the lower mol. wt. form was synthesized, suggesting that inhibition was complete and that lectin binding was likely due to the GPI anchored sugars. Removal of the oligosaccharides from *L. major* and *L. mexicana amazonensis* promastigotes using endo-β-N-acetylglucosamidase F, caused gp63 to shift to a lower molecular weight, but again did not affect the proteolytic activity. However, this deglycosylated enzyme continued to bind Con A until subsequently treated with periodate. The latter oxidation reaction resulted in complete loss of Con A binding without inhibiting the protease activity or the substrate specificity of gp63. Removal of the oligosaccharides also did not affect the susceptibility of gp63 to proteolytic digestion by either autolysis or cathepsin D. These findings indicate that the N-linked oligosaccharides of gp63 are not essential for folding, transport, maintenance of enzyme activity or resistance to proteolysis.

There was, however, evidence suggesting that the N-linked oligosaccharides of *Leishmania* act as virulence factors. This possibility lead to the structural characterization of the *Leishmania* N-linked oligosaccharides. Treatment of *L. major* gp63 with anhydrous hydrazine resulted in the release of 2 glycans with hydrodynamic volumes of 10.5 and 9.6 glucose units. However, the hydrazinolysis procedure also caused side reactions to occur at the reducing terminus of G9.6 which resulted in a by-product that co-eluted with G10.5 and interfered with data interpretation. To avoid this problem, the oligosaccharides were removed enzymatically using either endo-β-N-acetylglucosaminidase H or N-Glycanase F. These enzymes also released two N-linked oligosaccharides and sequential exoglycosidase digestion, fragmentation by acetolysis and methylation analysis revealed the complete structures as $GlcMan_6GlcNAc_2$ and $Man_6GlcNAc_2$. These two oligosaccharides were the predominant glycans on *L. major* promastigote gp63 regardless of cell culture stage. These oligosaccharides were also the predominant structures isolated from promastigotes of 6 different *Leishmania spp.* and strains. Analysis of oligosaccharides from amastigotes and promastigotes of *L. m. mexicana* and *L. donovani* LV9 indicated that changes in N-linked oligosaccharide structure, as a function of promastigote to amastigote transformation, may occur in a species specific manner.

The above investigations lead to the finding that *Leishmania mexicana mexicana* promastigotes and amastigotes contained large amounts of an unusual β1-2 linked mannose polymer (mannan) that is either unbranched or branched at a very low frequency. The mannan, which is markedly different from any host homopolysaccharide, varies in length from a minimum of 3 to 23 or more residues and is not covalently linked to protein. The β1-2 linked mannans were also abundant in *L. donovani* LV9, *L. donovani* LD3, *L. mexicana amazonensis*, *L. tropica* and *L. major* Neals, but were present at low levels in *L. donovani* NLB-065, *L. major* A2 and were absent in *T. b. rhodesiense*. The intracellular mannan pool of *L. m. mexicana* promastigotes was depleted by 50% following 6 h of glucose starvation, indicating that the mannans likely serve as energy storage molecules. Additional experimentation revealed that the mannans are cleaved by a mannan phosphorylase to produce mannose 1-phosphate which is further processed by

phosphomannomutase and phosphomannose isomerase to yield mannose 6-phosphate and fructose 6-phosphate, respectively. *In vitro* incubation of cytoplasmic supernatant with purified mannans results in the accumulation of glucose 6-phosphate. The possible significance of the mannans for parasite survival is discussed.

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GENERAL INTRODUCTION

The leishmaniases are a group of diseases that are caused by protozoan parasites belonging to the genus *Leishmania* (Protozoa: Trypanosomatidae). The latter are digenetic parasites having a flagellated promastigote stage that multiplies in the gut of a phlebotomid sandfly (Diptera: Phlebotominae) and a non-flagellated amastigote stage that multiplies within the phagolysosome of mammalian macrophages (Killick-Kendrick et al., 1974; Alexander and Vickerman, 1975; Chang and Dwyer, 1976, 1978). The promastigote is transmitted to a mammalian host when an infected sandfly takes a blood meal. Promastigotes are phagocytosed by macrophages and transformation to amastigotes as well as subsequent multiplication of amastigotes occurs within the phagolysosome (Chang and Dwyer, 1976, 1978). The disease caused by the various species of *Leishmania* results from the apparent differences in the tissue tropism of each species. For example, the clinical forms of the disease have been categorized as cutaneous (CL), diffuse cutaneous (DCL), mucocutaneous (MCL) and visceral leishmaniasis (VL). CL and DCL arise when amastigotes multiply within cutaneous macrophages, multiplication within macrophages of the oronasal pharynx results in MCL and VL results from parasitization of the macrophages within the reticuloendothelial system (Zuckerman and Lainson, 1977).

Leishmania species have been crudely divided into New World (western hemisphere) and Old World species (eastern hemisphere). Speciation was originally based on the clinical form of the disease but has since been supported by biological, geographical and epidemiological information. *Leishmania* of the western hemisphere, or New World, causing cutaneous diseases, are believed to be distinct from those in the Old World as the parasites were wide spread among wild animals in remote uninhabited areas of Brazil at an early date - a fact which precluded any possibility of man introducing Old World species to these areas (Zuckerman and Lainson, 1977). However, *L. donovani*, the causative agent of VL, is believed to have been introduced to the Americas from the Old World (Zuckerman and Lainson, 1977), a belief which is supported by studies comparing gene sequences among Old and New World species (Webb et al., 1991).

Disease Manifestations

CL of the Old World is cause by *L. major, L. tropica, L. aethiopica* and occasionally by *L. donovani*. The classical form of this disease starts as a nodule at the site of inoculation which later ulcerates. The incubation period is highly variable, ranging from one month to three years or longer but the lesions are usually self healing. *L. aethiopica* can also metastasize to give rise to DCL where multiple nodules occur on the face and limbs. This form of the disease does not heal spontaneously and tends to relapse after treatment (WHO, 1984; Marsden and Jones, 1985). CL of the New World (NWCL) is clinically similar to the disease of the Old World, but lesions are typically more severe, chronic and respond poorly to treatment. NWCL is caused by *L. peruviana* and by various subspecies within *L. mexicana* and *L. braziliensis*. *L. m. amazonensis* is most commonly associated with DCL in the New World but *L. m. pifanoi* and *L. b. guyanensis* are also known to cause this disease (WHO, 1984).

MCL occurs from the metastatic spread of parasites causing cutaneous lesions to the oronasal/pharyngeal mucosa. Ulceration and erosion progressively destroys the soft tissue and cartilage of this area resulting in severe suffering and mutilation. The lesions do not heal spontaneously and death often occurs as a result of malnutrition or secondary infection resulting in bronchopneumonia. In the Old World, *L. donovani* and *L. aethiopica* have occasionally been associated with this disease. MCL of the New World occurs from infection with subspecies of *L. braziliensis*. Onset of the mucosal disease following infection can vary from a few months to 30 years (Marsden and Jones, 1985).

VL, also known as kala azar, is caused by *L. donovani* in both the Old and New Worlds. Common symptoms include fever, malaise, weight loss, anorexia and discomfort in the left hypochondrium. The term kala azar, which means black sickness, originated in India where the disease causes the skin of the face, hands, feet and abdomen to darken. The common clinical signs of VL are marked non-tender splenomegaly, moderate hepatomegaly, and lymphodenopathy, as well as pallor of the mucous membranes and edema which conceals the extent of wasting. This disease is fatal if left untreated, and death is often the result of secondary infections such as pneumonia, dysentery, or

pulmonary tuberculosis. Development of the disease can begin ten days to two years after exposure (WHO, 1984; Marsden and Jones, 1985).

Treatment of the Leishmaniases

All forms of the leishmaniases are most commonly treated with pentavalent antimony in the form of either sodium stibogluconate (Pentostam) or N-methyl glucantime antimonate (Glucantime) (Berman, 1985). The mechanism leading to parasite clearance is not known but it is believed that formation of trivalent antimony is responsible for killing by inhibiting sulfhydryl glycolytic enzymes (Gutteridge and Coombs, 1977). The pentavalent form of antimony is administered because it is rapidly excreted in the urine compared to the slow excretion of the trivalent form and therefore, accumulation in the tissues is minimal. Selective killing of parasites over host cells is most likely due to the greater metabolic rate of parasites compared to mammalian cells as well as their to greater reliance on glycolysis for energy (Blum, 1993). Antimony is generally administered systemically, by either intravenous or intramuscular injection, and injections must occur daily for a minimum of 20 days. Simple CL can be treated with intralesional perfusion, provided detection is early and the lesions are not inflamed or nodular (WHO, 1984). All other types of lesions should be treated systemically (WHO, 1984). Common side effects of antimony treatment include anorexia, vomiting, nausea, malaise, myalgia, headache and lethargy (WHO, 1984). It should be noted that although antimony treatment has been quite successful in treating this spectrum of diseases, particularly the epidemics of kala azar, disease relapse or unresponsiveness to treatment occurs in all forms of leishmaniasis and represent 10-25% of all cases (c.f. Berman, 1985).

Patients who relapse after the first antimony treatment are generally given a second treatment that is extended to twice the length of the original treatment before trying alternative/second line drugs. Patients who are unresponsive to antimony and those who relapse after a second treatment of antimony, are treated with either Pentamidine or amphotericin B. Pentamidine is believed to exert its cytotoxic effects by inhibiting the polymerization of DNA and RNA by binding to DNA (Gutteridge and Coombs, 1977). The selectivity of this drug towards *Leishmania* results from the preferential uptake of the

drug by parasites compared to mammalian cells (Gutteridge and Coombs, 1977). Treatment using Pentamidine, like that of antimony, is very lengthy, being administered 3 times a week for 5-25 weeks or longer, depending on the response (WHO, 1984). Side effects caused by this drug are generally more severe than those caused by pentavalent antimony and include local pain, induration, nausea, vomiting, abdominal pain, hypotension, syncope, hypoglycemia and diabetes mellitus (WHO, 1984).

Amphotericin B is an antifungal agent and by analogy, it disrupts leishmanial membranes by binding membrane sterols making the membranes leaky to cations (Berman 1985; Gutteridge and Coombs, 1977). The treatment regime for Amphotericin B involves daily administration by intravenous infusion which takes several hours. Treatment continues for a minimum of 20 days depending on the response (WHO, 1984). Side effects of amphotericin B treatment, like Pentamidine, are more severe than treatment with pentavalent antimonials. Most patients suffer from anorexia, nausea, vomiting, local thrombophlebitis, fever, chills, elevation of blood urea and creatinine and anaemia.

An ideal chemotherapeutic agent should be administered orally, it should be effective, non-toxic and inexpensive. At the very least, the agent should be reasonably effective and the side effects caused by the drug should be less severe than the disease. Pentavalent antimony treatment meets the minimum requirements for a chemotherapeutic agent but the length of the treatment and the need for medical facilities make treatment expensive and geographically restrictive so that people in rural areas, which are often the people at most risk of contracting the disease, have difficulty receiving proper treatment. Second-line drugs are even less ideal than antimony because, in addition to the disadvantages of antimony treatment, Pentamidine and amphotericin B treatment cause side effects that are often worse than the disease. New chemotherapeutic agents are clearly needed.

Epidemiology

The leishmaniases are endemic in the tropical regions of America, Africa, and the Indian sub-continent, and in the subtropics of south-west Asia and the Mediterranean (Modabber, 1993). Endemic areas must have both vectors and reservoir hosts as

leishmaniasis in man is usually a zoonosis, i.e., transmission to man only occurs from a mammal so that transmission directly from man to man (anthroponosis) typically does not occur (WHO, 1984). There are, however, some exceptions. *L. donovani* causing Indian and Kenyan kala azar is often anthroponotic, and anthroponotic cutaneous leishmaniasis has been reported but is extremely rare (Zuckerman and Laison, 1977). A reservoir host can be any mammal, domestic or wild, but must be sufficiently abundant and long-lived to provide a significant food source for sandflies. A significant portion of the reservoir population must become infected and the infection must be long in duration and relatively non-pathogenic. The disease in the reservoir host must be characterized by parasites in the skin or blood so that infection of a sandfly during a blood meal can occur. Finally, the reservoir host and man are usually in close proximity so that transmission from the former to the latter is probable (WHO, 1984).

The importance of the leishmaniases as a public health problem is considerable in several countries. The total number of infected people world wide is estimated to be 12 million with approximately 3 million showing clinical symptoms (Modabber, 1993). VL or kala azar is the most significant form of this group of diseases from a public health perspective. Fatality rates of patients who are diagnosed and treated are between 5-10% and those for individuals who are not treated are 100%. Estimated deaths in 1991 due to VL was 75 000 (Modabber, 1993). CL of the old world are not considered an important health problem as the lesions are generally self healing. However, CL of the New and Old Worlds which have been shown to metastasize, giving rise to DCL and MCL, are of considerable importance due to the mutilating effects resulting in social and psychological trauma. Also, such diseases are often resistant to treatment and death resulting from secondary infection is common (WHO, 1984; Modabber, 1993).

Biology of *Leishmania*

Little is known of factors effecting virulence of the various *Leishmania spp.* For example, it is not known what governs the tissue tropisms which lead to the different forms of the disease, or why only some species (*L. aethiopica, L. braziliensis, L. mexicana amazonensis*) are capable of metastasizing giving rise to DCL and MCL.

However, progress is being made in understanding parasite development and virulence. *Leishmania* amastigotes taken up by a sandfly during a blood meal transform into non-infective promastigotes (nectomonads). This transformation occurs within 24 h of the blood meal (Davies et al., 1990). Most *Leishmania spp.* develop and multiply in the midgut of the sandfly within the chitinous peritrophic membrane that is secreted around the gut contents. The midgut of a sandfly contains many hydrolytic enzymes which are required for digestion of the blood meal and are capable of killing the parasites (reviewed by Schlein, 1993). It is important to note that *Leishmania spp.* are vector-specific so that a single species of *Leishmania* can only be transmitted by one species of sandfly. This specificity may be partly due to the ability of *Leishmania spp.* to reduce secretion of digestive enzymes in their specific vectors (Schlein, 1986; Schlein, 1993). However, other species of *Leishmania* (i.e. the *L. braziliensis* group) avoid digestive enzymes by migrating rapidly to the hind gut, which is low in protease activity, and undergo development and multiplication in this area (Schlein, 1986).

Promastigotes multiplying within the midgut, migrate to the anterior portion of the peritrophic cavity. It is in this area that haemoglobin from the blood meal first disappears (reviewed by Schlein, 1993), probably due to digestion by the promastigote surface metalloenzyme, gp63 which is expressed at this stage (Davies et al., 1990). Disappearance of haemoglobin is correlated to and required for the secretion of chitinase and N-acetylglucosaminidase by promastigotes (Schlein et al., 1991, 1992). These enzymes are responsible for degradation of the peritrophic membrane allowing the forward migration of parasites to the cardiac valve (Schlein et al., 1991, 1992). However, the proliferating promastigotes remain avirulent and must undergo a process known as metacyclogenesis (for review see Sacks, 1989) before migrating to the mouth parts of the insect vector as virulent parasites. Research using *L. major* promastigotes has shown that avirulent or log-phase promastigotes remain attached to the microvilli of the sandfly midgut and only virulent or metacyclic promastigotes migrate forward (Sacks and Perkins, 1984; Pimenta et al., 1992). The mechanism responsible for the differential adhesion of *L. major* promastigotes involves the stage-specific expression of the surface glycolipid, lipophosphoglycan (LPG) (Pimenta et al., 1992).

LPG is a predominant promastigote surface molecule (Handman et al., 1984; Turco et al., 1984) present at a density of approximately 1.25 - 6 x 10^6 copies per cell (Orlandi and Turco, 1987; McConville et al., 1991). It is a tripartite molecule composed of a lysoalkyl phospholipid that functions as a membrane anchor for the molecule (Orlandi and Turco, 1987; McConville et al., 1987), a carbohydrate core and a repeat structure. The repeat structure is composed of PO_4-6Galβ1-4Manα1- units that carry species and stage specific branches linked β1-3 to the galactose residue of the repeat unit (Turco et al., 1987; Turco, 1990; McConville et al., 1990; Ilg et al., 19[illegible]). LPG expressed by avirulent *L. major* promastigotes has side branches terminating with galactose residues (McConville et al., 1990; Sacks et al., 1990; Turco, 1990). The terminal galactose residues appear to mediate binding of promastigotes to the microvilli of the midgut epithelium (Pimenta et al., 1992). This specific lectin-ligand interaction may also contribute to the vector specificity of *Leishmania* promastigotes. As promastigotes undergo metacyclogenesis, the structure of LPG changes such that the side chains end predominantly with arabinose or glucose (McConville et al., 1990; Turco, 1990). As well, metacyclic LPG is approximately twice as long as the log phase form, forming a glycocalix (Sacks and da Silva, 1987; McConville et al., 1990; Sacks et al., 1990; Pimenta et al., 1991). The LPG expressed by metacyclic promastigotes, lacks the terminal galactose residues, do not bind to the midgut epithelium and are therefore, free to migrate forward to the cardiac valve as virulent parasites (Pimenta et al., 1992).

The next stage of the parasite lifecycle requires transmission to a vertebrate host. This occurs during a blood meal of an infected sandfly. Parasite movement out of the proboscis, against the flow of blood, is believed to occur as a result of a damaged cardiac valve (Schlein et al., 1991, 1992). The chitinolytic enzymes secreted by the promastigotes degrade the valve allowing movement of parasites into the mouth parts of the sandfly during suctioning and then into the mammalian host (Schlein et al., 1991, 1992).

Promastigote virulence depends on the parasites' ability to avoid being killed by the host's defences. The first line of defence the parasite has to overcome, is the lytic complement system. Virulent metacyclic promastigotes have increased resistance to

complement killing relative to log-phase promastigotes (Franke et al., 1985; Howard et al., 1987; Sacks and da Silva, 1987; Puentes et al., 1988, 1990; Bandyopadhyay et al., 1991). Puentes et al. (1990) reported that complement resistance in *L. major* was due to a spontaneous shedding of the C5b-9 complex. However, other workers have proposed that resistance to complement is due to the elongated metacyclic form of LPG acting as a physical barrier that prevents insertion of the attack complex (C5b-9) into the plasma membrane of the promastigote (McConville et al., 1990; Sacks et al., 1990).

Parasite survival next rests on successful entry into macrophages. There has been much work targeted towards identifying the macrophage receptor(s) and parasite ligand(s) responsible for attachment and internalization of *Leishmania* parasites, but no consensus exists. Parasite-macrophage binding studies have been carried out in the presence and absence of serum - presumably to investigate complement-dependent and -independent binding mechanisms. However, because macrophages secrete complement components which lead to the opsonization and binding of particles to macrophages via the type 3 complement receptor (CR3) (Ezekowitz et al., 1983), the presence or absence of serum does not allow any conclusions regarding the nature of such interactions. Investigations of parasite-macrophage binding have indicated that both the mannosyl-fucosyl receptor (MFR) and CR3 can mediate binding and phagocytosis of promastigotes (Blackwell, 1985; Blackwell et al., 1985; Mosser and Edelson, 1985; Wilson and Pearson, 1986, 1988). These studies assessed the importance of specific receptors by plating macrophages on coverslips coated with ligands causing specific receptor modulation and by monitoring parasite binding in the presence of soluble ligands or monoclonal antibodies against ligands or receptors. In some cases, binding to CR3 was shown to be dependent on the fixation of macrophage-derived C3 (Yamamoto et al., 1984; Blackwell et al., 1985; Wozencraft et al., 1986). Other researchers found that type 3 complement receptor (CR3) binding occurred in a complement-independent manner (Russell and Wilhelm, 1986; Russell and Wright, 1988). Russell and Wright (1988) and Russell et al. (1989) reported that gp63 was responsible for binding directly to the amino acid sequence, RGDS, which is the recognition site for iC3b (Wright et al., 1987). Soteriadou et al. (1992) later found that while this observation was essentially correct, the

actual gp63 ligand sequence responsible for the observed cross-reactivity with iC3b (Wright et al., 1989) and for binding CR3 was SYRD. Contrary to these results, da Silva et al. (1989) reported that promastigote binding to CR3 occurred at a site distinct from the iC3b binding site. CR3 has an additional binding site that behaves as a lectin (Ross et al., 1985) and has been shown to bind bacterial LPS (Wright et al., 1989). The leishmanial equivalent of LPS is LPG and Talamas-Rohana et al. (1990) reported that promastigote LPG mediated binding of promastigotes to macrophage via the lectin, or LPS binding site of CR3. Other macrophage receptors reported as being important in promastigote binding are the advanced glycosylation endproduct receptor (Mosser et al., 1987) and CR1 (da Silva et al., 1989). To add to the confusion, both gp63 (Chang and Chang, 1986; Russell and Wilhelm, 1986; Russell and Wright, 1988; Wilson and Hardin, 1988; Russell et al., 1989; Soteriadou et al., 1992) and LPG (Handman and Goding, 1985; Russell and Wright, 1988; Talamas-Rohana et al., 1990; Kelleher et al., 1992) have been reported as binding directly to macrophages, but they have also been reported as major acceptors of complement (C3) and as such, mediators of complement-dependent binding (Russell, 1987; Puentes et al., 1988).

Such varied findings may be a result of different strategies used by *Leishmania* isolates to gain entry into macrophages. However, they could also be due to the use of undefined promastigote cultures in terms of their life cycle stage. Membrane modulation experiments have shown that metacyclic *L. major* promastigotes enter macrophages through a pathway distinct from log-phase promastigotes and that binding and entry was complement-dependent and occurred through the type 1 complement receptor (CR1) (da Silva et al., 1989). These results are consistent with the findings that C3b is the major form of C3 deposited on the surface of promastigotes (Russell, 1987; Puentes et al., 1988). Wozencraft and Blackwell (1987) showed that complement fixation and macrophage binding increased with promastigote culture age, but that binding was via CR3. Since complement-dependent binding to either CR1 or CR3 does not elicit a respiratory burst (Wright and Silverstein, 1983; Yamamoto et al., 1984; Ross et al., 1985), increased complement-dependent binding of metacyclic promastigotes would explain the lower RB activity elicited by opsonized promastigotes (Mosser and Edelson,

1987) and may account for the higher survival rate and increased binding of metacyclic promastigotes (Mosser and Edelson, 1984, 1987; Wozencraft and Blackwell, 1987; da Silva et al., 1989).

Another indirect line of evidence suggesting that log-phase promastigotes and metacyclic promastigotes utilize distinct modes of entry into macrophages, was the finding that the LPG repeat structure of *L. major* log-phase promastigotes could inhibit uptake of promastigotes by macrophages (Kelleher et al., 1992). Because this structure is found only on log-phase promastigotes, it should only inhibit binding of log-phase promastigotes. These results suggest that metacyclic promastigotes bind via a distinct ligand. In addition, metacyclic promastigotes and amastigotes express a heparin receptor which appears to enhance parasite-macrophage interactions by binding cell surface heparan sulfate proteoglycans (Butcher et al., 1992; Love et al., 1993).

Amastigotes also appear to differ in their method of macrophage entry from metacyclic promastigotes, but the receptor(s) and ligand(s) involved in binding distinctly to the amastigote stage remain uncharacterized (Channon et al., 1984; Blackwell, 1985; Blackwell et al., 1985; Wozencraft and Blackwell, 1987; Guy et al., 1993).

Once inside the macrophage, the parasite must avoid the killing mechanisms - most importantly, the respiratory burst. Although amastigotes are comparatively more resistant to toxic oxygen metabolites than promastigotes, both forms are killed by H_2O_2 alone (Murray, 1981, 1982). Parasite survival rate, as mentioned previously, is at least partially dependent on the receptor utilized for entry as it is the receptor that activate a respiratory burst (Berton and Gordon, 1983). The MFR and the lectin binding site of CR3 trigger respiratory burst activity while complement-dependent binding to either CR3 or CR1 does not (Wright and Silverstein, 1983; Yamamoto et al., 1984; Ross et al., 1985; Mosser and Edelson, 1987).

Leishmania LPG is also important for parasite survival within the macrophage phagolysosome (Handman et al., 1986; Elhay et al., 1990; McNeely and Turco, 1990; Shankar et al., 1993). LPG is capable of scavenging the toxic oxygen metabolites, hydroxyl radical and superoxide anion (Chan et al., 1989) but because H_2O_2 alone is capable of killing *Leishmania* (Murray, 1981, 1982) it is unlikely that LPG functions in

this manner to allow parasite survival. Indeed, Murray (1982) reported that scavengers of hydroxyl radicals and superoxide anion did not prevent parasite killing by macrophages. It is more likely that LPG prevents parasite killing by inhibiting respiratory burst activity (McNeely and Turco, 1990). LPG inhibits protein kinase C activity (McNeely et al., 1987, 1989; Frankenburg et al., 1990; Descoteaux et al., 1991, 1992) which is required for activation of the respiratory burst (Wilson et al., 1986).

Murine macrophages have also been shown to kill intracellular *Leishmania* by production of nitric oxide from L-arginine (Green et al., 1990a; Liew et al., 1009a). Macrophages that have been activated by IFNγ and LPS, IFNγ and amastigotes (Green et al., 1990b) or IFNγ and IL-2 (Belosevic et al., 1990) produce TNFα which triggers production of nitric oxide (Belosevic et al., 1990; Green et al., 1990b; Liew et al., 1990b; Roach et al., 1991).

The present work was undertaken to further our understanding of *Leishmania* virulence factors. The protease activity of the predominant promastigote surface glycoprotein, gp63, was investigated in an attempt to determine its role in parasite survival and virulence. Second, since there is not yet information regarding tissue tropisms of different *Leishmania spp.*, the N-linked oligosaccharides at different lifecycle stages were structurally characterized and compared with the objective of finding a correlation between structure and virulence or disease manifestation. Last, the intermediary metabolism of a unique mannose energy storage molecule, discovered during the previous studies, was elucidated.

CHAPTER 1

Characterization of *L. major* and *L. mexicana amazonensis* gp63 enzyme activity

INTRODUCTION

The surface membrane of parasitic organisms is important in understanding parasite survival and virulence as it represents the functional interface between the parasite and host. Surface molecules are important at many levels of a parasitic infection as they are involved both in establishment and maintenance of infection. They may induce the host's immune response leading to the demise of the parasite or they may provide an immune evasion mechanism via antigenic variation.

One of the *Leishmania* promastigote membrane molecules, a 63 kDa glycoprotein, is the dominant protein structure on the cell surface (Fong & Chang, 1982; Bouvier et al., 1985; Colomer-Gould et al., 1985; Etges et al., 1985; Chang et al., 1986; Bouvier et al., 1987). Gp63 is expressed on the surface of the promastigote stage of all *Leishmania spp.* (Bouvier et al., 1987) as well as on the surface of the closely related monogenetic parasites *Crithidia fasciculata* and *Herpetomonas samuelpessoai* (Etges, 1992; Schneider and Glaser, 1993). In all cases, the protein is anchored to the membrane via a glycosyl-phosphatidyl inositol (GPI) lipid anchor (Etges et al., 1986b, Schneider and Glaser, 1993). The amount of gp63 present on the surface of *Leishmania* is highly variable among different strains (Bouvier et al., 1987) but can reach levels of 500,000 copies per cell - representing 1% of total cellular protein (Bouvier et al., 1985). It has been proposed that the GPI anchor allows gp63 to attain a high copy number without disrupting the lipid bilayer (Etges et al., 1986b).

The presence of gp63 on the surface of *Leishmania* amastigotes varies among species and strains and investigations from different laboratories have resulted in contradictory findings. For example, Frommel et al. (1990) and Pimenta et al. (1991) reported that gp63 was present on the surface of *L. major* amastigotes while other workers have reported results to the contrary (Chang et al., 1986; Davies et al., 1990; Schneider et al., 1992). *L. mexicana* amastigotes do express gp63 and although some may be present as an integral membrane protein, located primarily in the flagellar pocket (Medina-Acosta et al., 1989), it is accepted that most of the gp63 is secreted as an aqueous soluble form to the large lysosomal vacuoles (megasomes) that are characteristic

of this species (Bahr et al., 1993; Ilg et al., 1993; reviewed in Medina-Acosta et al., 1993b).

Gp63 is encoded by a varying number of tandemly linked isogenes (Button et al., 1989b; Murray et al., 1990) and some species also have copies at sites removed from the tandem array, as has been shown for *L. donovani* (Webb et al., 1991). The number of tandemly repeated gp63 isogenes can vary widely among strains of a single *Leishmania spp.* and does not correlate with the level of gp63 expression (Murray et al., 1990). *L. mexicana* contains ten gp63 genes in tandem array (Medina-Acosta et al., 1993a), *L. major* has 5, 6 or 8 repeats depending on the strain (Button et al., 1989b; Murray et al., 1990) while *L. donovani* LV9 was found to contain 7 genes in a tandem array with an additional 3 genes removed from the array (Webb et al., 1991). *L. donovani* LV9 has 2 distinct gp63 gene classes, both of which are transcribed throughout the promastigote life-stage (Webb et al., 1991). *L. mexicana* and *L. d. chagasi* encode three distinct classes of gp63 genes which are differentially transcribed as a function of lifecycle stage (Ramamoorthy et al., 1992; Medina-Acosta et al., 1993a). *L. d. chagasi* log- and stationary-phase promastigotes transcribe distinct gp63 gene classes as recognized by Northern blot analysis using probes specific for their 3'-untranslated regions. The third gene class appears to lack the GPI signal sequence and is constitutively expressed in the promastigote life-stage (Ramamoorthy et al., 1992). Expression of gp63 in the amastigote was not investigated. Expression of gp63 gene classes in *L. mexicana* was also found to be developmentally regulated (Medina-Acosta et al., 1993a). *L. mexicana* promastigotes and amastigotes contained the same levels of gp63 mRNA but the mRNAs differed qualitatively. Amastigotes only transcribed the class of genes having the unique 3' sequence which could give rise to an aqueous soluble or an integral membrane form of gp63 (Medina-Acosta et al., 1993a; reviewed by Medina-Acosta et al., 1993b). Expression of this gp63 gene class in the amastigote stage may explain the presence of an aqueous soluble gp63 in the megasomes. The mechanism responsible for the differential expression of the tandemly arrayed gp63 genes is unknown but evidence suggests that mRNA levels are controlled at the level of mRNA processing and degradation (Muhich et al., 1988; Ullu et al., 1993).

Evolution of tandemly arrayed genes appears to be common in trypanosomatids (Thomashow et al., 1983; Tschudi et al., 1985). Such gene organizations could have arisen by one of two possibilities. In the case of gp63, the *Leishmania* ancestor could have possessed a tandem array of genes which diverged during speciation, but remained homologous within a species through a process of gene homogenization, such as gene conversion (Button et al., 1989b; Webb et al., 1991). Alternatively, a single ancestral gene may have been present during speciation, diverged, and then multiplied by parallel gene amplification and/or uneven cross over during mitosis (Button et al., 1989b; Murray et al., 1990; Webb et al., 1991). The latter theory best accounts for the presence of distinct gp63 gene classes in *Leishmania* species (Webb et al., 1991).

Gp63 is a zinc endoproteinase (Etges et al., 1986a; Chaudhuri et al., 1988; Bouvier et al., 1989) with a broad substrate specificity (Bouvier et al., 1990). The pH optimum of this enzyme remains in dispute. Chaudhuri et al. (1989) reported that gp63, isolated from *L. mexicana amazonensis*, displayed maximal activity at pH 4.0 while Bouvier et al. (1989) and Ip et al. (1990), using gp63 from *L. major* and *L. mexicana amazonensis*, respectively, reported a pH optimum of 7.0 - 8.0. Results of Tzinia and Soteriadou (1991) indicated that gp63 isolated from 7 strains of *Leishmania* all behaved similarly, but that the pH optimum varied from 6.0 - 8.5 depending on the substrate. The possibility of an acidic pH optimum was generally refuted when sequencing data (Button et al., 1988, 1989a; Miller et al., 1990; Webb et al., 1991; Steinkraus and Langer, 1992) showed similarity with zinc metalloproteases (Kester and Mathews, 1977a, 1977b; Bertini et al., 1985) and by inference, therefore, the same reaction mechanism. The proposed mechanism of hydrolysis requires that the H_2O molecule occupying the fourth position of the zinc atom attacks the carbonyl of the scissile peptide bond. In order for this attack to occur, the H_2O molecule requires zinc and a deprotonated glutamate residue. The requirement of a deprotonated glutamate residue means that the pH of the reaction must exceed the pK_a of glutamic acid (pK_a=4.0-4.25) by at least one pH unit for hydrolysis to occur at a reasonable rate (Bouvier et al., 1989).

The function of the endoproteinase activity of gp63 remains unknown but its presence in all New and Old World *Leishmania spp.* (Bouvier et al., 1987; Murray et al.,

1990) suggests that it is important for parasite survival. The question of how and when it is important remains unresolved. Although gp63 has been proposed to be an important virulence factor (Kweider et al., 1987; Wilson et al., 1989, 1990), the presence of gp63 in the monogenetic parasites, *Crithidia* and *Herpetomonas*, as well as its apparent pH optimum of $\geq$ 6.0, would suggest that it is important some time prior to macrophage entry as the pH of *Leishmania* infected phagolysosomes is approximately 5.0 (Antoine et al., 1990). However, the fact that the gut of the sandfly is likely acidic following a blood meal, suggests that gp63 would have to function at a lower than optimal pH in this environment as well. This work was undertaken to clarify the contradictory data regarding the pH optimum of gp63 as well as to elucidate kinetic data and substrate specificity as part of an investigation into the role of this protease.

MATERIALS AND METHODS

Parasite cultivation

Leishmania major A2 (Neva et al., 1979) and *Leishmania mexicana amazonensis* M2269 promastigotes were cultured at 26°C in M199 medium supplemented with hemin (5 mg l^{-1}) and folic acid (23 μM). Penicillin/streptomycin (Sigma Chemical Company, St. Louis, MO) and Basal Eagle Medium vitamin solution (Gibco, Burlington, Ontario) were added to the medium at a dilution of 1:100 (v/v) and fetal bovine serum (FBS) was added to a final concentration of 5%. Parasites were grown to late log phase and harvested by centrifugation.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were electrophoresed according to the method of Laemmli (1970), using a 12.5% running gel and a 5% stacking gel. Samples were boiled in a equal volume of Laemmli 2X sample buffer for 2.0 min prior to gel loading. The gels were stained with Coomassie brilliant blue to visualize the protein bands.

Purification of gp63

Cells were harvested by centrifugation and washed 4 times with phosphate buffered saline (PBS) before lysis by freeze/thaw in 50 mM Tris, pH 7.2. The membranes were washed 4 times with lysis buffer and resuspended in 0.5 ml 25 mM Hepes, pH 7.2 containing 2.0 mM EDTA and 0.1% CHAPS. Phosphatidylinositol specific phospholipase-C (PI-PLC) (EC 3.1.4.10) from *Bacillus cereus* (Boehringer Mannheim) was added to a concentration of 100 mU ml^{-1} and digestion proceeded overnight at 37°C with stirring. Toluene (10 μl) was added to prevent bacterial growth. The delipidated gp63 was purified to homogeneity from supernatant by gel permeation HPLC using a Beckman ultraspherogel SEC 3000 column (dp 5 μm, 7.5 mm x 30 cm). Proteins were eluted with 100 mM Tris, pH 7.5, containing 150 mM NaCl at a flow rate of 0.5 ml min^{-1}. Gp63 was identified by the presence of protease activity and SDS-PAGE analysis. The protease activity was assayed using insulin as a substrate as described in the following section.

Insulin assay for gp63 protease activity

Aliquots (10 µl) of peaks eluting from the SEC 3000 column were incubated for 30 min at 37°C in 100 µl of 100mM Tris HCl, pH 7.0 buffer containing 60 µM B-insulin. The reaction was stopped by addition of 5 µl anhydrous trifluoroacetic acid (TFA). Twenty µl aliquots were run on a C_8 reverse-phase HPLC column (Aquapore RP-300, 100 x 22.1 mm, Applied Biosystems Inc.) using a 0.1% TFA/acetonitrile (ACN) step gradient at a flow rate of 1.0 ml min^{-1}. The column was equilibrated with 0.1% TFA and the apolar gradient was applied as follows: acetonitrile was ramped from 0 to 30% in 5 minutes, followed by an increase to 44% in 8 min and a final ramp to 100% in 1 min. Elution of intact B-insulin occurred at 13 min and was monitored at 230 nm.

Comparison of the specific activity of gp63 isolated from *L. major* and *L. mexicana amazonensis* promastigotes at pH 5.0, 7.0 and 8.0

The concentration of purified gp63 from *L. major* and *L. m. amazonensis* promastigotes was determined by quantitative amino acid analysis using an Applied Biosystems Inc., Model 420H instrument (Foster City, CA) assuming 65 moles alanine per mole gp63. The specific activity of gp63 at pH 5.0, 7.0 and 8.0 was determined using insulin B-chain (Sigma) as the substrate. The digests consisted of 130 µl of 100 mM MES buffer (pH 5.0) or 100 mM HEPES buffer (pH 7.0 and 8.0) containing an initial substrate concentration of 160 µM. *L major* gp63 concentration was 32 nM and *L. m. amazonensis* gp63 concentration was 88 nM. The reaction was carried out at 37°C and each reaction mixture was sampled after 0, 10 and 20 min. In each case, the reaction was stopped in a boiling water bath. The amount of insulin remaining in each sample was determined spectrophotometrically after separation by reverse-phase HPLC. Twenty ml aliquots were chromatographed as described above and the 230 nm absorbance of intact insulin was determined. A standard curve of [insulin] vs. $A_{230\,nm}$ was constructed to determine the insulin concentration - the molar extinction coefficient at 230nm was 2.05 x 10^4 l $mole^{-1}$ cm^{-1} under these conditions.

The effect of pH and substrate denaturation on gp63 protease activity

Reaction mixtures were set-up to assess the ability of gp63 to digest native and heat denatured human transferrin (HT) (Sigma) and bovine serum albumin (BSA) (Sigma) at pH 5.0, 7.0 and 8.0. The buffers were the same as described above with an enzyme concentration of approximately 0.15 mg ml^{-1} and initial substrate concentration was 0.25 mg ml^{-1}. The reactions were carried out overnight at 37°C in the presence of toluene vapours to prevent bacterial growth. The samples and controls were then analyzed by SDS-PAGE.

Determination of gp63 substrate specificity using a 13 residue peptide and insulin B-chain

One mg of a 13 amino acid peptide (pep13) having the sequence, STYTQTKYPIVLA, was dissolved in 200 µl of 100 mM Tris, pH 7.0 and digested for 30 min at 37°C with 5 µg of *L. major* and *L. m. amazonensis* gp63. Digest products were separated by reverse-phase chromatography on a C_{18} column (Aquapore ODS 20 micron, 250 x 10 mm, Applied Biosystems Inc.) using a 0.1% TFA and ACN linear gradient elution system. Approximately 400 µg of digested starting material was injected and the peptides were eluted using a flow rate of 3.0 ml min^{-1} with a ramp from 0 to 10% ACN in 40 min followed by a ramp to 100% ACN over 30 min. The elution profile was monitored at 230 nm and peak fractions were collected for automated amino acid analysis.

Insulin B-chain (1 mg ml^{-1}) (Sigma) was also used as a substrate for both *L. m. amazonensis* and *L. major* gp63. Digest products from 100 µg of starting material were injected onto a C_8 reverse-phase column and the peptides were separated using a 0.1% TFA/ACN linear gradient (0-40% ACN in 60 min.). The amino acid composition of the peptides was determined as described above.

Determination of K_m for *L. major* gp63 using pep13 as the substrate

L. major gp63 (13 nM) was incubated at 37°C in 100 mM Tris HCl, pH 7.0, with pep13 ranging in concentration from 20 - 640 µM. The specific activity was determined over two consecutive time periods. Pep13 and the digestion products were

chromatographed as described above for insulin. The intact form of pep13 eluted at 9.5 min and a standard curve of [pep13] vs. absorbance at 230nm was constructed to calculate the rate of pep13 hydrolysis: the molar extinction coefficient at 230 nm was 2.5 x 10^4 l cm^{-1} $mole^{-1}$.

The data was plotted using the linearized form of the Michaelis-Menten equation described by Hanes (1932) and recommended by Wilkinson (1961) for an accurate determination of K_m and V_{max}. In this type of plot, s/V_o is plotted against s where s represents the initial substrate concentration. The slope represents $1/V_{max}$, the y intercept gives K_m/V_{max} and the intercept of the x-axis is equal to $-K_m$. A regression analysis was employed to determine the best fit line.

RESULTS

Purification of *L. major* and *L. m. amazonensis* promastigote gp63

Gp63 released from *L. major* and *L. m. amazonensis* promastigote membranes using PI-PLC was separated from other proteins by gel permeation HPLC (Figure 1-1 and 1-2). The fraction containing gp63 was identified enzymatically using the insulin assay and SDS-PAGE analysis demonstrated a highly purified sample of gp63 (Figure 1-3).

Comparison of the specific activity of *L. major* and *L. m. amazonensis* promastigote gp63 at pH 5.0, 7.0 and 8.0 using insulin as the substrate

The specific activity of *L. major* and *L. m. amazonensis* gp63 at pH 5.0, 7.0 and 8.0 is reported in Table 1-1. *L. m. amazonensis* gp63, with insulin B-chain as the substrate, was more active at pH 5.0 than pH 7.0 while *L. major* gp63 was more active at pH 7.0. It is, however, important to note that *L. major* gp63 was still very active at pH 5.0.

Table 1-1: Specific activity of *L. major* and *L. m. amazonensis* gp63 at pH 5.0, 7.0 and 8.0 using insulin B-chain as the substrate.

	pH	Specific activity (moles insulin digested*min^{-1}* mole $gp63^{-1}$)
L. m. amazonensis gp63	5	278 ± 6 (n=2)
	7	110 ± 7 (n=2)
	8	0
L. major gp63	5	240 ± 34 (n=3)
	7	430 ± 40 (n=2)
	8	139 ± 2 (n=2)

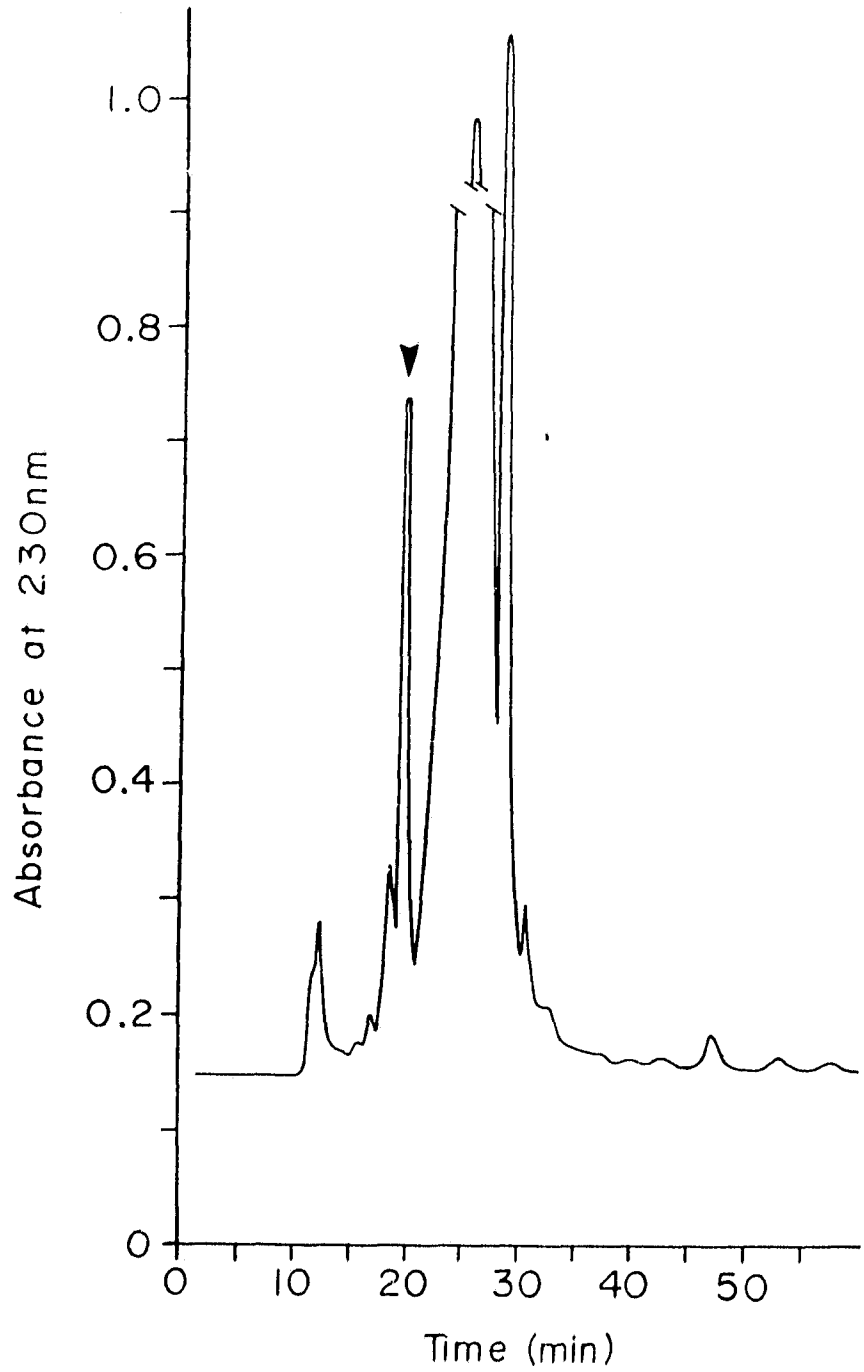


Figure 1-1. Gel permeation-HPLC separation of *L. major* membrane proteins released by PI-PLC. Arrow indicates gp63.

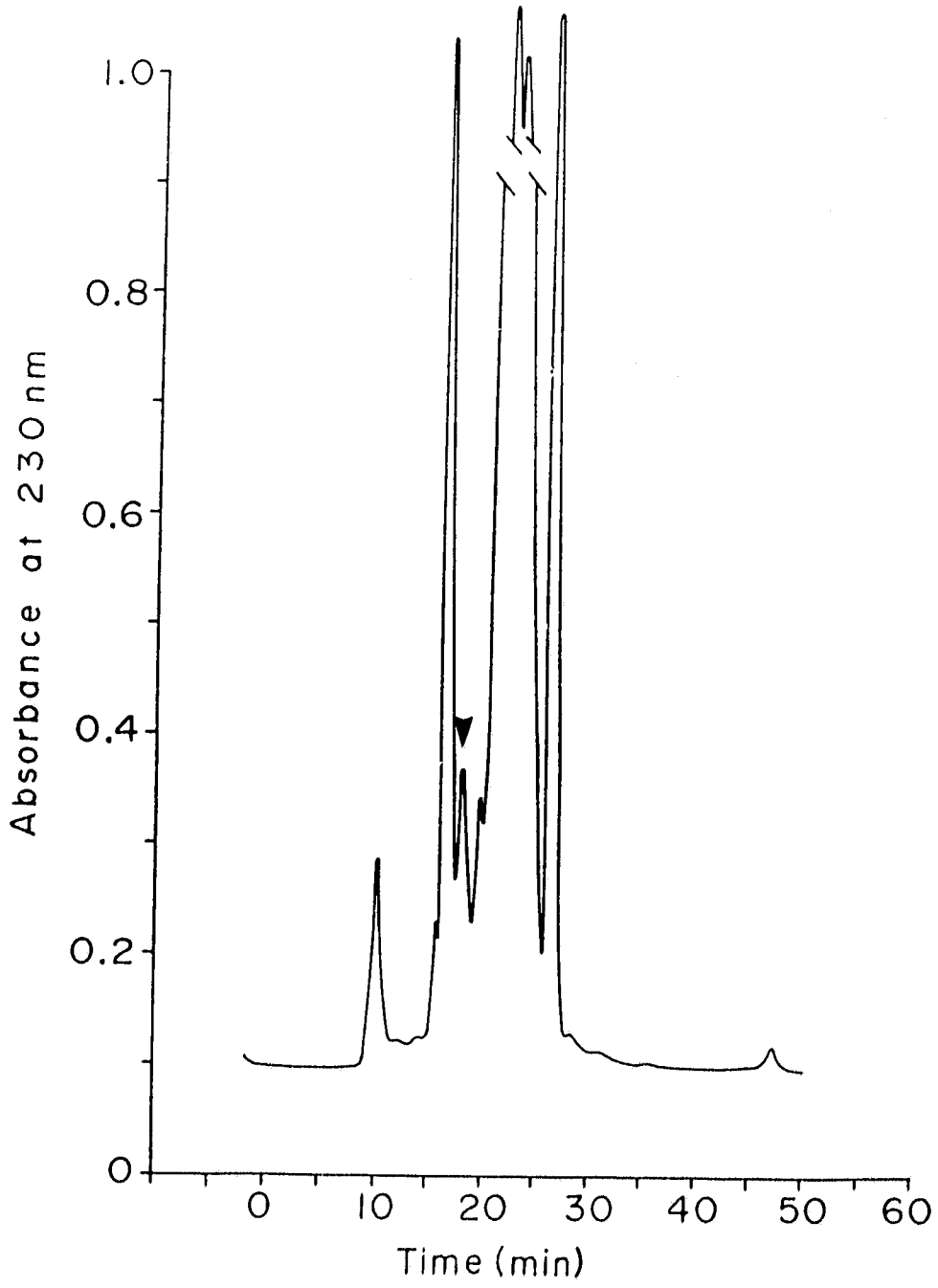


Figure 1-2. Gel permeation-HPLC separation of *L. m. amazonensis* membrane proteins released by PI-PLC. Arrow indicates gp63.

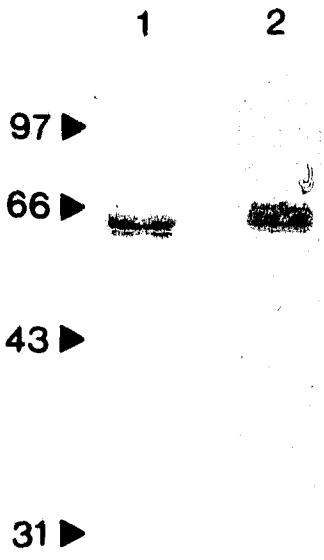


Figure 1-3. SDS-PAGE analysis of GP-HPLC fractions displaying protease activity. Lane 1, *L. major* gp63; lane 2, *L. m. amazonensis* gp63.

The effect of pH and heat denaturation of the substrate on the ability of gp63 to digest large proteins

To further assess the importance of gp63 as a protease in either the sandfly gut following a blood meal or the phagolysosome of macrophage, the ability of gp63 to digest large substrates at varying pH's was determined. Native and heat denatured HT and BSA were incubated with gp63 from *L. major* promastigotes at pH 5.0, 7.0 and 8.0. Heat denatured HT and BSA were readily digested by *L. major* gp63 regardless of the pH (Figure 1-4A and B, lanes 4-6). However, native HT and BSA were only minimally digested by gp63 (Figures 1-4A and B, lanes 10-12) with the exception of HT at pH 5.0 (Figure 1-4A, lane 10) which quite likely occurred as a result of denaturation of HT at acidic pH. Similar results were obtained using gp63 isolated from *L. m. amazonensis* promastigotes (results not shown).

Determination of gp63 cleavage sites for pep13 and insulin B-chain

Reverse-phase HPLC separation of pep13 fragments, generated by digestion with *L. major* and *L. m. amazonensis* gp63, revealed 3 peaks. Amino acid analysis identified the fragments as STYT, QTKYP and IVLA from the original sequence STYTQTKYPIVLA.

Similar analysis using insulin B-chain as the substrate identified 3 cleavage sites: FVNQHLCG - SHL - VEALY - LVCGERGFFYTPKA. The relative abundance of the latter fragments suggest that the first and third cleavage sites were preferred by both *L. major* and *L. m. amazonensis* gp63.

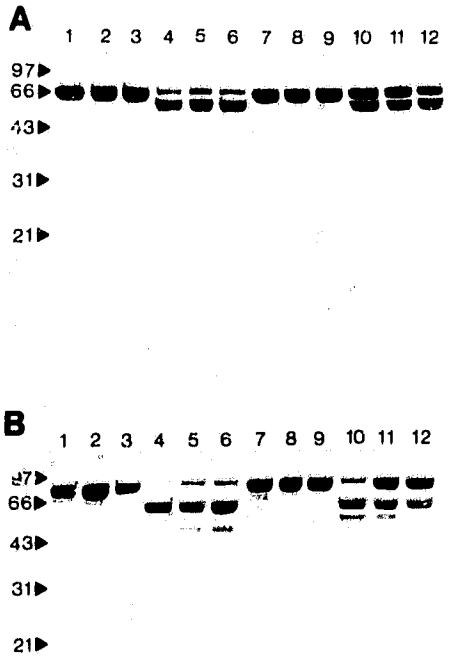


Figure 1-4. SDS-PAGE analysis showing digestion by *L. major* gp63 of native and heat denatured BSA and HT at different pH's. (A) Gp63 digestion of BSA. The upper band having a molecular weight of 66 kDa is BSA. (B) Gp63 digestion of HT. The upper band having an apparent molecular weight of 72 kDa is HT. Lanes 1-3 and 7-9 represent the level of heat denatured and native substrate present following overnight incubation at pH 5.0, 7.0 and 8.0 in the absence of gp63. Lanes 4, 5 and 6 show the digestion of heat denatured substrate that occurred at pH 5.0, 7.0 and 8.0, respectively, by overnight incubation with gp63. Lanes 10, 11 and 12 represent overnight digestion of native protein by gp63 at pH 5.0 7.0 and 8.0.

Determination of a Km value for *L. major* gp63 using pep13 as a substrate

The initial cleavage rates of the 13 residue peptide by gp63 at various substrate concentrations are reported in Table 1-2. Regression analysis of the Hanes plot resulted in a regression coefficient of 0.986 and the K_m was calculated as 58 μM (Figure 1-5).

Table 1-2: *L. major* gp63 enzyme activities at varying concentrations of the substrate, pep13. All reactions were carried out at pH 7.0.

pep13 concenration (μM)	*L. major* gp63 Specific Activity (min^{-1})
22	166
44	240
48	289
67	289
88	343
123	350
134	350
238	331
271	373
550	486
645	486

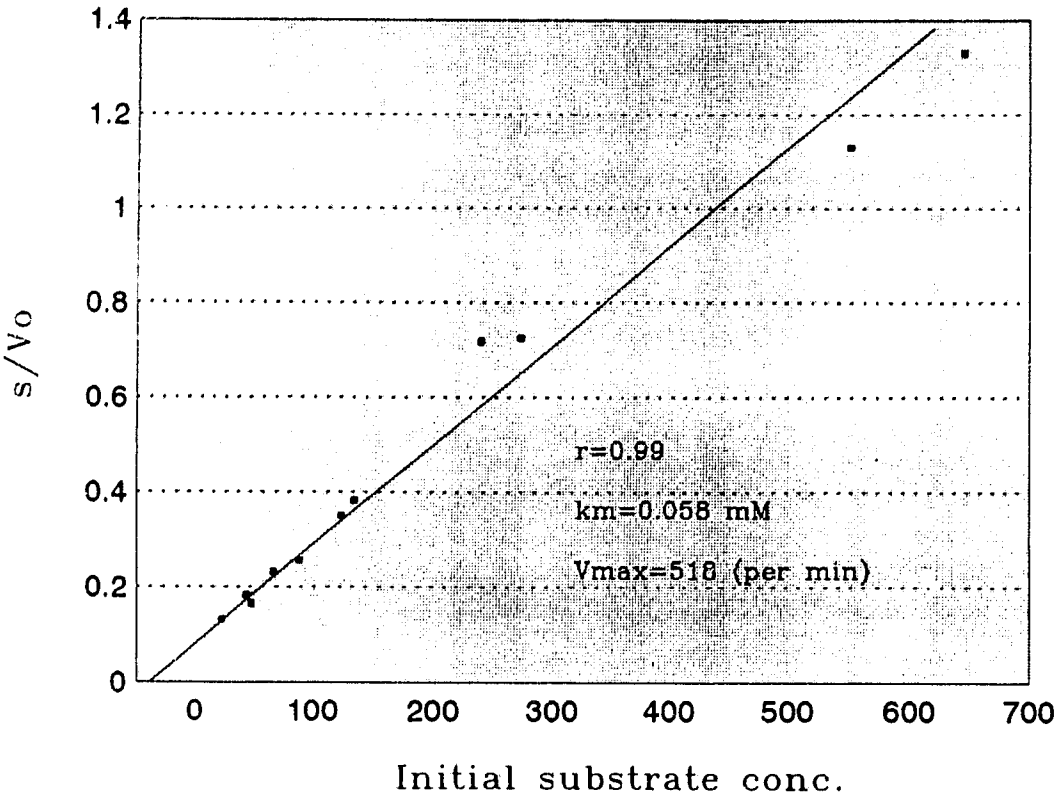


Figure 1-5. Hanes plot enzyme activity of *L. major* gp63 using pep13 as the substrate: $[S]/V_o$ vs. [S], where [S] is initial substrate concentration and V_o is the initial enzyme velocity.

DISCUSSION

Purification of gp63 was greatly facilitated by cleavage of its GPI anchor, providing a soluble and easily chromatographed product. The data regarding the pH optimum of gp63 isolated for *L. major* and *L. m. amazonensis* supports the present understanding of gp63 enzyme activity. This work demonstrated that the substrate specificity of gp63 remained constant among *Leishmania spp.*, as shown by digestion of pep13 and insulin. However, the relative activities of gp63 from *L. major* and *L. m. amazonensis*, at various pH's, differed despite the high degree of sequence homology. It was surprising to find that *L. m. amazonensis* gp63 was more active at pH 5.0 than pH 7.0 given that the proposed mechanism of action requires that the glutamate residue in the active site must be ionized for hydrolysis to occur (Kester and Mathews, 1977a, 1977b; Bertini et al., 1985). It has been argued that gp63 must be more active at pH 7.0 than pH 5.0 because the active site glutamate would not be fully deprotonated at pH 5.0 as the pKa of this residue is approximately 4.4 (Bouvier et al., 1989). However, basic residues around the active site glutamate could stabilize the negative charge of this residue and thereby decrease its pKa. Although little can be said regarding the pH optimum of these two enzymes due to insufficient data points, the results of Tzinia et al. (1991) clearly indicated that the pH optimum of *L. m. amazonensis* gp63 was substantially lower than the pH optimum of gp63 from other *Leishmania spp.* In addition, Tzinia et al. (1991) found that the substrate affected the pH optimum of gp63. Results reported here as well as those of Tzinia et al. (1991) indicate that gp63 can function in either the hydrolytic environment of the sandfly gut following a blood meal or in the macrophage phagolysosome. Further support of gp63 functioning as an endoproteinase at acidic pH were the results showing that *L. major* gp63 could digest HT and BSA at pH 5.0.

The peptide sequence specificity of gp63 was highly variable with hydrolysis occurring on the amino side of polar and hydrophobic amino acids. This is consistent with the results of Bouvier et al. (1990) and Ip et al. (1990) who published shortly after this work was initiated. Ip et al. (1990) found that gp63 favoured cleavage at the amino side of serine or threonine while Bouvier et al. (1990) reported that hydrophobic residues at this site were favoured for hydrolysis. The reported preference for cleavage at the

carboxy side of tyrosine (Bouvier et al., 1990) is not an absolute requirement for hydrolysis as cleavage of pep13 by gp63 generated the fragment STYT. Generation of this fragment by gp63 indicates that the specificity rules suggested by Ip et al. (1990) and Bouvier et al. (1990) must be taken in a very loose sense. For example, gp63 was also found to cleave at the carboxyl side of proline. Such a cleavage is interesting because is suggests that the active site of gp63 holds only one residue on the amino side of the cleavage site otherwise the bend in the peptide chain caused by the proline residue would prevent the peptide from entering the active site. The gp63 cleavage sites of insulin B-chain reported here were the same as those reported by Bouvier et al. (1990) but we also found a third, less favoured site. Evidently, gp63 is an endoproteinase with a broader substrate specificity than previously suggested.

An enzyme with such broad a substrate specificity would be expected to display varying Km values that are dependent on the substrate. Bouvier et al. (1990) and Ip et al. (1990), report Km values of 2.0×10^{-2} M for the substrate AY/LKK and 9.4×10^{-4} M for the substrate LV/TKK, respectively. The present work reported a Km an order of magnitude lower (6.3×10^{-5} M) when pep13 was the substrate. Such a low Km value indicates that gp63 can function at low protein concentrations.

The function of this proteolytic activity in parasite virulence remains unknown but results indicate that it would definitely be capable of functioning in lysosomes suggesting that its presence in the megasomes of *L. m. mexicana* amastigotes (Bahr et al., 1993; Ilg et al., 1993) has functional significance. However, the fact that gp63 is not consistently expressed on the surface of *Leishmania* amastigotes (Chang et al., 1986; Medina-Acosta et al., 1989; Davies et al., 1990; Frommel et al., 1990; Pimenta et al., 1991; Schneider et al., 1992; Bahr et al., 1993; Ilg et al., 1993) and that it is expressed on the surface of the monogenetic insect parasites, *Crithidia* and *Herpetomonas* (Etges, 1992; Schneider and Glaser, 1993), suggests that gp63 is primarily important in the gut of the sandfly. These data support the proposal that gp63 functions in the hydrolytic environment of the insect gut to digest hemoglobin - a process which is essential for the subsequent secretion of chitinolytic enzymes and forward migration of virulent promastigotes (Schlein et al., 1991, 1992).

CHAPTER 2

An investigation into the significance of the N-linked oligosaccharides of *Leishmania* gp63

INTRODUCTION

As discussed in the previous chapter, the enzyme activity of gp63 is possibly of primary importance in the gut of the sandfly. However, since gp63 is a glycoprotein, it could serve several roles, some of which may depend on its protease activity while others may be structural in nature. The proposal that N-linked oligosaccharides may be important in the enzymatic function of gp63 was intriguing as such functions for oligosaccharides are rare (Semenkovich et al., 1990; Riederer et al., 1991). N-linked oligosaccharides have been proposed to serve many different functions in biological systems, but the most common role is believed to be in protecting the protein from proteolytic degradation (Chu et al., 1978; Olden et al., 1978; Trowbridge et al., 1978; Peng Loh et al., 1979; Prives et al., 1980). More recently, N-linked oligosaccharides in multicellular organisms have been shown to be important in protein secretion and intracellular processing (Matzuk et al., 1988; Semenkovich et al., 1990; Grinnell et al., 1991; Riederer et al., 1991; Yamaguchi et al., 1991) for the activation of T cells (Thomas et al., 1991) and for modulating the plasma half-life of circulating glycoproteins - a process occurring through the asialoglycoprotein receptor of hepatocytes (Chen et al., 1982) and a unique SO_4-GalNAc receptor on hepatic reticuloendothelial cells. The latter appears to be specific for the glycoprotein hormones, luteinizing and thyroid stimulating hormone (Fiete et al., 1991; Baenziger et al., 1992). Finally, the N-linked oligosaccharides of lipoprotein lipase (Semenkovich et al., 1990) and yeast secreted acid phosphatase (Riederer et al., 1991) have been proposed to be essential for the enzymatic function of these proteins. This latter function may be effected by oligosaccharide mediated stabilization of protein conformation both during and following initial folding of the nascent polypeptide chain (Semenkovich et al., 1990; Riederer et al., 1991).

The present investigation was initiated to define the role of the N-linked glycans of gp63 with particular interest toward their reported requirement for enzyme activity. *L. major* gp63 has 3 potential sites for N-linked glycosylations (Button et al., 1988, 1989a; Miller et al., 1990) and also carries a glycan tail attached to the carboxyl terminal amino acid which serves to link the protein to the parasite surface via a

glycosyl-phosphatidylinositol membrane anchor (Etges et al., 1986b). In this paper, deglycosylated *L. major* gp63 was obtained by metabolic inhibition using tunicamycin (TM) as well as by enzymatic cleavage. For comparison purposes, *L. mexicana amazonensis* gp63 was also enzymatically deglycosylated. Using these modified enzymes, we have investigated the role of oligosaccharides in cellular targeting of gp63, protein stability toward autolysis and proteolysis, protein folding and enzyme activity.

MATERIALS AND METHODS

Parasite cultivation.

Leishmania major A2 (Neva et al., 1979) and *Leishmania mexicana amazonensis* M2269 promastigotes were cultured at 26°C in M199 medium supplemented with hemin (5 mg l^{-1}) and folic acid (23 μM). Penicillin/streptomycin (Sigma Chemical Company, St. Louis, MO) and Basal Eagle Medium vitamin solution (Gibco, Burlington, Ontario) were added to the medium at a dilution of 1:100 (v/v) and fetal bovine serum (FBS) was added to a final concentration of 5%. Parasites were grown to late log phase and harvested by centrifugation (3,000 xg) at room temperature.

Enrichment for gp63 using phosphatidylinositol specific phospholipase C (PI-PLC).

Pelleted parasites were frozen and thawed in 50 mM Tris, pH 8.0 and sonicated (45 Watts) on ice for 3 x 10 sec to lyse the cells. To dissociate peripheral membrane proteins, membranes were resuspended in lysis buffer containing 3 M NaCl and washed 3 times with intervening centrifugation at 19,000 xg for 20 min. In order to provide a highly soluble enzyme preparation in a simple aqueous buffer system, gp63 was released from the membranes by cleaving the glycosyl-phosphatidylinositol membrane anchor with phosphatidylinositol specific phospholipase-C (PI-PLC) (a gift from Dr. Sam Turco, Department of Biochemistry, University of Kentucky Medical Center, Lexington, Kentucky, 40536). Membranes from 6.0 x 10^9 parasites were resuspended in 1 ml of 25 mM HEPES buffer, pH 7.2, with 2.0 mM EDTA and 0.1% CHAPS (3-[(3-cholamidopropyl)dimethylammonlo]propanesulfonate) (Sigma) and digestion proceeded overnight at 37°C using PI-PLC at a concentration of 20 mU ml^{-1}.

Purification of Gp63.

Washed membranes were stirred overnight in 1% octylthioglucoside, 100 mM Tris, pH 8.0 and the resulting extract after dilution with concanavalin A (Con A) buffer A (20 mM Tris, pH 7.4, 1 mM $CaCl_2$, $MnCl_2$ and $MgCl_2$ and 0.1% CHAPS) was applied to a Con A Sepharose column (1.5 x 6.0 cm) at a flow rate of 0.2 ml min^{-1}. The column was

washed with one bed volume of buffer A to remove non-specifically bound material followed by elution with 3 bed volumes of a 500 mM α-methylmannoside and 400 mM NaCl solution to elute bound ligands. The Con A eluate was dialyzed and further purified using a preparative isoelectric focusing apparatus (BioRad Laboratories Inc., Mississauga, Ontario). The proteins were focused in a 0.75% ampholyte gradient, pH 3-10, using 12 Watts constant power for 4.0 h. Purity of the resulting fractions was assessed by SDS-PAGE.

Inhibition of N-linked glycosylation by treatment with tunicamycin (TM).

TM treatment. Log-phase parasites (4×10^6 per ml) were grown in M199, 10% FBS at 26°C for 100 h with or without 5 μg ml^{-1} TM.

Titration of TM activity. Log-phase *L. major* A2 promastigotes (6×10^9 cells) were harvested, divided into 3 flasks and resuspended to a concentration of 4.0×10^7 cells ml^{-1} Dulbecco's Modified Eagle Medium (D-MEM) (Gibco) supplemented with vitamins, folic acid and hemin as described for M199. Adenosine (0.1 mM), pyruvate (1.0 mM), β-mercaptoethanol (50 μM), glutamine (2.0 mM), gentamycin (1 ml l^{-1}) (Sigma) and 10% FBS were also added. Parasites were exposed to either 0, 5 or 10 μg ml^{-1} TM and ^{35}S-methionine (100 μCi) was added to each flask 1h after addition of TM. Cultures were incubated for 24 h.

Localization of gp63 produced in the presence of TM.

L. major promastigotes (2×10^9 cells) were grown with or without 5 μg ml^{-1} TM for 100 h. Cells were concentrated by centrifugation (10 min at 2000 xg), washed twice with PBS containing 1% glucose and resuspended in the same buffer to a final concentration of 2×10^8 cells ml^{-1}. Cell surface proteins were biotinylated by addition of 100 μg ml^{-1} Sulfo-NHS-Biotin (Pierce, Rockford, IL) to each flask followed by incubation at room temp. for 20 min. After washing twice with PBS to remove residual labelling reagent, gp63 was released from membranes with PI-PLC as described above. The released protein was electrophoresed on SDS-PAGE, Immunoblotted, probed with gp63

antiserum as described below and finally probed with an Avidin-HRP conjugate (Pierce) at a dilution of 1:1000.

Enzymatic removal of N-linked oligosaccharides.

1 U of endoglycosidase-F/N-glycosidase F (EC 3.2.1.96/EC 3.2.2.18) (Endo-F) (Boehringer Mannheim, Laval, Quebec) was added to 200 µl PI-PLC cleaved gp63 in 50 mM potassium phosphate buffer, pH 7.0, with 0.5% octylthioglucoside and 10 mM EDTA. The digest was incubated at 37°C and sampled after 24 and 72 h. A control sample containing 200 µl of gp63 in the above buffer was incubated at 37°C in the absence of Endo-F. Decreases in molecular weight due to loss of oligosaccharides were assessed by SDS-PAGE.

Determination of gp63 endoproteinase activity.

Zymograms. SDS-PAGE was performed according to the method of Laemmli (1970) using a 12.5% running gel with a 5% stacking gel. Fibrinogen substrate was incorporated into the running gel at a concentration of 0.3 mg ml^{-1} and all samples were electrophoresed in non-reducing sample buffer without prior boiling, to maintain protease activity. Following electrophoresis, the gel was rinsed twice in 50 mM Tris, pH 8.0, incubated overnight at 37°C with shaking and finally stained in 0.1% Coomassie Brilliant Blue.

Quantitation of gp63 protease activity using a synthetic peptide, pep13, as substrate. Native and Endo-F deglycosylated *L. major* gp63 were incubated at 37°C in 100 mM Tris HCl, pH 8.0, with pep13 (STYTQIKYPIVLA) (220 µM). The gp63 concentration employed (4.4 µM) was determined by quantitative amino acid analysis using an Applied Biosystems Inc. Model 420H instrument (Mississauga, Ont.). Values were normalized to alanine, assuming 65 moles of alanine per mole gp63 (Button et al., 1988, 1989a; Miller et al., 1990). Conditions of unlimited substrate were ensured by using a high substrate to enzyme ratio (50:1) and by determining the specific activity over two consecutive 10 min periods. The reaction was stopped by addition of 5 µl anhydrous trifluoroacetic acid

(TFA). Twenty µl aliquots were run on a C_8 reverse-phase HPLC column (Aquapore RP-300, 100 x 22.1 mm, Applied Biosystems Inc.) using a 0.1% TFA/acetonitrile (ACN) step gradient at a flow rate of 1.0 ml min^{-1}. The column was equilibrated with 0.1% TFA and the apolar gradient was applied as follows: acetonitrile increased from 0 to 30% in 5 minutes, 30-44% in 8 min and 44-100% in 1 min. Pep13 and the digestion products were detected at 230 nm using a detector sensitivity of 0.5 AUFS. The intact form of pep13 eluted at 9.5 min as a sharp peak and a standard curve of [pep13] vs. absorbance at 230 nm was constructed to calculate the rate of pep13 hydrolysis.

The activity of native and Endo-F deglycosylated *L. m. amazonensis* gp63 was also estimated using a procedure analogous to that described above and pep13 as substrate. The initial substrate concentration was 350 µM and *L. m. amazonensis* gp63 was added to a final concentration of approximately 1.4 µM, making the substrate to enzyme ratio 250:1 on a molar basis. The hydrolysis mixture was sampled after 30 and 60 min incubation at 37°C in 100 mM Tris HCl, pH 7.0.

Determination of gp63 proteolytic specificity.

One mg of pep13 was dissolved in 200 µl of 100 mM Tris, pH 8.0 and digested for 48 h at 37°C with 5 µg of *L. major* gp63. The digest products were separated by reverse-phase chromatography on a C_{18} column (Aquapore ODS 20 micron, 250 x 10 mm, Applied Biosystems Inc.) using a 0.1% TFA and ACN linear gradient elution system. Approximately 400 µg of digested starting material was injected and the peptides were eluted using a flow rate of 3.0 ml min^{-1} with a ramp from 0 to 10% ACN in 40 min followed by a ramp to 100% ACN over 30 min. The elution profile was monitored at 230 nm and peak fractions were collected for amino acid analysis on an Applied Biosystems instrument.

Insulin B-chain (1 mg ml^{-1}) (Sigma) was also digested as described above with native gp63 and Endo-F deglycosylated/periodate oxidized gp63. Digestion products from 100 µg of starting material were injected onto a C_8 reverse-phase column and the peptides were separated using a 0.1% TFA/ACN linear gradient (0-40% ACN in 60 min.). The amino acid composition of the peptides was determined as described above.

Periodate oxidation.

Endo-F deglycosylated *L. major* gp63 was dissolved in 1 ml of 100 mM sodium acetate buffer, pH 5.0 and periodic acid was added to a final concentration of 12 mM. The sample was incubated overnight at 4°C in the dark. Excess periodate was scavenged by addition of 2.0 µl of 50% glycerol and dialyzed against distilled water overnight at 4°C. Reactive aldehydes on gp63 were reduced at room temperature for 1.5 h in 0.33 M NH_4OH using 300 mM $NaBD_4$ and the product dialyzed against distilled water before use.

N-acetylglucosamine (GlcNAc) (0.1 mM) was treated with periodate as described above and subsequently run on a PA-1 HPLC column using a pulsed amperometric detector (PAD) (Dionex Corporation, Mississauga, Ont.). Standard monosaccharides and control GlcNAc were resolved isocratically using 16 mM NaOH.

Polyclonal antibodies.

Potential B-cell epitopes were predicted from the *L. major* gp63 amino acid sequence (Button et al., 1988, 1989a; Miller et al., 1990) using computer programs to predict regions of high accessibility, flexibility and hydrophilicity (Parker et al., 1986). Also, areas having a predicted secondary structure of turns or coils were determined using a Garnier plot (Garnier et al., 1978). Four peptides, corresponding to the predicted amino acid sequences 114-138, 173-197, 321-338 and 516-538 (Figure 2-1), were synthesized using a Model 430A peptide synthesizer (Applied Biosystems Inc.). The peptides were conjugated to Keyhole Limpet hemocyanin (KLH) (Reichlin, 1980) to be used as a mixed antigen. Briefly, the peptides were solubilized at a concentration of 5 mg/ml in phosphate buffered saline and KLH was added to attain a molar ratio of 18 moles of peptide per mole of KLH. An equal volume of 0.2% glutaraldehyde in PBS was slowly added to the peptide/protein solution with constant stirring and incubated for 1 h. Glycine was then added to a final concentration of 200 mM and incubated, with stirring, for 1 h. Uncoupled peptides were removed by dialysis. Anti-gp63 antibodies were raised in New Zealand White rabbits by an initial subcutaneous injection of 800 µg antigen with Freund's complete adjuvant, followed by similar injections of antigen with Freund's

incomplete adjuvant. The optimal working dilution of the serum was determined as 1:2000 by an enzyme-linked immunosorbent assay (ELISA).

Enzyme-linked immunosorbent assay (ELISA)

Wells were coated with approximately 1 μg of gp63. Remaining binding sites in the wells were blocked by adding 200 μl of 3% milk powder and incubating for 1 h at 37°C. The wells were rinsed with PBS/0.05% TWEEN 20 and then the rabbit serum was added to duplicate wells at dilutions ranging from 1:100 to 1:12,800. Binding of the first antibody occurred for one hour at 37°C with shaking. The wells were then rinsed with PBS/0.05% TWEEN 20 three times. Each rinse consisted of 3 quick rinses followed by a 10 min incubation period at room temp. The second antibody, commercial goat anti-rabbit IgG/IgM conjugated to horse radish peroxidase (Pierce, Rockford, IL), was added at a dilution of 1:2000 and incubated for one hour at 37°C with shaking. The wells were again rinsed 3 times as described above. The second antibody was detected by incubating the wells in 100 μl of ABTS substrate for 20 min in the dark. Absorbances were read at 405nm in a Dynatech MR5000 ELISA plate reader.

Pre-immune rabbit serum was used as a negative control and partially purified gp63 was used as the antigen. Western blot analysis was also carried out using a crude preparation of membrane proteins from various *Leishmania spp.* to ensure that no cross-reactivity was occurring with other leishamanial proteins.

ABTS substrate

The ABTS substrate consisted of 50 ml of citrate buffer (100 mM), 250 μl ABTS stock (274 mg ABTS in 12.4 ml distilled water) and 200 μl hydrogen peroxide stock (0.5 ml of 30% hydrogen peroxide in 7.5 ml distilled water).

Gel electrophoresis and immunoblotting.

SDS-PAGE was performed according to the method of Laemmli (1970), using a 12.5% running gel and a 5% stacking gel. Proteins were transferred to Immobilon (BioRad Laboratories Inc.) as described by Matsudaira (1987) and the membrane blocked with 10% FBS in PBS, 0.5% Tween 20. The blots were incubated for 2.0 h at 37°C with gp63 polyclonal antiserum diluted 1:2000 in PBS containing 0.5% Tween 20, 10% FBS, 10% glycerol and 1.0 M glucose and finally given 6 ten min washes. The second antibody (HRP-conjugated goat anti-rabbit IgG/IgM, Pierce) was applied at a dilution of 1:2000 for 1 h at 37°C in PBS/0.5% Tween 20, 10% FBS, 1% BSA and 0.1% Nonidet P-40 and the blot was washed 3 times prior to development. The blot was developed using 30 mg $CoCl_2$ and 50 mg diaminobenzidine (Sigma Chemical Company, St. Louis, MO) in 100 ml PBS, 1% BSA with a final addition of 1.0 ml of 30% H_2O_2.

Detection of gp63 Concanavalin A binding activity.

Con A was biotinylated by incubating 5mg Con A in 1.0 ml of 50 mM Tris HCl buffer, pH 7.2 with 100 µg of Sulfo-NHS-biotin for 30 min at room temperature (Hurley et al., 1985). The mixture was dialyzed against 20 mM Tris, pH 7.4 containing 1mM each of $CaCl_2$, $MgCl_2$ and $MnCl_2$ and titrated by ELISA against 1µg human transferrin (HT). The optimal dilution was 1:1000.

After electroblotting the proteins to Immobilon, the membranes were blocked with 3% periodate treated BSA in PBS as described by Glass et al. (1981) and probed with biotinylated Con A at a dilution of 1:1000 in the presence and absence of 200 mM α-methylmannoside to control for nonspecific binding. Incubation was carried out for 1.0 h at 37°C. The second antibody, Avidin-HRP, was used at a dilution of 1:1000 for 1.0 h at 37°C and developed with $CoCl_2$/DAB as described above.

Resistance of deglycosylated gp63 to autolysis.

Both *L. major* gp63, isolated from promastigotes grown in the presence of 5 μg ml^{-1} of TM, as well as native gp63, were incubated at room temperature in 100 mM Tris, pH 8.0 at a concentration of 0.3 mg ml^{-1}. Subsamples were taken after 0, 2, 4 and 6 days of incubation, electrophoresed by SDS-PAGE, Western blotted and probed with anti-gp63 antiserum. The same experiment was carried out using Endo-F deglycosylated gp63.

Resistance of gp63 to cathepsin D.

Native, Endo-F deglycosylated and Endo-F/periodate treated gp63 (0.5 mg ml^{-1}) were incubated for 24 h at 37°C in 100mM sodium citrate buffer, pH 4.5, with 500 mU ml^{-1}cathepsin D (EC 3.4.23.5) from bovine spleen (Sigma). Gp63 was also inhibited by overnight incubation in the presence of 50 mM 1, 10-phenanthroline prior to incubation with cathepsin D to ensure any resistance to proteolysis was not a result of gp63 degrading cathepsin D. HT was used as a positive control for cathepsin D activity in the presence and absence of 1, 10-phenanthroline. All samples were run on SDS-PAGE and stained with Coomassie Brilliant Blue.

RESULTS

Inhibition of N-linked glycosylation by treatment of *L. major* A2 promastigotes with tunicamycin.

When *L. major* gp63 was isolated from TM treated cells, SDS-PAGE and Western blotting showed two distinct molecular weight forms (Fig. 2-2A, lane 4). The higher molecular weight band corresponded to gp63 derived from untreated control membranes (Fig. 2-2A, lane 2) and presumably represented glycosylated gp63, synthesized prior to addition of TM. Evidence in support of this was obtained in metabolic labelling experiments described later. The lower mol. wt. band was assumed to represent non-N-glycosylated gp63. Both molecular weight forms of gp63 were enzymatically active as demonstrated by a zone of clearing in SDS-PAGE zymograms (Fig. 2-2B). The possible presence of contaminating proteases was addressed by comparing the cleavage pattern of a synthetic peptide (pep 13, STYTQTKYPIVLA) following digestion with either purified gp63 or PI-PLC released gp63. In both cases the cleavage sites were identical, resulting in production of 3 peptides isolated by reverse-phase HPLC: STYT, QTKYP and IVLA. Similar findings were observed for digests carried out using insulin B–chain as the substrate (data not shown). Thus, the zone of clearing at the lower molecular weight position was due to a protease with identical cleavage specificity to gp63. Since this proteolytic activity only appeared following culture in TM, it was concluded that the enzyme activity was derived from a nonglycosylated form of gp63 and that the absence of N-linked sugars had no apparent affect on the enzyme activity. These data also indicated that N-linked oligosaccharides were not essential for correct folding of the nascent polypeptide chain as the tertiary structure necessary for enzymatic activity was attained.

MSVDSSSTHRRRCVAARLVRLAAAGAAVTVAVGTAAAWAHAGALQ 45
HRCVHDAMQARVRQSVADHHKAPGAVSAVGLPYVTLDAAHTAAAA 90
DPRPGSARSVVRDVNWGALRIAVSTEDLTDPAYHCARVGQHVKDH 135
AGAIVTCTAEDILTNEKRDILVKHLIPQAVQLHTERLKVQQVQGK 180
WKVTDMVGDICGDFKVPQAHITEGFSNTDFVMYVASVPSEEGVLA 225
WATTCQTFSDGHPAVGVINIPAANIASRYDQLVTRVVTHEMAHAL 270
GFSGPFFEDARIVANVPNVRGKNFDVPVINSSTAVAKAREQYGCD 315
TLEYLEVEDQGGAGSAGSHIKMRNAQDELMAPAAAAGYYTALTMA 360
IFQDLGFYQADFSKXEVMPWGDNAGCAFLTNKCMEQSVTQWPAMF 405
CNESEDAIRCPTSRLSLGACGVTRHPGLPPYWQYFTDPSLAGVSA 450
FMDYCPVVVPYSDGSCTQRASEAHASLLPFNVFSDAARCIDGAFR 495
PKATDGIVKSYAGLCANVQCDTATRTYSVQVHGSNDYTNCTPGLR 540
VELSTVSNAFEGGGYITCPPYVEVCQGNVQAAKDGGNTAAGRRGP 585
RAAATALLVAALLAVAL

Figure 2-1. Sequence of *L. major* gp63. Underlined sequences indicate synthesized peptides used for production of anti-gp63 polyclonal antiserum (copied from Miller et al., 1990).

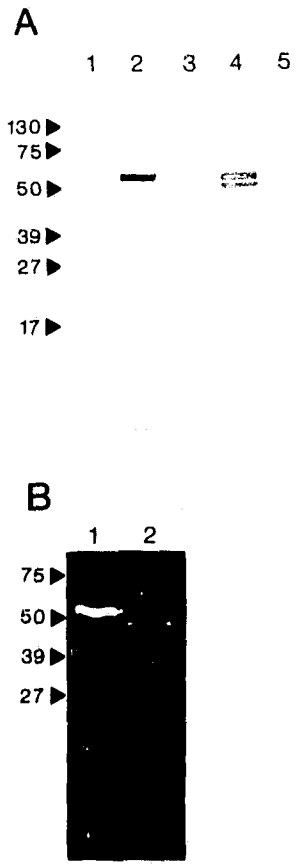


Figure 2-2. (A) Western blot analysis of Con A-Sepharose separated *L. major* gp63 produced in the absence (lanes 1 and 2) and presence (lanes 3 and 4) of TM. Lanes 1 and 3 are Con A voids while lanes 2 and 4 represent column eluates. HT was run in lane 5 as a negative control for antibody binding. Blots were probed with gp63 polyclonal antiserum. (B) Zymogram of *L. major* gp63 produced in the absence (lane 1) and presence (lane 2) of TM. Numbers on the left represent mol. wt. standards in kDa.

In an attempt to prove that inhibition of N-linked glycosylation was complete, the control and TM treated extracts were applied to a Con A-Sepharose column. Results of this experiment demonstrated that both molecular weight forms of gp63 were still capable of binding Con A (Fig. 2-2A). However, it was ambiguous as to whether these data indicated only partial inhibition of N-linked glycosylation in the presence of TM or whether lectin binding was due to the remaining intact glycan tail from the GPI anchor. The oligosaccharide core of the latter structure contains an α1,2-linked mannose residue (Schneider et al., 1990) which is a known Con A ligand (Beeley, 1985).

Titration of TM activity.

In order to resolve some of the preceding ambiguity, the concentration range of TM required for complete inhibition of gp63 glycosylation was investigated to ensure that 5μg ml^{-1} TM was sufficient to completely inhibit the transfer of UDP-GlcNAc to dolichol monophosphate. Parasites were metabolically labelled with ^{35}S-methionine in the presence of 0, 5 and 10 μg ml^{-1} TM as described in the Methods section. The autoradiogram of SDS-PAGE separated proteins demonstrated that in the presence of 5 and 10 μg ml^{-1} TM, all newly synthesized gp63 migrated to a lower mol. wt. compared to the control (Fig. 2-3) supporting the conclusion that 5 μg ml^{-1} TM was sufficient to completely inhibit addition of N-linked oligosaccharides.

Non-glycosylated gp63 is surface oriented.

The previous results demonstrated that non-glycosylated gp63 remained susceptible to cleavage by PI-PLC. However, because gp63 was released from sonicated washed membranes, the exact cellular location of the non-glycosylated gp63 could not be established. Thus, live *L. major* promastigotes were biotinylated and analyzed by Western blotting of PI-PLC released protein. The results clearly showed that both mol. wt. forms of gp63 were labelled with biotin and, therefore, located on the cell surface (Fig. 2-4). Thus, these data indicated that N-linked glycosylation was not important for intracellular targeting of gp63 to the plasma membrane.

Figure 2-3. Metabolic labelling of *L. major* gp63 produced in the presence of 0, 5 and 10 μg/ml TM using ^{35}S-methionine. Autoradiogram of metabolically labelled gp63 separated by SDS-PAGE. Lanes 1, 2 and 3 represent proteins from promastigotes grown in the presence of 0, 5 and 10 μg ml^{-1} TM, respectively. Markers on the left of the figure represent standards having molecular weights of 75, 50, 39, 27 and 17 kDa; the arrow on the right of the figure indicates non-glycosylated gp63.

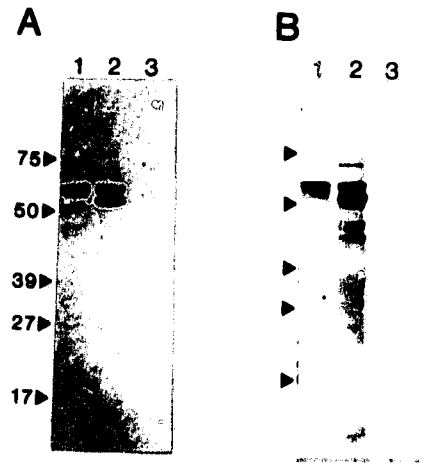


Figure 2-4. Western blot analysis of *L. major* membrane proteins after biotinylation of live promastigotes. (A) Western blot probed with gp63 antiserum. (B) Western blot probed with Avidin-HRP. Lane 1 in parts A and B represents protein from parasites grown the absence of TM, lane 2 represents protein produced in the presence of TM and lane 3 is HT, run as a negative control. Numbers on the left indicate mol. wt. standards in kDa.

Enzymatic removal of N-linked oligosaccharides.

The importance of N-linked oligosaccharides for enzyme activity was further assessed by removing the oligosaccharides enzymatically. Following 24 h digestion with Endo-F, *L. major* gp63 showed a quantitative shift to a lower mol. wt. compared to the untreated control (Fig. 2-5A, lanes 1 and 2). A further reduction in mol. wt. was not observed following digestion for an additional 48 h (Fig. 2-5A, lane 3). The Endo-F deglycosylated form of gp63 had the same electrophoretic mobility on SDS-PAGE as gp63 produced in the presence of TM. Both native and Endo-F treated gp63 were subsequently electrophoresed on substrate containing gels and results clearly showed that the two mol. wt. forms of gp63 were enzymatically active (Fig. 2-5B; lanes 1 and 2). The endoproteinase activity of both the glycosylated and deglycosylated forms of gp63 was then assayed to determine if deglycosylation affected the rate of proteolysis. Analysis showed no significant differences in the specific activity of the two samples at two consecutive time periods (Table 2-1). However, quantitative binding of Endo-F treated gp63 to a Con A Sepharose column was observed, indicating that, similar to gp63 generated in the presence of TM, deglycosylation was not complete following this treatment (data not shown).

Table 2-1. Determination of the specific activity of *L. major* and *L. m. amazonensis* gp63 before and after deglycosylation using Endo-F.

	Specific activity during first time interval	Specific activity during second consecutive time interval
L. major native gp63	1.4[1]	1.7[1]
L. major Endo-F treated gp63	2.0[1]	1.7[1]
L. m. amazonensis native gp63	3.6[2]	1.6[2]
L. m. amazonensis Endo-F treated gp63	3.32	2.0[2]

[1] Specific activity of *L. major* gp63 expressed as moles pep13 hydrolysed min^{-1} $mole^{-1}$ gp63 and were determined over a 10 min incubation period.

[2] Specific activity of *L. m. amazonensis* gp63 expressed as moles pep13 hydrolysed min^{-1} $mole^{-1}$ gp63. Values were determined over a 30 min period.

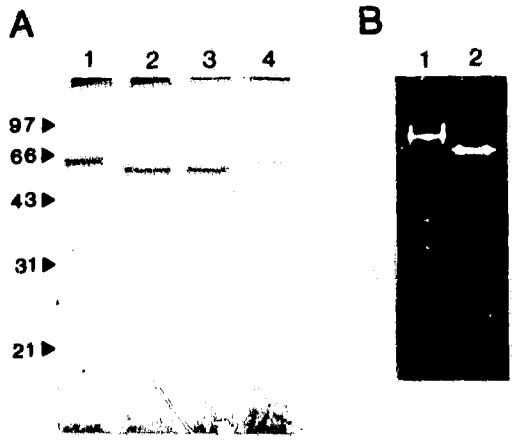


Figure 2-5. (A) Coomassie blue stained SDS-PAGE of *L. major* promastigote gp63 treated with Endo-F. Lane 1, 0 h digestion; lane 2, 24 h digestion; lane 3, 72 h digestion and lane 4 is after 72 h in the absence of Endo-F. Numbers on the left indicate mol. wt. standards in kDa. (B) Zymogram of gp63 before (lane 1) and after (lane 2) digestion with Endo-F.

Periodate oxidation of Endo-F treated gp63.

The binding of gp63 to Con A following either TM exposure or Endo-F cleavage, prevented an unequivocal conclusion regarding the quantitative removal of sugars and, therefore, the importance of N-linked oligosaccharides for the proteolytic activity of gp63. Although Endo-F deglycosylated gp63 would carry one residual GlcNAc residue from each N-linked oligosaccharide, only the remaining glycan tail had potential Con A ligands. Therefore, in an attempt to chemically degrade residual carbohydrate and eliminate the Con A binding activity, Endo-F deglycosylated gp63 was oxidized with periodate. The remaining GlcNAc residue would be susceptible to oxidation by periodate between C3 and C4 of the pyranose ring and the glycan tail would also be oxidized at many sites. Destruction of the former under the oxidation conditions used was verified by HPLC-PAD chromatography of oxidized GlcNAc. Following oxidation of Endo-F cleaved gp63, the Con A binding activity was assessed by probing Western blots with biotinylated Con A. A Western blot probed with gp63 antiserum showed the presence of deglycosylated gp63 before and after periodate oxidation (Fig. 2-6A). When this blot was probed with biotinylated Con A, the oxidized form of gp63 was completely unreactive (Fig. 2-6B). All Con A binding was inhibited by the presence of 200mM methylmannopyranoside verifying the specificity of the lectin interaction (data not shown). These results demonstrated complete degradation of the lectin-binding oligosaccharides following periodate oxidation of Endo-F treated gp63.

The periodate treated gp63 was then tested for protease activity by employing an SDS-PAGE zymogram. Figure 2-6C, lane 2 shows that the enzyme activity was retained after both Endo-F and periodate treatment. The band broadening of the oxidized protein is consistent with the non-quantitative generation of oxidized forms of Trp, Cys and Met in the protein. Western blot analysis of a gel run under the same conditions and subsequently probed with anti-gp63 antiserum, identified the enzyme as gp63 (data not shown).

To ensure that the oligosaccharides were not important in determining the substrate specificity of gp63, the cleavage products of a synthetic substrate, pep 13, and oxidized insulin B-chain were also determined following digestion with periodate

oxidized gp63. Amino acid analysis of the pep13 showed that the cleavage sites were identical to those produced by the native molecule. Moreover, both native and periodate oxidized gp63 generated the same peptides by digestion of insulin. The three primary products, FVNQHLCG, SHLVEALY and LVCGERGFFYTPKA, were in agreement with the cleavage sites reported by Bouvier et al. (1990). These data demonstrated that the N-linked oligosaccharides of gp63 were not important in the enzyme function or in the substrate specificity of gp63.

Enzymatic deglycosylation of *L. mexicana amazonensis* gp63.

L. m. amazonensis gp63 was also treated with Endo-F to ensure that it was not unique in its proposed requirement for N-linked oligosaccharides (Chaudhuri and Chang, 1988). The PI-PLC cleaved protein from *L. m. amazonensis* consisted of 2 major proteins, gp63 and a second protein presumed to be gp46 (Lohman et al., 1990; Rivas et al., 1991). Treatment of these proteins for 24 h with Endo-F resulted in a quantitative shift of gp63 to a lower mol. wt. (Fig. 2-7A). A zymogram showed the lower mol. wt. form to be enzymatically active (Fig. 2-7B) and kinetic studies showed that the rate of pep13 hydrolysis by the two forms of gp63 were similar (Table 2-1). Although the substrate concentration may have become limiting during the second consecutive incubation period of 30 min, as shown by the decrease in specific activity (Table 2-1) both the glycosylated and deglycosylated forms of gp63 were affected to the same extent, further supporting the conclusion that the N-linked oligosaccharides of *L. m. amazonensis* gp63 do not affect the rate of substrate hydrolysis.

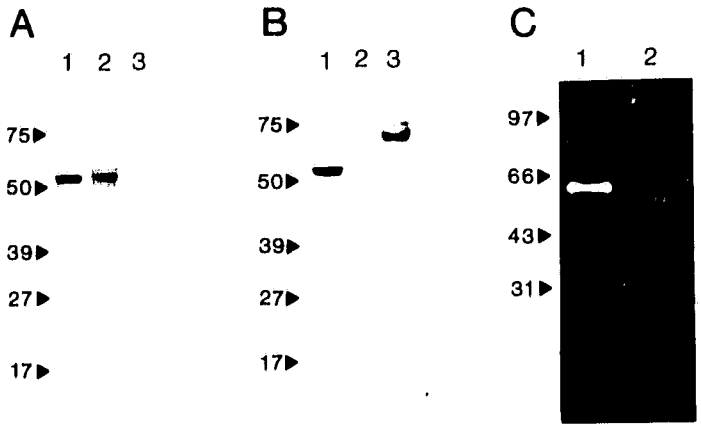


Figure 2-6. Endo-F deglycosylated *L. major* gp63 treated with periodate to destroy remaining susceptible carbohydrate residues. (A) Western blot analysis of an SDS-PAGE probed with gp63 polyclonal antiserum. (B) Western blot analysis of an SDS-PAGE probed with biotinylated concanavalin A. (C) Zymogram of deglycosylated gp63 before and after periodate oxidation. Lane 1 in (A), (B) and (C) represents *L. major* gp63 deglycosylated with Endo-F; lane 2 represents deglycosylated gp63 after periodate oxidation; and lane 3 is the control human transferrin. Markers on left side represent mol. wt. standards expressed in kDa.

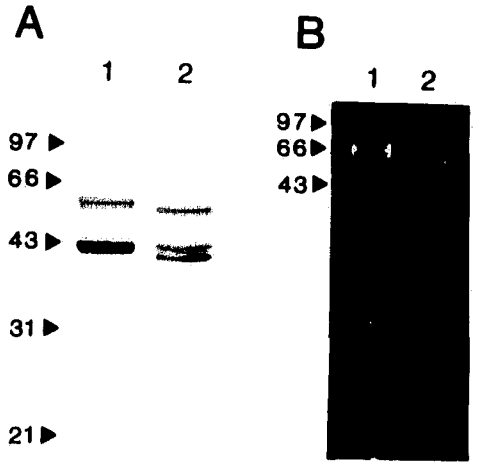


Figure 2-7. (A) Coomassie blue stained SDS-PAGE of PI-PLC released proteins from *L. m. amazonensis* promastigotes before and after treatment with Endo-F. Lane 1, untreated protein; lane 2, 24 h digestion with Endo-F. (B) Zymogram of *L. m. amazonensis* gp63 before (lane 1) and after (lane 2) treatment with Endo-F.

Resistance of deglycosylated gp63 to enzymatic degradation.

Chaudhuri and Chang (1988) suggested that the observed decrease in gp63 enzyme activity following treatment with PNGase F was a result of increased autolysis due to deglycosylation. The hypothesis that the N-linked oligosaccharides of gp63 protect the protein form proteolytic digestion, both from autolysis and lysosomal proteolysis was further investigated. Gp63 synthesized in the presence of TM and gp63 deglycosylated using Endo-F were incubated at room temperature to determine if these forms of gp63 were more susceptible to autolysis than fully glycosylated gp63. Following 6 days at room temperature at a concentration of 0.3 mg ml^{-1}, there was no observable loss of native or TM treated gp63 (Fig. 2-8A). *L. major* gp63 which had been deglycosylated with Endo-F was also resistant to autolysis during the 6 day incubation period (Fig. 2-8B) and analysis of this protein on a substrate containing gel clearly demonstrated that gp63 remained active (Fig. 2-8C).

The apparent resistance of gp63 to proteolysis was further investigated by determining its susceptibility to cathepsin D, a lysosomal proteinase. The resistance of gp63 to proteolysis by cathepsin D was assessed using active, inactive and heat denatured gp63 as described in the Methods section. Digestion of HT by cathepsin D in the presence of 1, 10-phenanthroline demonstrated that the concentration of inhibitor used had no affect on cathepsin D activity (Fig. 2-9B). Both active and inactive gp63 were found to be resistant to degradation by cathepsin D regardless of the level of glycosylation (Fig. 2-9A, lanes 2, 3, 6, 7, 10 and 11). However, the various forms of gp63 were readily degraded by cathepsin D following heat denaturation (Fig. 2-9A, lanes 4, 8 and 12). These data indicated that the observed resistance to proteolysis was dependent on the tertiary structure of gp63 and independent of N-linked oligosaccharides.

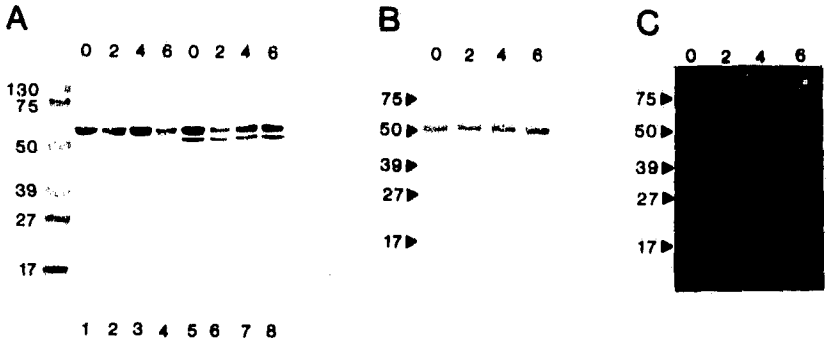


Figure 2-8. Resistance of deglycosylated gp63 to autolysis. (A) Western blot analysis demonstrating the resistance of native and TM generated gp63 to autolysis. Numbers along the top of the figure represent incubation time in days. Lanes 1-4 are native gp63; lanes 5-8 represent gp63 generated in the presence of TM. (B) Western blot analysis of Endo-F treated gp63 after incubation for 0, 2, 4 and 6 days at room temperature. (C) Zymogram of Endo-F treated gp63 demonstrating that it remained active throughout the incubation period.

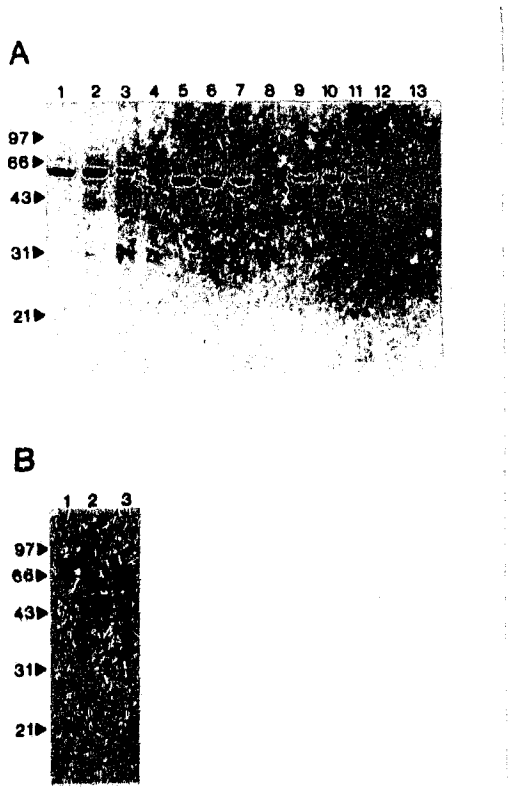


Figure 2-9. (A) The susceptibility of native (lanes 1-4), Endo-F deglycosylated (lanes 5-8) and Endo-F/periodate treated (lanes 9-12) gp63 to hydrolysis by cathepsin D. Lanes 1, 5 and 9 are the above forms of gp63 after 24 h incubation with out cathepsin D. Lanes 2, 6 and 10 represent active gp63 incubated with cathepsin D for 24h. Lanes 3, 7 and 11 represent gp63 inhibited with 1, 10-phenanthroline and then exposed to cathepsin D for 24 h. Finally, lanes 4, 8 and 12 represent heat denatured gp63 after 24 h incubation with cathepsin D. Lane 13 is cathepsin D. (B) Digestion of HT by cathepsin D in the presence of 1,10-phenanthroline. Lane 1 is the substrate control incubated over night in the presence of inhibitor. Lane 2 is cathepsin D and lane 3 shows digestion of HT by cathepsin D in the presence of 1,10-phenanthroline.

DISCUSSION

The objective of this study was to investigate whether any of the established roles for glycoprotein oligosaccharides could be ascribed to the N-linked sugars of *L. major* gp63. Three distinct methods of eliminating or degrading these sugars, under conditions which maintained enzyme activity, produced results contrary to earlier findings and in support of the conclusion that N-linked oligosaccharides are not required for the protease activity of gp63. Although both enzymatic deglycosylation and inhibition of N-linked glycosylation with tunicamycin left an intact Con A ligand on gp63, we have shown that periodate oxidation, which cleaves all sugars having vicinal hydroxyl groups, destroys the structure responsible for this interaction without affecting the protease activity. This independence of enzyme activity from carbohydrate did not appear to be a function of species as deglycosylated *L. m. amazonensis* gp63 remained active in zymograms and rates of proteolysis for this enzyme did not decrease following enzymatic deglycosylation. Finally, it was shown that the substrate specificity of gp63 toward the synthetic peptide, pep13, and oxidized insulin B-chain was similarly unaffected by enzymatic deglycosylation and periodate oxidation. Thus, no evidence could be obtained for any requirement for glycosylation in the enzymatic function of gp63. Wilson and Hardin (1990) characterized an enzymatically inactive gp63 derived from TM-resistant mutants which was suggested to be inactive due to the absence of N-linked oligosaccharides. In light of the present findings, it appears likely that some other change, apart from the absence of N-linked oligosaccharides, must have occurred during the extended culture of the parasites for mutant selection (5-24 months). It was of interest that the enzymatically inactive gp63 described by the previous investigators, retained Con A binding activity, as was found in the present study. Although it was suggested by the previous workers that the presence of O-linked glycosylations might be an explanation, this also seems unlikely as Con A binding requires a terminal glucose, mannose, or α2-linked mannose (Beeley, 1985) - all rare in O-linked oligosaccharides (Sadler, 1984). Moreover, to-date it has not been possible to isolate an O-linked sugar from any gp63, despite characterization of structures from several species (Olafson et al., 1990). It appears likely that Con A binding activity is

more readily explained by the presence of the α2-linked mannose residue present in the glycan tail (Schneider et al., 1990) which remains intact in the presence of TM and after endoglycosidase F treatment but is readily destroyed by periodate.

The function of the gp63 N-linked oligosaccharides remains unsolved. Not only do these data show that sugars are unessential for maintenance of enzyme activity but it can be concluded that N-linked glycosylation is not required to initiate proper folding of the enzyme. In addition, biotinylation of intact *L. major* promastigotes, grown in the presence of TM, demonstrated the surface orientation of non-glycosylated gp63, thus indicating that the oligosaccharides were not required for correct cellular targeting. It is interesting to note that the TM treated promastigote membranes appeared to have released more biotinylated proteins into the PI-PLC buffer than the untreated membranes. A possible explanation of this is that the accessibility of primary amines may have been increased in the absence of N-linked oligosaccharides resulting in the increased level of biotinylation. This was supported by SDS-PAGE analysis, with Coomassie blue staining, revealing no qualitative or quantitative differences between the PI-PLC extracts from treated verses untreated membranes.

Perhaps most surprising of all, these data have demonstrated that the N-linked oligosaccharides do not protect gp63 from autolysis or proteolysis by lysosomal proteases. Rather, it is possibly the tertiary structure which renders gp63 inherently resistant to proteolytic attack. It is of interest that the only apparent testable biological role for the N-linked oligosaccharides of gp63 remaining, is one which has largely fallen out of favour, in the context of the mammalian host phagocytes, and that is as a ligand for the mannosyl-fucosyl receptor (Russell and Wilhelm, 1986; Wilson and Hardin, 1990). However, the role of these structures as lectin ligands in the insect gut remains unexplored.

ACKNOWLEDGEMENTS

I would like to thank Darryl Hardie for making the gp63 peptides and for conjugating them to KLH.

CHAPTER 3

Structure and glycosylation fidelity of the *L. major* gp63 N-linked oligosaccharides - comparison with the predominant glycoprotein glycans in the genus *Leishmania* and with life cycle stage.

INTRODUCTION

The molecular basis underlying *Leishmania* infectivity and virulence is poorly understood but changes in the composition of surface carbohydrates have been implicated with an increase in virulence (Sacks et al., 1985; Saraiva et al., 1986; da Silva et al., 1987; Howard et al., 1987; Davies et al., 1990; Jacobson et al., 1990). The only characterized glycoform known to undergo structural variation during metacyclogenesis is the cell surface lipophosphoglycan (LPG) (Sacks and da Silva, 1987; Sacks et al., 1990). These changes have been correlated with increased promastigote survival in mononuclear phagocytes (Handman et al., 1986; Elhay et al., 1990; McConville and Homans, 1992; Shankar et al., 1993; McNeely and Turco, 1990).

N-linked oligosaccharides have also been implicated in parasite virulence (Nolan and Farrell, 1985; Kink and Chang, 1988; Wilson and Hardin, 1990) but unlike LPG, there has been only limited structural information reported (Parodi et al., 1984; Olafson et al., 1990, reviewed by Parodi, 1993). With respect to individual glycoproteins, only the N-linked oligosaccharides of *L. mexicana amazonensis* gp63 have been well characterized (Olafson et al., 1990). The glycans of this protease were found to consist of high mannose type structures in addition to a unique, terminally glucosylated species. To what extent these glycoforms are utilized in other leishmanial proteins or in gp63 of other species is unknown. The glycosylation fidelity of leishmanial proteins also remains unexplored - that is, are all the putative N-linked sites of a particular protein occupied and are they always occupied by the same glycan(s)? This type of information is important for the evaluation of the potential significance of these molecules to parasite pathogenicity - particularly as it pertains to species differences and changes occurring during the parasite life cycle. Where possible emphasis was placed upon the ubiquitous major surface protease gp63 (Etges et al., 1985; Bouvier et al., 1987; Etges, 1992), reported in some species to be a potential virulence factor (Kweider et al., 1987; Wilson et al., 1989) and whose N-linked oligosaccharides have also been implicated as virulence factors (Wilson and Hardin, 1990). In order to allow a more generalized comparison of *Leishmania* N-linked oligosaccharides, the present work describes the predominant oligosaccharides

from promastigotes of several *Leishmania spp.* and compares them with those found on the amastigote life-stage.

MATERIALS AND METHODS

Parasite cultivation

Promastigotes were cultured at 26°C in M199 medium supplemented with hemin (5 mg l^{-1}) and folate (23 μM). Penicillin/streptomycin (Sigma Chemical Company, St. Louis, MO, USA) and Basal Eagle Medium vitamin solution (Gibco, Burlington, Ontario) were added to the medium at a dilution of 1:100 (v/v). *L. m. mexicana* (MNYC/BZ/62/M379) promastigotes, were grown in 10% fetal bovine serum (FBS), *L. mexicana amazonensis* (MHOM/BR/73/M2269), *L. major* A2 (Neva et al., 1979), *L. tropica* (MHOM/SU/74/k27), and *L. donovani* LV9 promastigotes were grown in 5% FBS while *L. donovani donovani* LD3 promastigotes were maintained in the absence of FBS. All parasites were harvested by centrifugation at late log phase. *L. major* parasites were cultured for 5 days following stabilization in cell number at approximately 1 x 10^7 cells ml^{-1} and then harvested as late stationary-phase promastigotes. Log-phase promastigotes were collected when they reached a density of 5 x 10^6 cells ml^{-1}.

L. donovani LV9 amastigotes were isolated from the spleens of hamsters as described by Channon et al. (1984) while *L. m. mexicana* (MNYC/BZ/62/M379) amastigotes were grown axenically as described by Bates et al. (1992).

Purification of *L. major* gp63 for oligosaccharide analysis.

Pelleted *L. major* A2 promastigotes were frozen and thawed in 50 mM Tris, pH 7.5 and sonicated on ice at 45 Watts for 3 x 10 s to lyse cells. Peripheral membrane proteins were dissociated by sequentially resuspending the membranes in lysis buffer containing 3 M NaCl and centrifuging at 20 000 x g for 20 min. After 3 repetitions, the integral membrane proteins were extracted by stirring for 24 h at 4°C in 1% octylthioglucoside (Sigma). The resulting extract was diluted 5 fold with buffer A (20 mM Tris, pH 7.2, 1 mM each of $CaCl_2$, $MgCl_2$ and $MnCl_2$ and 0.1% CHAPS (Sigma) and applied to a concanavalin A (Con A) Sepharose column (Pharmacia, Baie d'Urfe, Que.) at a flow rate of 0.2 ml min^{-1}. The column was washed with 2 bed volumes of buffer A to remove non-specifically bound protein followed by elution with 3 bed volumes of buffer B

(buffer A with 500 mM methylmannopyranoside and 400 mM NaCl) to elute bound ligands. The Con A eluate was dialyzed, lyophilized and resuspended in SDS sample buffer (60 mM Tris, pH 6.8, 3% SDS, 10% glycerol, 0.2% β-mercaptoethanol) and the proteins separated using a model 230A micropreparative electrophoresis system (Applied Biosystems Inc., Foster City, CA). Electrophoresis was performed using a 7.5% polyacrylamide gel (2.5 x 50 mm) with a constant current producing a voltage range of 180-260V. Proteins were detected at 280 nm at a sensitivity of 0.5 AUFS. The upper buffer consisted of 25 mM Tris/HCl, 192 mM glycine, 0.1%SDS, while the lower and elution buffers were 25 mM Tris/HCl, pH 8.3. Fractions were assessed for purity by standard SDS-PAGE using a 12.5% gel (Laemmli, 1970). The purified gp63 was dialyzed, lyophilized and SDS was extracted by washing the precipitate 3 times with cold acetone/water (20:1 v/v) allowing 20 min incubation on dry ice to maximize precipitation of protein. Centrifugation was carried out at 20,000 x g for 20 min. A final wash with 100% acetone facilitated drying of the sample.

Isolation of neutral oligosaccharides using anhydrous hydrazine

Oligosaccharides were release from purified, anhydrous gp63 (1-2 mg) by treatment with 0.5 ml anhydrous hydrazine (Oxford GlycoSystems Ltd., Rosedale, NY) for 4 h at 95°C under argon. The hydrazine was then removed under reduced pressure and residual reagent was expelled by repeated evaporation in the presence of 0.5 ml toluene. The glycans were re-N-acetylated by adding 0.4 ml of saturated $NaHCO_3$ and 20 µl acetic anhydride. The samples were reacted on ice for 10 min and for an additional 40 min at room temperature following addition of a further aliquot of acetic anhydride. Sodium ions were removed by ion exchange on a 0.5 ml column of Ag50- X12 [+H] (BioRad). Samples were then loaded onto 3 mm Whatman paper that had been prewashed for 24 h with water and subsequently dried. The samples were washed for 36 h with N-butanol, ethanol, water (4 : 1 : 1 v/v) by descending chromatography. The area including the origin and 5 cm in the direction of flow was cut from the paper and the oligosaccharides eluted with water. Residual hydrazides were removed by incubation in 0.5 ml 1 mM cupric acetate at 27°C for 30 min and the copper subsequently removed

using a 1.0 ml mixed column of Ag50-X12[+H]/Chelex resin (BioRad). The glycans were then taken to dryness and 4 mCi of ^{3}H-$NaBH_4$ (American Radiolabeled Chemicals Inc., St. Louis, MO), resuspended in 200 µl of 300mM borate buffer, pH 11.0, was added to each sample. Reduction occurred for 4 h at 30°C. To ensure that all glycans were reduced, samples were incubated for a further 2 h in the presence of 1.0 M Na $B^{2}H_4$. Excess borohydride was expelled by successive additions of 20 µl of 1 M acetic acid. Sodium ions were removed by ion exchange chromatography on a 0.5 ml column of Ag50-X12[+H] and then dried repeatedly under vacuum after additions of 0.5 ml acidified methanol (5% acetic acid in methanol). After washing again in n-butanol, ethanol, water as previously described, neutral species were isolated by high voltage electrophoresis (HVE) performed as follows. Samples were spotted onto prewashed 3 mm Whatman chromatography paper which was then wet with the electrophoresis buffer containing 7.7 ml pyridine and 2.5 ml acetic acid per 1.0 l of water. Samples were electrophoresed at 2000 V for 20 min. Neutral oligosaccharides were recovered from the origin and separated by gel permeation chromatography on Bio-gel P-4 (BioRad Laboratories Inc., Mississauga, Ontario) prior to sequential exoglycosidase digestion (Parekh et al., 1987).

N, N' diacetylchitobiose (Sigma) and human transferrin (HT) were used as standards. Oligosaccharides released from HT were desialylated with neuraminidase from *Arthrobacter ureafaciens*, EC 3.2.1.18 (Boehringer Mannheim) in 50 mM sodium acetate, pH 5.0, prior to HVE.

<u>Isolation of neutral oligosaccharides using endoglycosidases endo-β-N-acetylglucosaminidase H (Endo-H) and N-glycanase F</u>

The N-linked oligosaccharides from 2.0 mg of *L. major* gp63 were released from the protein enzymatically using 60 mU ml^{-1} of Endo-H (EC 3.2.1.96) (Boehringer Mannheim) in 50 mM phosphate buffer, pH 5.5. Oligosaccharides were also removed from 1.0 mg of gp63 using N-glycanase F (EC 3.5.1.52) (New England Biolabs, Mississauga, ON) at a concentration of 40,000 U ml^{-1}. Gp63 was incubated in the presence of these enzymes for 16 h at 37°C and subsequent analysis by SDS-PAGE showed that deglycosylation had occurred. Protein and ions were then removed using a 3.0 ml mixed column of Ag

50-X12[+H]/Ag 3-X4[OH-]. The column eluate, containing the released glycans, was taken to dryness and the glycans were subsequently reduced with ^{3}H-$NaBH_4$ and purified as described above.

Exoglycosidases

Jack Bean α-mannosidase, EC 3.2.1.24 (Boehringer Mannheim) was purified according to the method of Li and Li (1972). The activity was measured using 3 mM p-nitrophenyl- α-D-mannopyranoside as the substrate in 200 mM citrate, pH 4.5 with 0.2 mM zinc acetate at an enzyme concentration of 10 U ml^{-1}. β-galactosidase from bovine testes, EC 3.2.1.23 (Boehringer Mannheim) was used at a concentration of 500 mU ml^{-1} in the presence of 100 mM citrate phosphate, pH 4.3 with 0.5% Triton-X-100 while β-mannosidase from *Helix pomatia*, EC 3.2.1.35 (Oxford GlycoSystems Ltd.) was used at a concentration of 2 U ml^{-1} in 100 mM citrate, pH 4.0 and the α1-2 specific mannosidase from *Aspergillus saitoi*, EC 3.2.1.24 (Oxford GlycoSystems Ltd.) was used at a concentration of 250 x 10^{-6} U ml^{-1} in 10 mM sodium acetate, pH 5.0. Jack Bean β-hexosaminidase, a gift from Oxford, Glycobiology unit was used at a concentration of 2 U ml^{-1} in 100 mM citrate, pH 5.0. Glucosidase II was a gift from Dr. Terry Butters (Glycobiology Unit, Oxford). All enzymes were checked for extraneous enzyme activities.

Partial acetolysis

Acetolysis was performed after the method of Kocourek and Ballou (1969), with the following modifications. Samples were peracetylated in a 1:1 mixture of acetic anhydride and pyridine for 20 min at 100°C, then evaporated under reduced pressure. Acetolysis of the acetylated product was carried out in 30 µl of acetic anhydride, acetic acid, concentrated sulfuric acid in a ratio of 10:10:1 (v/v) for 6 h at 37°C, followed by addition of 10 µl pyridine to neutralize the solution. The sample was dried under reduced pressure, and the acetolysis products were dissolved in 0.4 ml of chloroform and washed 4 times with 0.6 ml water. The chloroform phase was taken to dryness and evaporated under reduced pressure in the presence of toluene to remove traces of acetic acid.

Deacetylation was performed at 37°C for 18 h in a solution containing equal parts of methanol and 35% NH_4OH (aqueous). Re-N-acetylation was carried out for 10 min on ice in the presence of 200 µl saturated $NaHCO_3$ and 20 µl acetic anhydride. Samples were incubated at room temperature for an additional 40 min after the addition of a further aliquot of acetic anhydride. Sodium ions were removed by ion exchange on a 1 x 2 cm column of AG 50-X12 [H+].

Linkage analysis

Partially permethylated alditol acetates (PMAA) were made using a modified method of Anumula and Taylor (1992). The NaOH-methyl sulfoxide (DMSO) reagent was prepared by mixing 0.1 ml of 50% NaOH with 0.2 ml methanol and diluting with 6 ml DMSO. The suspension was vortexed vigorously and the NaOH precipitate collected after brief centrifugation. The NaOH precipitate was washed 5 times with DMSO and finally resuspended in 2 ml of this solvent. DMSO (100µl) was added to each oligosaccharide sample and sonicated for 15 min at room temperature. After addition of 100 µl of the NaOH-DMSO reagent, samples were stirred for 20 min, followed by addition of 50 µl of iodomethane, an additional 5 min sonication and 5 min of stirring. Another aliquot of iodomethane was then added and the sample allowed to react for a further 30 min. The reaction was stopped by the addition of 0.4 ml of water and the methylated oligosaccharides were partitioned into 0.4 ml of chloroform, washed 5 times with 0.5 ml water and finally taken to dryness under reduced pressure. The methylated oligosaccharides were then hydrolyzed in 150 µl of formic acid, water, trifluoracetic acid (TFA) in a ratio of 10:2:1 for 16 h at 100°C. Following addition of 150 µl of freshly prepared 5% pyridine in 50% acetonitrile/water to neutralize residual acids, samples were dried under reduced pressure. The partially methylated monosaccharides were then reduced for 16 h at room temperature with 150 µl sodium borodeuteride (12 mg ml^{-1}) in 30% methanol containing 30 mM NaOH and the reaction was stopped by addition of 2N acetic acid. Acetylation was achieved by the addition of 100 µl acetonitrile containing 5 mg ml^{-1} dimethylaminopyridine, 100 µl acetic anhydride and 33 µl pyridine to the dry

sample followed by reaction at room temperature for 4 h. After terminating the reaction by the addition of 300 µl water, the PMAA were partitioned into 400 µl dichloromethane, the organic layer washed 6 times with water and then taken to dryness under a stream of nitrogen.

Analysis of the PMAA was performed on a Hewlett-Packard 5890 series II gas chromatography system interfaced with a Kratos Concept-H double sector magnetic mass spectrometer (GC/MS) and flame ionization detection. PMAA were separated using a J & W DB-1 column (0.25 mm x 30 m) (Altech, Deerfield, IL) with helium as the carrier gas (2.0 ml min^{-1}). Samples were loaded using a splitless injector and developed using the following temperature program: 60°C for 2.0 min, followed by a linear increase to 140°C at 40° per min, held for 4.0 min and then another linear increase to 250°C at 4° per min whereupon the temperature was held for 20 min. Separation of the PMAA corresponding to terminal-linked glucose, and mannose was achieved by capillary gas-liquid chromatography on a SP-2380 (0.32 mm X 60 m, Chrompak, London) with helium as the carrier gas. Direct on-column injection was employed with a temperature program of 90°C (held for 1 min), followed by a linear increase to 170°C at 30°C min^{-1} and then 170°C to 230°C at 5°C min^{-1}, 20 min hold and then to 231°C at 5°C min^{-1} and hold 15 min.

Time of flight mass spectral analysis (TOF MS) and Fast atom bombardment mass spectral analysis (FAB-MS)

TOF-MS samples were analyzed in a matrix of DHB/Fucose using a Kratos Kompact MALDI 3 V2.0.1.

Positive ion FAB-MS were obtained using magnetic sector 1 of a JEOL JMS-SX/SX102A tandem mass spectrometer operated at 10 kV accelerating voltage. The FAB gun was operated at 6 kV accelerating voltage with an emission current of 10 mAmps using xenon as the bombarding gas. Spectra were scanned at a speed of 30 seconds for the full mass range specified by the accelerating voltage used and were recorded and averaged on a Hewlett packard HP9000 data system running JEOL COMPLEMENT software.

Collision induced dissociation mass spectra (CID-MS) were recorded using the same machine, with helium as the collision gas in the third field free region collision cell, at a pressure sufficient to reduce the parent ion to one third of its original intensity.

Underivatized oligosaccharides were dissolved in 5 µl 5% acetic acid (aqueous), and 1.5 µl aliquots of sample solution were loaded into a matrix of mono-thioglycerol.

Compositional analysis

Isolated oligosaccharides were hydrolyzed in 2N HCl at 100°C for 3 h, neutralized with NH_4OH and the Cl^{-1} removed using AG 3-X4 (OH^-) resin. Monosaccharides were separated by anion exchange chromatography using a PA-1 HPLC column developed isocratically with 16 mM NaOH and monitored with a pulsed amperometric detector (Dionex Corporation, Mississauga, Ont.).

Metabolic labelling of gp63 with ^{3}H-mannose

L. major promastigotes (1×10^{10}) were resuspended in 100 ml of glucose free Dulbecco's Modified Eagle Medium (Gibco) and incubated in the presence of 0.5 mCi ^{3}H-mannose (Du Pont) for 16 h at 26°C. The media was supplemented with vitamins, folic acid and hemin as described for M199. Adenosine (0.1 mM), pyruvate (1.0 mM), β-mercaptoethanol (50 mM), glutamine (2.0 mM), gentamycin (1 ml l^{-1}) and 5% FBS were also added.

The radiolabelled cells were harvested by centrifugation and washed 4 times with phosphate buffered saline (PBS) before lysis by freeze/thaw in 50 mM Tris/HCl, pH 7.2. The membranes were washed 4 times with lysis buffer and resuspended in 0.5 ml 25 mM HEPES, pH 7.2 containing 2.0 mM EDTA and 0.1% CHAPS. Phosphatidylinositol specific phospholipase-C (PI-PLC) (EC 3.1.4.10) from *Bacillus cereus* (Boehringer Mannheim) was added to a concentration of 100 mU ml^{-1} and digestion proceeded overnight at 37°C with stirring. Toluene (10 µl) was also added to prevent bacterial growth. The supernatant, enriched with radiolabelled delipidated gp63, was pooled with approximately 1.4 mg of unlabelled gp63 prepared in an identical manner.

Preparation, isolation and identification of gp63 glycopeptides

The gp63 in the pooled PI-PLC supernatant was purified to homogeneity by gel permeation HPLC using a Beckman Ultraspherogel SEC 3000 column (7.5 mm x 30 cm). Proteins were eluted with 100 mM Tris, pH 7.5, containing 150 mM NaCl at a flow rate of 0.5 ml min^{-1}. Gp63 was identified by an HPLC based protease assay (Chapter 1, Methods) and by SDS-PAGE analysis. The purified gp63 was then heat denatured to prevent autolysis and dialyzed. Sufficient NH_4HCO_3 was then added to make a 100 mM solution, pH 7.6. Disulfides were reduced over night at room temperature with stirring in 10,000 molar excess of β-mercaptoethanol and then pyridylethylated using 4-vinylpyridine in a molar ratio of 3:1 to β-mercaptoethanol. The solution was allowed to react for 15 min at room temperature with stirring. Excess 4-vinylpyridine was removed by partitioning with water saturated butanol.

The pyridylethylated gp63, in 1.0 ml of 100 mM NH_4HCO_3, pH 7.6, was digested with 600 mU of endoproteinase Lys-C from *Lysobacter enzymogenes* (Boehringer Mannheim) at 37°C. After 2 h incubation, another aliquot of 600 mU of Lys-C was added and digestion proceeded overnight. Digestion was stopped and HCO_3^{-1} expelled by addition of anhydrous TFA. The peptides were resolved using a C_8 reverse-phase HPLC column (Aquapore RP-300, 100 x 22 mm, Applied Biosystems Inc., Foster City, CA) developed using a 0.1% TFA/acetonitrile gradient from 0 - 60% acetonitrile over 75 min at a flow rate of 0.5 ml min^{-1}. The peptides were detected at 230 nm using a detector sensitivity of 0.2 AUFS and the resulting fractions were dried in a speed-vac and resuspended in 100 µl 10% methanol in water. Twenty microlitres of each fraction was counted in a liquid scintillation counter (Beckman LS8100) and the fractions containing radioactivity were spotted onto Immobilon membrane (Biorad Laboratories Inc., Mississauga, Ont.) and sequenced using an Applied Biosystems, Model 475, protein sequencer. An aliquot of the column void was subjected to amino acid analysis using an Applied Biosystems Inc., Model 420H instrument to determine if any peptides were present in this fraction.

Identification of gp63 glycopeptides by binding to Concanavalin A Sepharose

Lys-C peptides of *L. major* gp63, generated as described above, were incubated with 0.2 ml Con A Sepharose (Pharmacia) in 0.6 ml 50 mM Tris/HCl, pH 7.4 containing 1 mM $CaCl_2$, $MnCl_2$ and $MgCl_2$ with 0.1%CHAPS. The samples were rotated overnight at 4°C to allow binding of glycosylated peptides. Unbound peptides were washed off the gel by rinsing the gel 5 times with 0.5 ml of buffer. The bound peptides were then released by incubating the gel in 0.5 ml buffer containing 0.5 M methylmannoside and 0.4 M NaCl for 2 h at 4°C. Peptides from the bound eluate were chromatographed on a reverse-phase HPLC column and the resulting peptides were sequenced as described above. The glycopeptides were then treated with hydrazine and the released glycans analyzed by P-4 chromatography as described above.

Isolation of predominant *Leishmania* surface glycoprotein glycans

Washed membranes from 1 x 10^{10} parasites were stirred for 48 h at 4°C in 100 mM Tris, pH 8.0 with 1% octylthioglucoside to extract membrane proteins. The homogenate was centrifuged at 20,000 x g for 30 min and the supernatant dialyzed for 72 h at 4°C against several changes of water. The samples were then lyophilyzed and residual detergent removed by washing with acetone as described above. Neutral oligosaccharides were then isolated as previously described.

RESULTS

Structural analysis of *L. major* gp63 oligosaccharides released by hydrazinolysis

Gp63 isolated from *L. major* A2 promastigotes, in the log or stationary phase of their growth cycle, carried two predominant neutral oligosaccharides having hydrodynamic volumes corresponding to 10.5 and 9.6 glucose units (g.u.) (G10.5 and G9.6) (Fig. 3-1A). Digestion of G9.6 with the α1-2 specific mannosidase resulted in a decrease in hydrodynamic volume of 2.1 g.u. (Fig 3-1B). Incomplete digestion with this exoglycosidase produced two products with hydrodynamic volumes of 8.5 and 7.5 confirming that the reduction of 2.1 g.u. represented the loss of two mannose residues. Partial acetolysis of G9.6 produced an oligosaccharide of 7.9 g.u. (Fig. 3-1C) indicating the presence of 2 mannose residues extending from C6 of the β-linked mannose. Digestion of the latter product with α1-2 specific mannosidase again resulted in the loss of 2.0 mannose residues (data is summarized in Table 3-2) and digestion with Jack Bean α-mannosidase (JBAM) yielded a product having a hydrodynamic volume of G5.2 (Fig. 3-1D). These results indicated that 3 mannose residues remained linked to the 3 position of the β-linked mannose following acetolysis and further, the terminal and penultimate mannose residues were linked α1-2. It is important to note that because these oligosaccharides are not linear but rather branched and therefore globular, all of the mannose residues do not contribute equally to the hydrodynamic volume. For example, removal of residues from the 6-arm of the β-linked mannose results in a smaller decrease in the hydrodynamic volume compared to removal of mannose residues from the 3-arm. The G5.2 product, resulting from treatment with JBAM, co-eluted with the standard Manβ1-4GlcNAcβ1-4GlcNAc-ol, obtained from human transferrin and results from subsequent digestions with β-mannosidase and β-hexosaminidase were consistent with this structure. GC/MS analysis of the PMAA derived from G9.6 (Table 3-1) confirmed the linkages of the structure proposed for G9.6 (Figure 3-2). Finally, FAB^+ MS analysis revealed an intense $M + H^+$ pseudomolecular ion at 1400 a.m.u. (Figure 3-3) which is consistent with this structure.

The oligosaccharide eluting at G10.5 was resistant to digestion by JBAM suggesting terminal residues other than mannose. G10.5 was also resistant to digestion by α-glucosidase II. Analysis of G10.5 by FAB^+ MS produced an intense $M + H^+$ pseudomolecular ion that was only 38 a.m.u. larger than G9.6 (Figure 3-4). CID MS-MS analysis of the two $M+H^+$ pseudomolecular ions from G10.5 and G9.6 yielded identical A^+-type ions at m/z 1176 (data not shown). This type of ion arises by glycosidic cleavage between the two residues at the reducing terminus, with charge retention on the second GlcNAc. The presence of an intense A^+-type ion at m/z 1176 from both G10.5 and G9.6 indicated that these glycans differed only at the reduced GlcNAc-ol. An increase in molecular weight of 38 a.m.u. is consistent with a molecular rearrangement reported by Michalski et al. (1984) which occurs during the hydrazinolysis procedure. A 1-deoxyfructose hydrazone is produced which becomes diacetylated during the re-N-acetylation procedure. TOF mass spectral analysis of G9.6 and G10.5 also showed that the two glycans differed by 38 amu. Figures 3-5 and 3-6 show intense $M+Na^+$ pseudomolecular ions at m/z 1424 and 1462 for G9.6 and G10.5, respectively. The difficulty with these results is that the 1-deoxyfructose hydrazone would not become labelled with ^3H. Therefore, the G10.5 fraction must contain two distinct molecules - a radiolabelled molecule and a 1-deoxyfructose hydrazone derivative of G9.6 which originated during the hydrazinolysis procedure and co-eluted with the radiolabelled unknown oligosaccharide (G10.5). Detection of only one molecule during FAB^+ MS and TOF MS can be rationalized because the diacetylated 1-deoxyfructose hydrazone derivative is more easily ionized than the normal alditol.

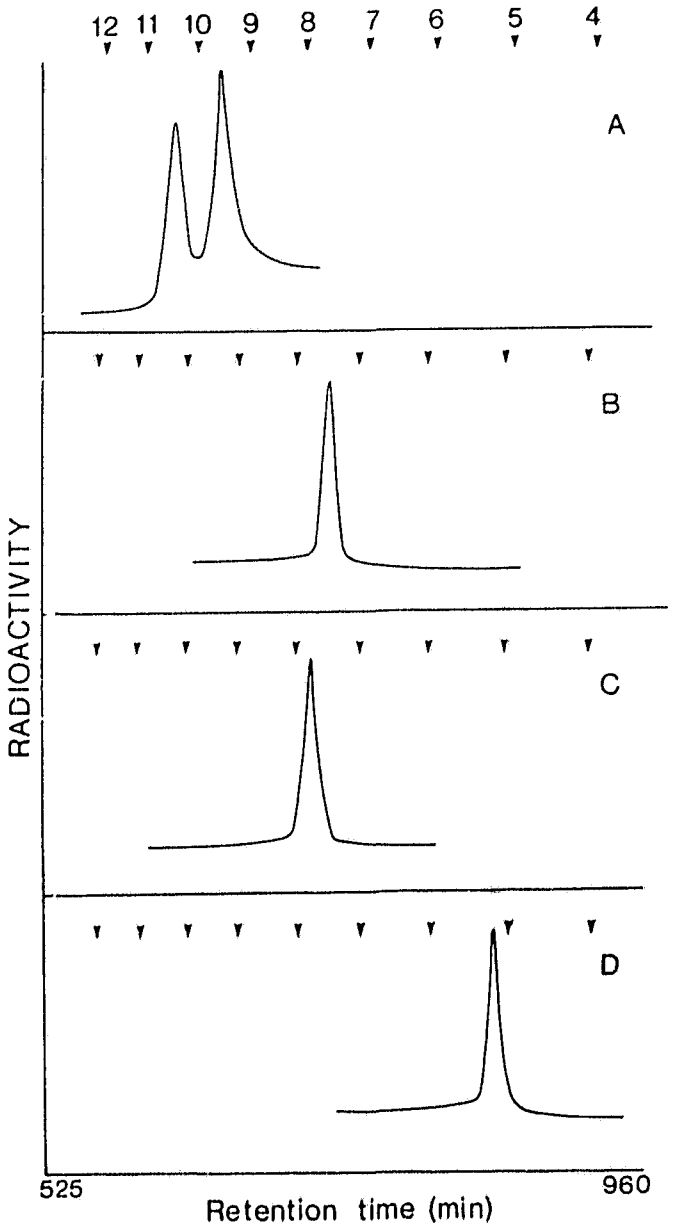


Figure 3-1: Bio-Gel P-4 chromatography of neutral oligosaccharides from *L. major* gp63. A) Neutral oligosaccharides from *L. major* gp63 eluted at 10.5 and 9.6 glucose units (g.u.). B) Treatment of G9.6 with *Aspergillus saitoi* α1-2 specific mannosidase produced a product of 7.5 g.u. C) Partial acetolysis of G9.6 resulted in an oligosaccharide eluting at G7.9. D) The product of acetolysis (G7.9) eluted at G5.2 after digestion with JBAM and coeluted with Manβ1-4GlcNAcβ1-4GlcNAc-ol from human transferrin. Arrows along the top of each figure represent the elution position of partially hydrolyzed dextran polymers and the numbers indicate the number of residues in each polymer.

Structural analysis of oligosaccharides released enzymatically

Enzymatic removal of the oligosaccharides from gp63 precluded the possibility of contamination of the larger oligosaccharide with by-products of the hydrazinolysis procedure. The oligosaccharides of gp63 were released using Endo-H and N-glycanase F. Endo-H was used for 2 reasons. Firstly, cleavage by this enzyme provided structural information due to its substrate specificity: it cleaves only those oligosaccharides having the structure Manα1-3Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc (Tarentino et al., 1978). Secondly, Endo-H was efficient at removing the oligosaccharides from native gp63 and therefore, provided high yields. This was in contrast to the second endoglycosidase used, N-glycanase F, which required that gp63 be heat denatured to allow efficient removal of oligosaccharides. This resulted in lower yields compared to Endo-H because a substantial amount of gp63 precipitated during heat denaturation - even in the presence of detergent (results not shown). However, SDS-PAGE analysis showed that all the gp63 remaining in solution following heat denaturation, moved to a single, lower molecular weight band after digestion with N-glycanase F (results not shown). These results are consistent with complete removal of glycans. The use of N-glycanase F, an enzyme capable of removing all known N-linked oligosaccharide structures (Plummer et al., 1981, 1984; Tarentino et al., 1985), was necessary to ensure that the Endo-H released glycans represented all the glycans on gp63 and not just a subset due to the specificity of the latter enzyme.

Two oligosaccharides were released from gp63 using Endo-H which cleaves high mannose type glycans between the two N-acetylglucosamine residues at the reducing terminus. The resulting radiolabelled glycans had hydrodynamic volumes of G8.5 and G7.6. These glycans corresponded in size to the G10.5 and G9.6 oligosaccharides released after treatment with anhydrous hydrazine, taking into account the loss of one GlcNAc with a nominal hydrodynamic volume of 2.0 g.u. Enzymatic and chemical analysis of G7.6 confirmed that this molecule was identical to G9.6 with the exception of having one less GlcNAc at the reducing terminus. Endo-H G7.6 lost 2 mannose residues due to treatment with α1-2 mannosidase, eluting at G5.5. Treatment with JBAM resulted in a shift to G3.0 which was then susceptible to digestion by β-mannosidase and

produced a molecule which co-eluted with reduced GlcNAc. Removal of the 6-arm by acetolysis caused a shift in the hydrodynamic volume to G6.0 indicating the presence of 2 mannose residues extending from C6 of the β-linked mannose (results summarized in Table 3-2). removal of this oligosaccharide and G8.5 by Endo-H indicated that the second mannose on the 6-arm was in an α1-3 linkage. This linkage as well as all the linkages proposed for the structure of G7.6 (Fig. 3-2) were confirmed by GC/MS analysis of PMAA (Fig. 3-7).

G8.5 was resistant to digestion by α1-2 mannosidase but digestion with JBAM resulted in a shift to G6.9, representing a loss of two mannose residues. An identical shift also occurred after acetolysis. Resistance of this product to digestion by both JBAM and α1-2 mannosidase indicated that two mannose residues extend from C6 of the β-linked mannose. Treatment of G8.5 with α-glucosidase II resulted in a shift to G7.6 indicating the presence of a terminal glucose residue linked α1-3 on the 3-arm of the β-linked mannose (results summarized in Table 3-2). The presence of a terminal glucose residue was further confirmed by separation of PMAA on a Sp-2380 GC column. The G8.5 sample contained a residue that co-eluted with the terminal glucose standard (Fig. 3-8A) and was absent in the G7.6 sample (Fig. 3-8B). Once the glucose residue was removed, the product could be digested by α1-2 mannosidase and JBAM to produce G5.5 and G3.0, respectively (Table 3-2). The structure proposed for G8.5 is shown in Fig. 3-2 and the linkages were confirmed by GC/MS (Fig. 3-9).

Removal of the oligosaccharides using N-glycanase F, which cleaves the oligosaccharide between the proximal GlcNAc and the Asn residue, resulted in isolation of 2 radiolabelled glycans having hydrodynamic volumes of G10.5 and G9.6. G10.5 was susceptible to digestion by α–glucosidase II, causing a shift to G9.6. This product and the original G9.6 were found to be identical to the G9.6 isolated by anhydrous hydrazine and corresponded to the Endo-H generated G7.6.

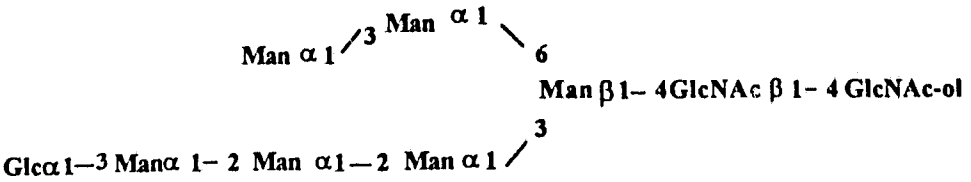
We propose that the radiolabelled molecule in the G10.5 sample, generated by treatment of gp63 with hydrazine, is the same oligosaccharide as the Endo-H generated G8.5 and the N-glycanase F generated G10.5 (Fig. 3-2).

Table 3-1: Identification of partially permethylated alditol acetates (PMAA) from G9.6 which was isolated from *L. major* gp63 following treatment with hydrazine.

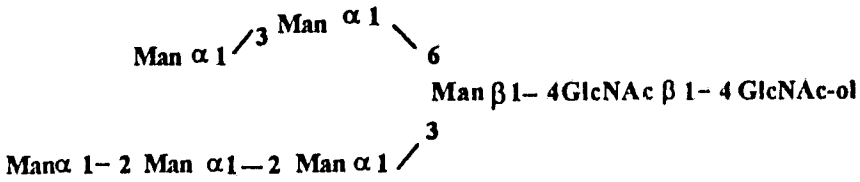
Methylated monosaccharide	Linkage	G9.6
2,3,4,6-Tetra-O-methyl (1,5-di-O-acetyl) hexose	terminal	+
3,4,6-Tri-O-methyl (1,2,5-tri-O-acetyl) hexose	2	+
2,4,6-Tri-O-methyl (1,3,5-tri-O-acetyl) hexose	3	+
2,4-Di-O-methyl (1,3,5,6-tetra-O-acetyl) hexose	3,6	+
3,6-Di-O-methyl (1,4,5-tri-O-acetyl) 2-(N-methylacetamido)-2-deoxyhexitol	4	+
1,3,5,6-Tetra-O-methyl (4-mono-O-acetyl) 2-(N-methylacetamido)-2-deoxyhexitol	4OL	+

Table 3-2: Summary of enzymatic and chemical analysis of oligosaccharides isolated from *L. major* gp63.

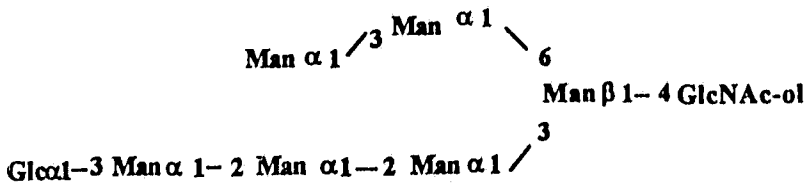
Oligosaccharide	Treatment	Product	Proposed structure
Hydrazine G9.6	α1-2 mannosidase	G7.5	Manα1-3(Manα1-3Manα1-6)Man β1-4GlcNAcβ1-4GlcNAc-ol
	acetolysis	G7.9	Manα1-2Manα1-2Manα1-3Manβ1 -4GlcNAcβ1-4GlcNAc-ol
G7.9	JBAM	G5.2	Manβ1-4GlcNAcβ1-4GlcNAc-ol
Endo-H G7.6	JBAM	G3.0	Manβ1-4GlcNAc-ol
	α1-2 mannosidase	G5.5	Manα1-3(Manα1-3Manα1-6)Man β1-4GlcNAc-ol
	acetolysis	G6.0	Manα1-2Manα1-2Manα1-3Manβ1 -4GlcNAc-ol
Endo-H G8.5	JBAM	G6.9	Glcα1-3Manα1-2Manα1-2Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc-ol
	α1-2 mannosidase	G8.5	
	acetolysis	G6.9	Glcα1-3Manα1-2Manα1-2Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc-ol
	α-glucosidase II	G7.6	Manα1-2Manα1-2Manα1-3(Manα 1-3Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc-ol
G7.6 (from G8.5)	JBAM	G3.0	Manβ1-4GlcNAc-ol
	α1-2 mannosidase	G5.5	Manα1-3(Manα1-3Manα1-6)Man β1-4GlcNAc-ol
N-glycanase F G10.5	α-glucosidase II	G9.6	See Fig. 3-2



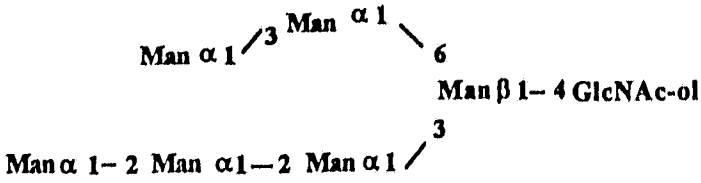
G10.5



G9.6



G8.5



G7.6

Figure 3-2: The proposed structures of the predominant neutral oligosaccharides from *L. major* gp63. G10.5 and G9.6 were isolated from *L. major* gp63 using either anhydrous hydrazine or N-glycanase F. G8.5 and G7.6 were released from gp63 using Endo-H.

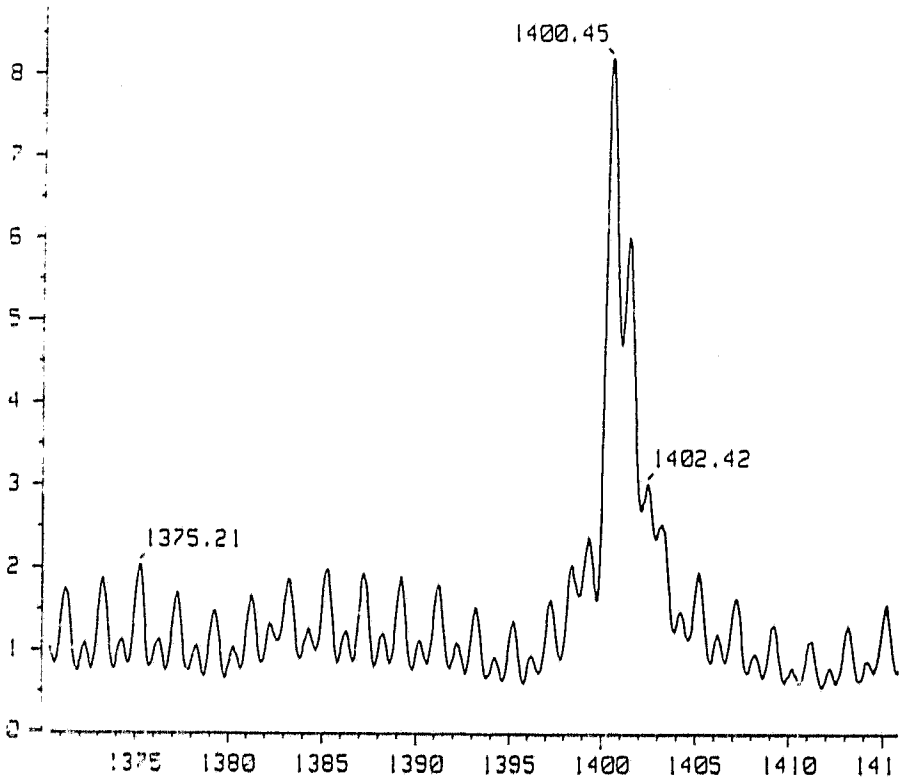


Figure 3-3: Positive ion FAB mass spectrum of G9.6 showing an intense $M+H^+$ pseudomolecular ion at m/z 1400, with a less intense $M+Na^+$ ion at m/z 1422. The ion at m/z 1400 corresponds to a deuterium-reduced $Hex_4HexNAc_2$.

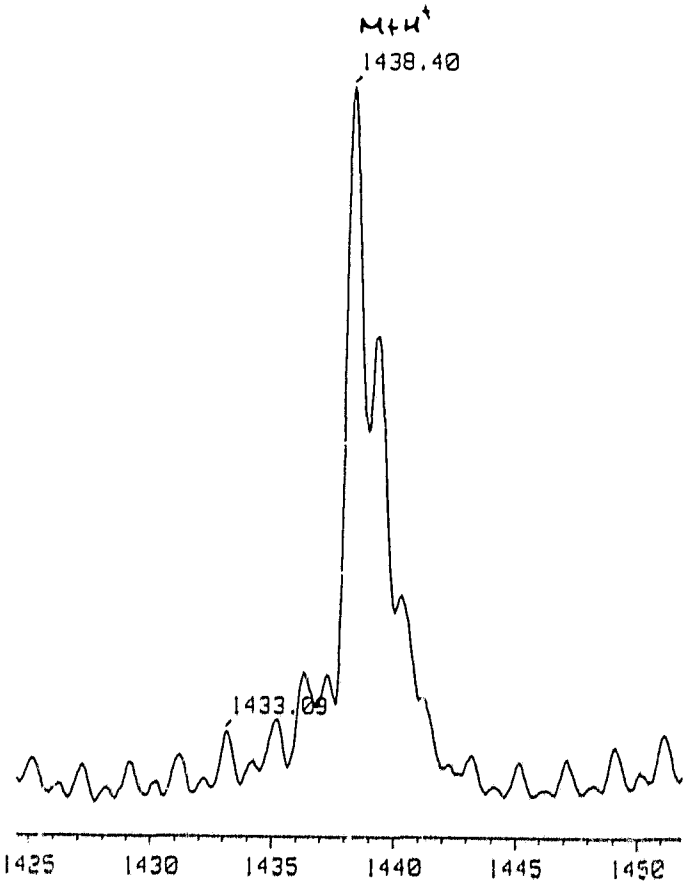


Figure 3-4: Positive ion FAB spectrum obtained from G10.5 contains $M+H^+$ and $M+Na^+$ pseudomolecular ions at m/z 1438 and 1460, respectively.

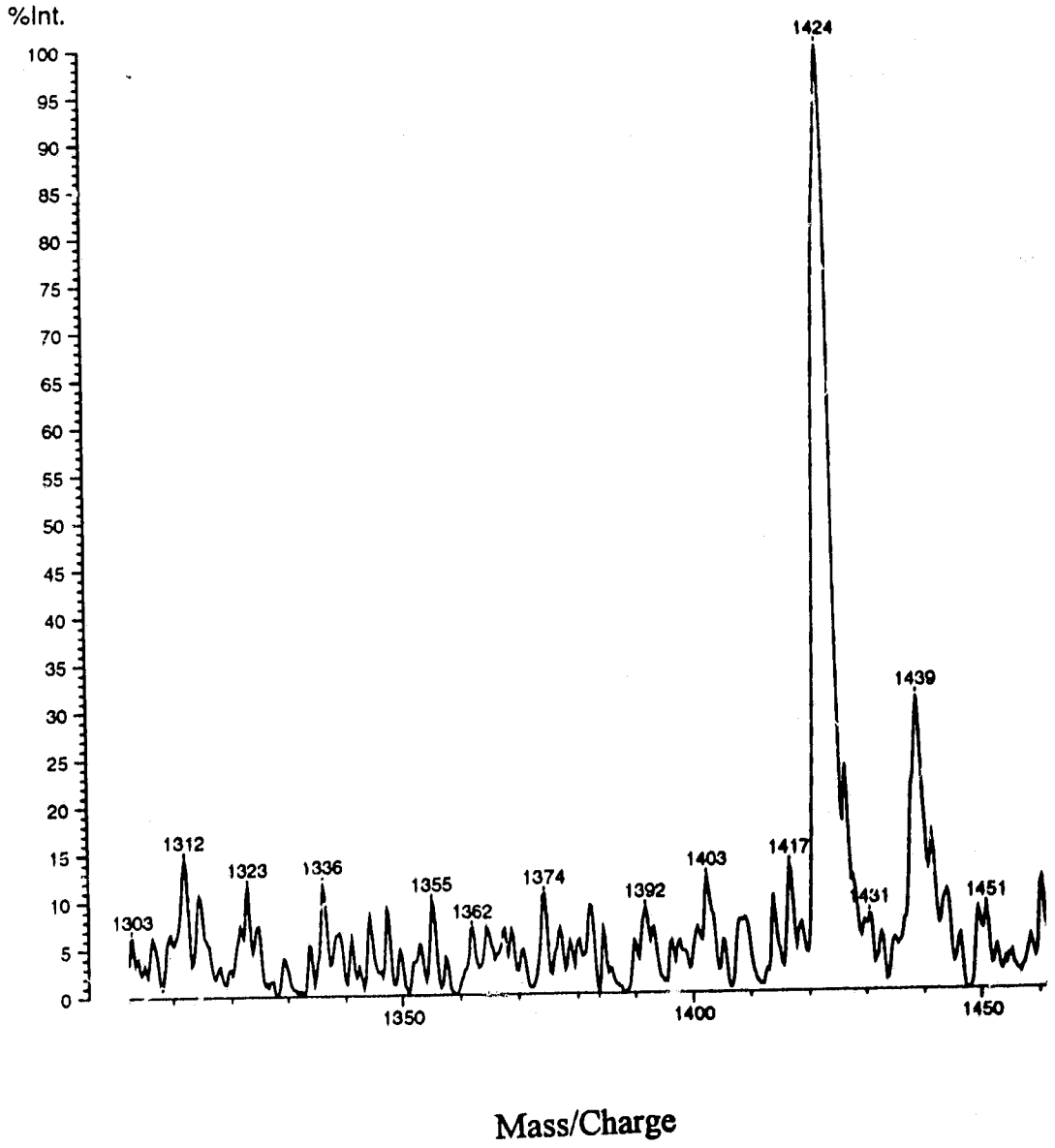


Figure 3-5: Molecular weight determination of G9.6 by time of flight MS. One intense $M+Na^+$ pseudomolecular ion at m/z 1424 corresponding to a deuterium-reduced $Hex_6HexNAc_2$.

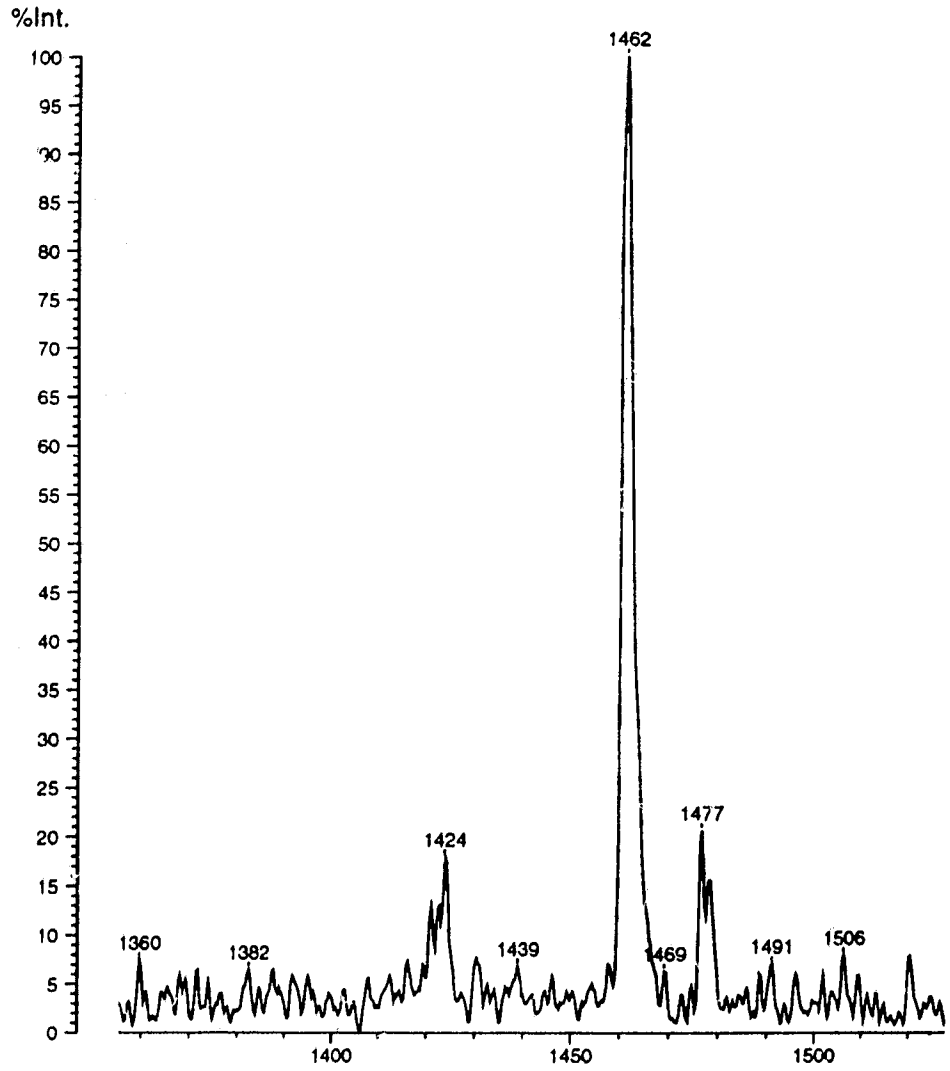


Figure 3-6: Molecular weight determination of G10.5 by time of flight MS. One intense $M+Na^+$ pseudomolecular ion at m/z 1462.

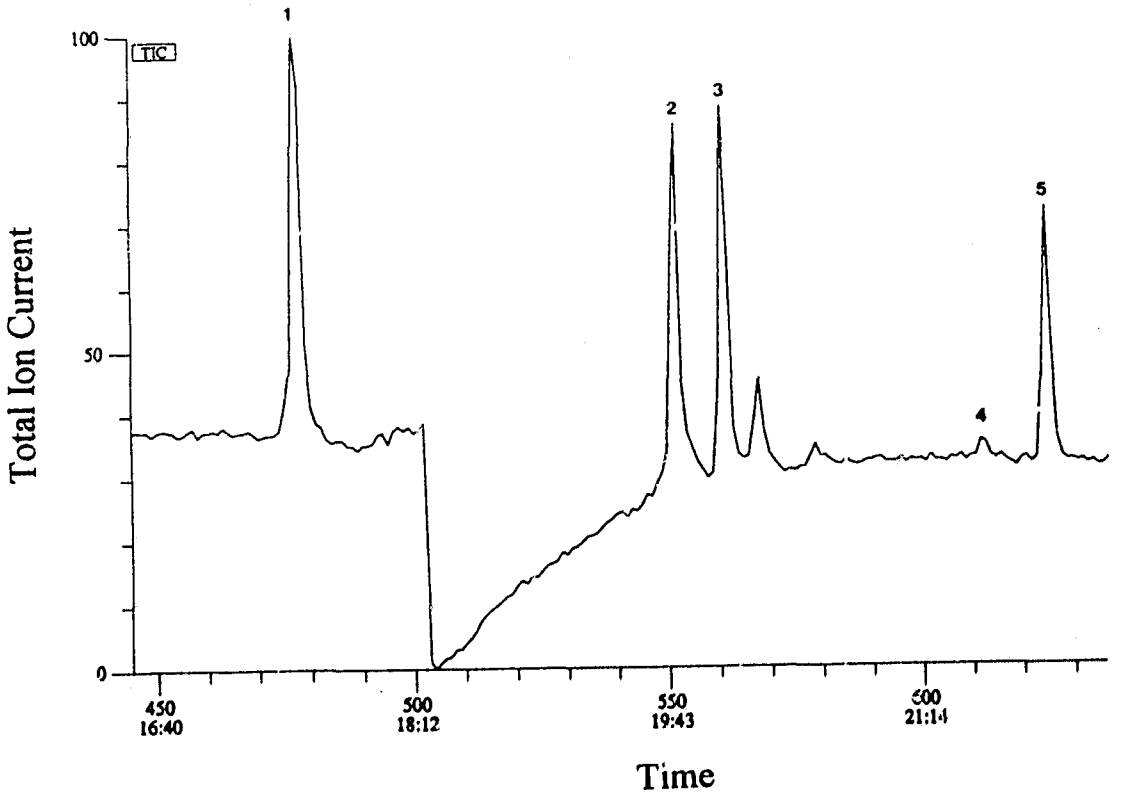


Figure 3-7: GC separation of PMAA derived from Endo-H cleaved G7.6 using a J & W DB-1 column. Peak 1, terminal hexose; peak 2, 2-linked hexose; peak 3, 3-linked hexose; peak 4, reduced N-acetylhexosamine; peak 5, 3,6-linked hexose.

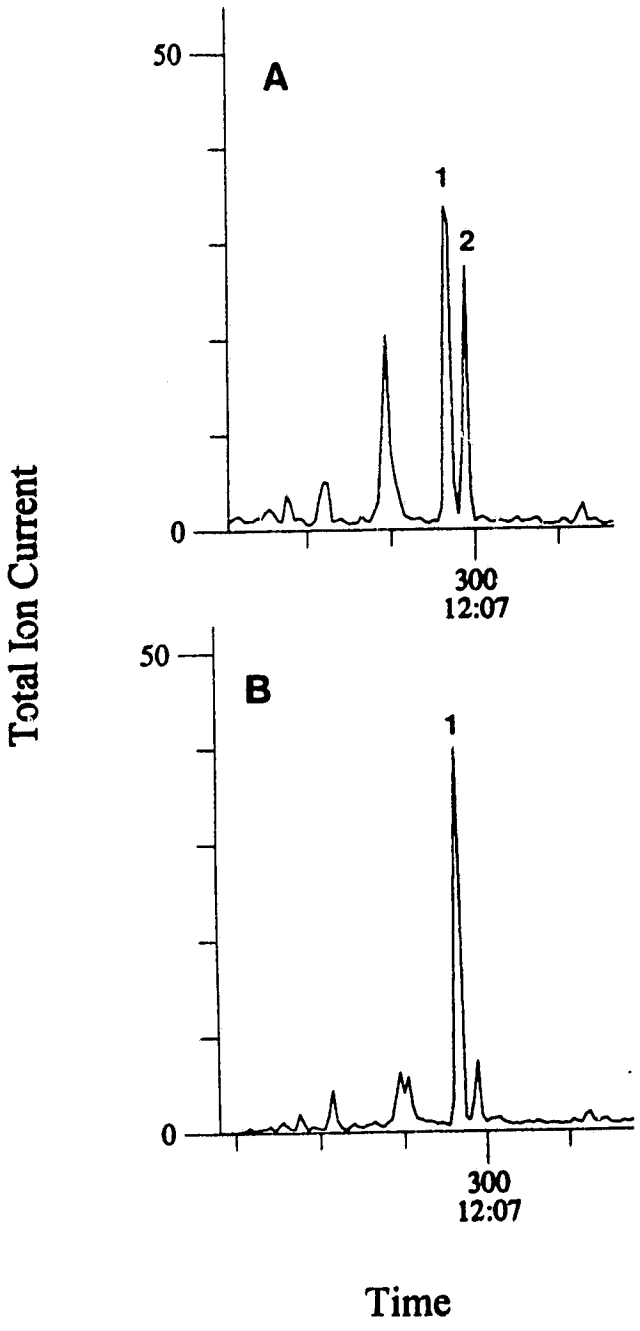


Figure 3-8: GC separation of Endo-H derived G8.5 PMAA (A) and 7.6 PMAA (B) using a SP-2380 column. Peak 1, terminal mannose; peak 2, terminal glucose.

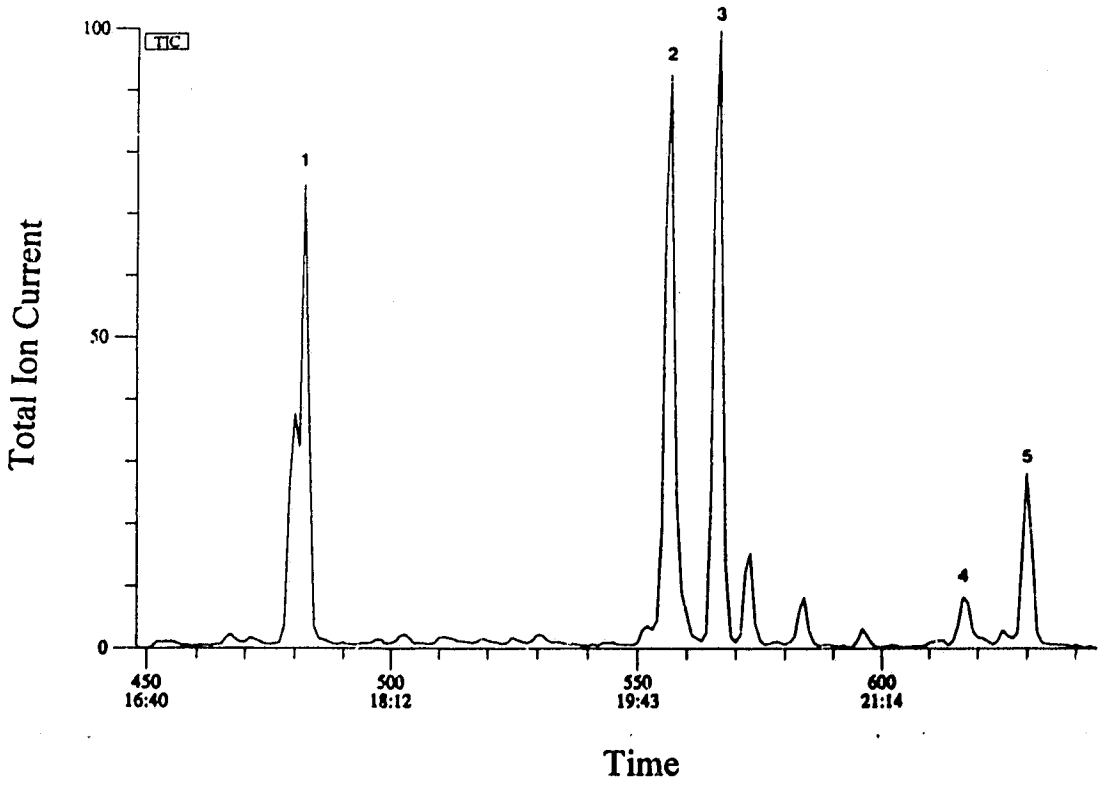


Figure 3-9: Separation of PMAA derived from Endo-H cleaved G8.5 using a J & W DB-1 column. Peak 1, terminal hexose; peak 2, 2-linked hexose; peak 3, 3-linked hexose; peak 4, reduced N-acetylhexosamine; peak 5, 3,6-linked hexose.

Identification of the oligosaccharide linkage sites on gp63

L. major glycoproteins were metabolically labelled with ^{3}H-mannose and gp63 was isolated as described in the methods section. Delipidated gp63 was used in these experiments to increase aqueous solubility of the glycoprotein, thereby increasing the efficiency of cleavage with Lys-C. In addition, removal of the lipid anchor prevented possible irreversible binding of the lipidated C-terminal peptide to the reverse-phase column. The reverse-phase chromatography profile of gp63 peptides is shown in Figure 3-10A. Only two peaks were found to contain the metabolically incorporated ^{3}H-mannose apart from the void. Amino acid analysis of the void fraction indicated that no protein was present. Sequence analysis of peak one identified the peptide NFDVPVIXSS... (Table 3-3), where the X represents a cycle with an undetermined modified residue. The modification occurred at a residue which had previously been determined as an asparagine in a putative attachment site for N-linked glycosylations - Asn 200 of the mature protein sequence (Button et al., 1988, 1989; Miller et al., 1990). The second peptide having incorporated radiolabel was identified as CMEQSVTQWPAMFCXES (Table 3-3), again showing a blank cycle at position 307 corresponding to a second putative attachment site for N-linked oligosaccharides. The absence of cysteine in cycle 1 and 14 of the sequence resulted from alkylation using 4-vinylpyridine (see Methods). In addition, low yields of ser, thr, met and trp are common due to destructive oxidation reactions occurring during derivatization.

Table 3-3: Sequence and quantification of phenythiohydantoin (PTH) derivatized amino acids for the putative glycopeptides from *L. major* gp63.

PEPTIDE 1			PEPTIDE 2		
Cycle #	PTH amino acid	pmoles	Cycle #	PTH amino acid	pmoles
1	asn	36.5	1	(cys)[1]	-
2	phe	41	2	met	61
3	asp	36.5	3	glu	72
4	val	38.9	4	gln	66
5	pro	21.3	5	ser	28
6	val	27.2	6	val	48
7	ile	17	7	thr	34
8	xxx[2]	-	8	gln	32
9	ser	0.81	9	trp	9
10	ser	1.34	10	pro	21
			11	ala	39
			12	met	13
			13	phe	16
			14	(cys)[1]	-
			15	xxx[2]	-
			16	glu	21
			17	ser	7

[1](cys), presumed pyridylethylcysteine

[2]xxx, presumed glycosylation site

Gp63 peptides were also applied to Con A Sepharose resulting in elution of two peptides which displayed reverse-phase chromatography retention times identical to those described above (Fig. 3-10A and B). Sequence analysis confirmed their identity. Subsequent carbohydrate analysis, using anhydrous hydrazine to release the glycans, resulted in purification of both G10.5 and G9.6 from each peptide indicating that the N-linked sites were occupied in a nonspecific manner. The third potential site for attachment of N-linked oligosaccharides was apparently unoccupied.

Determination of the predominant membrane associated oligosaccharides of six *Leishmania spp.*

The predominant oligosaccharides of *L. major*, *L. m. mexicana*, *L. m. amazonensis*, *L. donovani* LD3, *L. donovani* LV9 and *L. tropica* promastigotes were isolated using anhydrous hydrazine. In all cases, only two oligosaccharides, having hydrodynamic volumes of G10.5 and G9.6, were found. The glycans eluting at G9.6 were confirmed as being the same as that isolated from *L. major* gp63. However, the G10.5 oligosaccharide was resistant to digestion by JBAM as was observed previously for the G10.5 isolated in this manner. However, it is likely that the ^{3}H-labelled G10.5 corresponds to the terminally glucosylated $Man_6GlcNAc_2$ structure depicted in Figure 3-2.

L. m. mexicana amastigotes also carried two oligosaccharides having hydrodynamic volumes of G10.5 and G9.6 while preliminary investigations of *L. donovani* LV9 amastigotes revealed the presence of a much smaller glycan on this life-stage. The predominant oligosaccharide had a hydrodynamic volume of G5.2 and compositional analysis following acid hydrolysis indicated that it was composed of glucosamine and mannose residues in a ratio of 2 to 1.

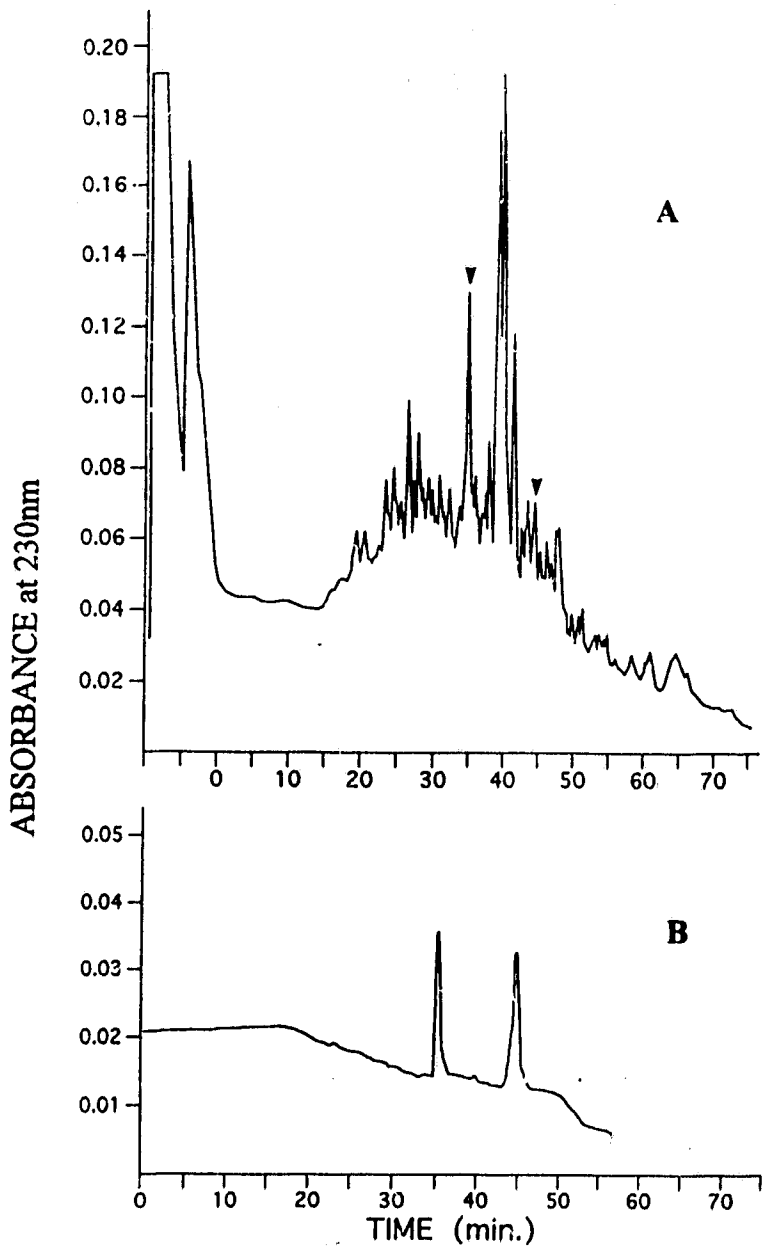


Figure 3-10: Identification of gp63 glycosylated peptides. A) HPLC profile of *L. major* gp63 peptides generated by digestion with Lys-C. Peptides were resolved using a C_8 reverse-phase column at a flow rate of 0.5 ml min^{-1}. The column was equilibrated in 0.1% TFA and the acetonitrile organic modifier concentration was increased linearly to 60% over 75 min. The radiolabelled peptides are indicated by arrows. B) Separation of gp63 peptides that bound to Con A Sepharose.

DISCUSSION

Results reported here indicate that *L. major* gp63 carries two predominant neutral oligosaccharides having the structures depicted in Figure 3-2. Tne G9.6 high mannose structure was found to be similar to that previously reported in other *Leishmania* species (Parodi et al., 1984; Olafson et al., 1990). Structural elucidation of G10.5 was complicated by the presence of a diacetylated 1-deoxyfructose hydrazone of G9.6 in this sample. Treatment of glycoproteins with anhydrous hydrazine was known to cause side reactions at the reducing terminus resulting in production of by-products (Takasaki et al., 1982; Michalski et al., 1984; Olafson et al., 1990), but such reactions were believed to be minimized by favourable conditions, including ultrapure hydrazine, anhydrous samples and mild reaction conditions (4 h at 95°C under argon). Unfortunately, these conditions were not successful at preventing the side reactions for unknown reasons and the presence of this product in all samples treated with hydrazine should make researchers consider alternative methods of isolating oligosaccharides.

The presence of only two biantennary high mannose oligosaccharides on 6 species and strains of *Leishmania* promastigotes, that differ only in the presence or absence of a terminal glucose, is best explained by proposing that these species lack N-linked oligosaccharide processing enzymes. Enzymes of the Golgi give rise to oligosaccharide microheterogeneity due to processing reactions occurring differentially on oligosaccharide species (reviewed by Schachter, 1986). The lack of heterogeneity in the high mannose structure reported here suggests that no processing occurs, otherwise, some variation would be expected. The structure of $Man_6GlcNAc_2$ determined in this study is consistent with the mammalian pathway for synthesis of high mannose oligosaccharides (Kornfeld and Kornfeld, 1985), again suggesting that processing has not occurred. This is in agreement with the results of Parodi et al. (1984) who found that in *L. mexicana* promastigotes, $Man_5GlcNAc_2$ was the only oligosaccharide transferred to protein and was the only glycan isolated from mature glycoproteins. However, this hypothesis does not account for the results of Olafson et al. (1990) who isolated $Man_{4,5}GlcNAc_2$ as well as $Man_6GlcNAc_2$ from *Leishmania mexicana amazonensis* promastigotes. The former two glycans likely arise as a result of processing of the $Man_6GlcNAc_2$ glycan by an

α-mannosidase. It is difficult to rationalize the two sets of results except to suggest that this discrepancy represents differences among strains of *Leishmania mexicana amazonensis*. Analysis of oligosaccharide structures in other trypanosomatids indicates that differences of this nature can occur within a genus (reviewed in Parodi, 1993),

The presence of the terminally glucosylated structure on all species and strains of *Leishmania* promastigotes examined is very interesting. It has long been recognized that organisms belonging to the order Kinetoplastida do not synthesize a glucosylated dolichol diphosphate intermediate (Parodi et al., 1981, 1982a) due to their inability to sythesize dolichol-P-Glc (Parodi et al., 1982a; Mendelzon and Parodi, 1986; de la Canal and Parodi, 1987). But glycoproteins become transiently glucosylated (Parodi et al., 1982b, 1983, 1984; Mendelzon and Parodi, 1986; Mendelzon et al., 1986) due to the sequential action of UDP-Glc: glycoprotein glucosyltransferase (Trombetta et al., 1989; Ganan et al., 1991) and glucosidase II (Bosch et al., 1988; Ganan et al., 1991). The presence of a terminal glucose on a mature glycoprotein as reported here and previously by Olafson et al. (1990), is contrary to the current dogma. It is possible that the species and strains carrying terminally glucosylated oligosaccharides do so because they lack a glucosidase II enzyme - α-glucosidase II activity has thus far only been reported to occur in *Trypanosoma cruzi*, *Crithidia fasciculata* and *Leptomonas samueli* (Bosch et al., 1988). The results of Parodi et al. (1984) are consistent with this hypothesis. They reported that proteins of *L. mexicana* promastigotes undergo transient glucosylation and presumably have an active glucosidase II responsible for removing terminal glucose residues prior to export of glycoproteins from the golgi. Characterization of putative RER and golgi processing enzymes is necessary to further address the legitimacy of this proposal.

Metabolic labelling of gp63 with ^{3}H-mannose identified two Lys-C generated peptides as having mannose containing oligosaccharides. Peptide sequencing and carbohydrate analysis of the tritiated peptides revealed the presence of both G9.6 and G10.5 oligosaccharides at both Asn 200 and 307 of the mature protein sequence indicating that these sites were not differentially glycosylated. The third putative attachment site for N-linked oligosaccharides (Asn 434) was unoccupied in *L. major* gp63, indicating some selectivity in attachment of N-linked oligosaccharides to proteins in

Leishmania. Two possibilities exist to explain the failure to glycosylate the third site, which had the amino acid sequence TNCTP. Roitsch et al. (1989) found that proline C-terminal to the recognition sequence interfered with oligosaccharide transfer. In addition, the cysteine within the recognition sequence may sterically hinder the transfer of oligosaccharides due to disulfide bond formation in the rough endoplasmic reticulum prior to oligosaccharide transfer. Testing these two possibilities using site-directed mutagenesis could prove useful.

The transformation of promastigote to amastigote is accompanied by large morphological (Bates, 1994) and surface compositional changes (McConville and Blackwell, 1991; Schneider et al., 1992; Bahr et al., 1993) compared to that occurring during metacyclogenesis (reviewed by Sacks 1989). The possibility that changes in N-linked oligosaccharide structures could also occur during this transformation was supported by the finding that the predominant N-linked oligosaccharides of *Trypanosoma cruzi*, another protozoan parasite, changed during the transformation of epimastigotes to amastigotes (Engel and Parodi, 1985). Analysis of *L. mexicana mexicana* promastigotes and amastigotes showed that oligosaccharide structure did not vary between these two forms. However, preliminary investigations comparing *L. donovani* LV9 amastigotes and promastigotes suggested that oligosaccharide structures can vary as a function of promastigote to amastigote transformation. These studies showed the presence of lower molecular weight oligosaccharides associated with *L. donovani* LV9 amastigotes compared to promastigotes and were consistent with previous observations (Olafson, unpublished data). The possibility that the G5.2 oligosaccharide isolated from LV9 amastigotes could have originated from the glycosylinositol phospholipids (GIPL's) is unlikely for several reasons. Firstly, compositional data of G5.2 showed a mannose to glucosamine ratio of 1:2. GIPL's isolated from *L. donovani* amastigotes would display mannose to glucosamine ratios of 2:1 and 3:1 (McConville and Blackwell, 1991). It is important to note that acid hydrolysis cleaves N-acetyl bonds, so that the glucosamine residue may represent N-acetylglucosamine. Secondly, glycolipids are extracted from membranes with chloroform:methanol:water (1:2:0.8, v/v) (McConville and Blackwell, 1991) so that it is likely these molecules were removed from the samples during the

acetone precipitation step (see Methods). Moreover, the glycolipids would have been expected to separate from the neutral oligosaccharides during chromatography with butanol:ethanol:water (4:1:1, v/v). Lastly, the fact that GIPL's were not found in any of the other *Leishmania* samples analyzed, strongly supports the contention that G5.2 is a glycoprotein glycan. In conclusion, these data suggest that changes in N-linked oligosaccharide structure as a function of life-stage is a species specific phenomenon. Further work is required to determine how wide spread such changes are among *Leishmania spp.* and to determine the importance such changes may have on parasite survival both in the mammalian host and the insect vector. These comparisons will be facilitated as *in vitro* culture techniques for amastigotes improve.

The function of the N-linked oligosaccharides of *Leishmania* remain unknown. Evidence from these studies has shown that the oligosaccharides of gp63 are not important for targeting gp63 to the plasma membrane and removal of the oligosaccharides does not in any way affect the enzyme activity of gp63. In addition, removal of the oligosaccharides does not increase the susceptibility of this protein to proteolysis (Funk et al., 1993; Chapter 2 of this document). Furthermore, the absence of structural changes in the N-linked oligosaccharides as a function of metacyclogenesis, reported here, indicates that they are not responsible for the increase in virulence observed during this transformation. Finally, the presence of the same two predominant N-linked oligosaccharide structures in 6 different *Leishmania* species and strains, which are responsible for a number of distinct disease manifestations, indicates that the N-linked oligosaccharides of *Leishmania* do not account for the differences observed in virulence or disease manifestations. However, it is intriguing that all *Leishmania spp.* studied here display the unique terminally glucosylated oligosaccharide first described to occur on mature glycoproteins by Olafson et al. (1990). That this terminal glucose may be important in parasite uptake via the macrophage advanced glycosylation endproducts receptor (AGE-protein receptor) was suggested by the finding that AGE-BSA inhibited parasite uptake by 50% (Mosser et al., 1987).

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CHAPTER 4

Structural characterization and metabolism of *Leishmania* cytoplasmic mannans - unique energy storage polymers.

INTRODUCTION

The intermediary metabolism of *Leishmania spp.* has received much attention over the last few years and several reviews have been published (Cazzulo, 1992; Blum, 1993; 1994). A number of interesting studies have indicated the presence of an endogenous carbon source comparable to glycogen (Simon et al., 1978; Darling et al., 1989; Keegan and Blum, 1990; Walsh and Blum, 1992) and NMR data have indicated the presence of carbohydrate polymers in *Leishmania* amastigotes and promastigotes (Rainey and MacKenzie, 1991). Keegan and Blum (1992) recently isolated large amounts of mannose, present as polymers, from *L. donovani* promastigotes and presented evidence consistent with the mannans functioning as an energy reserve (Keegan and Blum, 1992, 1993). The presence of such a unique energy store in *Leishmania* may offer an avenue for development of chemotherapeutic reagents.

While investigating the cytoplasmic pool of high mannose N-linked oligosaccharides, I was struck by the unusually high concentration of mannose polymers. This study is a report of the structural nature of the mannans present in *Leishmania spp.*, including polymer size, sugar composition and linkages. In addition, information is provided about the life cycle stage and species distribution, along with the intermediates of mannan catabolism and evidence for the presence of a mannan phosphorylase.

MATERIALS AND METHODS

Parasite cultivation.

Promastigotes were cultured at 26°C in M199 medium supplemented with hemin (5 mg l^{-1}) and folate (23 μM). Penicillin/streptomycin (Sigma Chemical Company, St. Louis, MO, USA) and Basal Eagle Medium vitamin solution (Gibco, Burlington, Ontario) were added to the medium at a dilution of 1:100 (v/v). *L. m. mexicana* (MNYC/BZ/62/M379) promastigotes, *L. major* (MRHP/SU/59/Neals strain) promastigotes and *T. brucei rhodesiense* were grown in 10% fetal bovine serum (FBS[1]). *L. mexicana amazonensis* (MHOM/BR/73/M2269), *L. major* A2 (Neva et al., 1979), *L. tropica* (MHOM/SU/74/k27), *L. donovani donovani* (NLB-065) and *L. donovani* LV9 promastigotes were grown in 5% FBS while *L. donovani donovani* LD3 promastigotes were grown in the absence of FBS. *L. m. mexicana* (MNYC/BZ/62/M379) amastigotes were also grown axenically as described by Bates et al. (1992). All parasites were grown to late log phase and harvested by centrifugation.

L. m. mexicana promastigotes were also maintained in D-MEM media without glucose (Gibco) and in the absence of FBS for a period of 6 h.

Isolation of cytoplasmic oligosaccharides.

Parasites were washed two times with phosphate buffered saline and then stored frozen at -80°C. Thawed parasites were suspended in 5 ml of water, sonicated on ice for 3 x 10 s at 45 W and then centrifuged at 100,000 x g for 1.5 h at 4°C to remove all membrane fragments. The cytoplasmic fraction was lyophilized, treated with anhydrous hydrazine (200 μl mg^{-1} protein)(Sigma) for 4.5 h at 95°C, reduced with $NaBH_4$ (250 mM) and subjected to high voltage electrophoresis as previously described (Parekh et al., 1987). The resulting neutral oligosaccharides were separated using a Bio-Gel P-4 (400 mesh) (BioRad, Mississauga, Ont.) gel permeation chromatography column (1.5 cm x 2.0 m) monitored by a Waters 410 differential refractometer (Millipore, Milford, MA). The column was calibrated using partially hydrolyzed dextran as standards and the relative molecular weights of unknown oligosaccharides expressed in glucose units.

Compositional analysis.

Isolated oligosaccharides were hydrolyzed in 2N HCl at 100°C for 3 h and the hydrolysate desalted using a mixed bed of AG 50W-X4 (H^+) and AG 3-X4 (OH^-). Monosaccharides were separated by anion exchange chromatography using a PA-1 HPLC column developed isocratically with 16 mM NaOH and monitored with a pulsed amperometric detector (PAD) (Dionex Corporation, Mississauga, Ont.).

Linkage analysis.

Partially permethylated alditol acetates (PMAA) were made using a modified method of Anumula and Taylor (1992). The NaOH-methyl sulfoxide (DMSO) reagent was prepared by mixing 0.1 ml of 50% NaOH with 0.2 ml methanol and diluting with 6 ml DMSO. The suspension was vortexed vigorously and the NaOH precipitate collected after brief centrifugation. The NaOH precipitate was washed 5 times with DMSO in this manner and finally resuspended in 2 ml DMSO.

DMSO (100µl) was added to each oligosaccharide sample and sonicated for 15 min at room temperature. The NaOH-DMSO reagent (100µl) was then added, stirred for 20 min, followed by addition of 50 µl of iodomethane and an additional 5 min sonication and 5 min of stirring. Another aliquot of iodomethane was added and the sample stirred for a further 30 min. The reaction was stopped by the addition of 0.4 ml of water and the methylated oligosaccharides were partitioned into 0.4 ml chloroform. The chloroform phase was washed 5 times with 0.5 ml water and finally taken to dryness under reduced pressure.

The methylated oligosaccharides were hydrolyzed in 150 µl of formic acid, water, TFA in a ratio of 10:2:1 for 16 h at 100°C. Samples were dried under reduced pressure following addition of 150 µl of fresh 5% pyridine in 50% acetonitrile/water to neutralize residual acids. The partially methylated monosaccharides were reduced for 16 h at room temperature with 150 µl sodium borodeuteride (12 mg ml^{-1}) in 30% methanol containing 30 mM NaOH. The reaction was stopped by addition of 2N acetic acid and the sample was taken to dryness. Acetylation was achieved by the addition of 100 µl acetonitrile

containing 5 mg ml^{-1} dimethylaminopyridine, 100 µl acetic anhydride and 33 µl pyridine. The samples were incubated at room temperature for 4 h. After terminating the reaction by the addition of 300 µl water, the PMAA were partitioned into 400 µl dichloromethane. The organic layer was washed 6 times with water and then taken to dryness under a stream of nitrogen.

Analysis of the PMAA was performed on a Hewlett-Packard 5890 series II gas chromatography system interfaced with a Kratos Concept-H double sector magnetic mass spectrometry system (GC/MS). PMAA were separated using a J & W DB-1 column (0.25 mm x 30 m) (Altech, Deerfield, IL) with helium as the carrier gas (2.0 ml min^{-1}). Samples were loaded using a splitless injector and developed using the following temperature program: 60°C for 2.0 min, followed by a linear increase to 140°C at 40° per min, held for 4.0 min and then another linear increase to 250°C at 4° per min where upon the temperature was held for 20 min.

Exoglycosidase digestion.

Jack Bean α-mannosidase (JBAM), EC 3.2.1.24 (Oxford GlycoSystems Ltd., Rosedale, NY) was used at a concentration of 10 U ml^{-1} in 200 mM citrate, pH 4.5 with 0.2 mM zinc acetate. Incubation proceeded for 24 h at 37°C.

NMR Spectroscopy.

NMR spectra were recorded at 25°C in D_20 solution on a Bruker AMX 500 FT-NMR spectrometer operating at 500.13 MHz for ^1H and 125.77 MHz for ^{13}C. In addition, one-dimensional ^{13}C experiments were recorded on a Bruker AM400 spectrometer at 100.6 MHz. All proton NMR spectra were obtained with presaturation of the residual water resonance at 4.8 ppm, and are referenced to internal DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) (0.0 ppm). The one-dimensional carbon-13 NMR spectra were proton decoupled using a bilevel composite pulse decoupling scheme (2 Watts during acquisition, otherwise 0.4 W). Carbon-13 shifts were referenced to internal dioxane (67.4 ppm). The two-dimensional NMR experiments were performed in the phase-sensitive mode (TPPI) using standard Bruker software. Typically, 48 to 112 scans

were accumulated per t1 increment with a recycle time of 2.0 s. Due to the low dispersion of the non-anomeric proton signals, double quantum filtered (DQF) COSY spectra were employed instead of conventional COSY spectra. For the double quantum filtered COSY experiments, the initial data matrix of 2K x 512 (sweep width of 2000 Hz) was zero-filled to 4K x 2K in the F2 and F1 dimensions prior to being apodized with a cosine squared function. The mixing time in the TOCSY and HMQC-TOCSY experiments varied from 44 ms to 87 ms, using a spin lock power of 5 W. The NOESY spectrum was recorded with an average mixing time of 100 ms which was varied in a random fashion from 95 to 105 ms in order to reduce the intensity of COSY type signals. For these two-dimensional experiments, the initial 2K x 400 matrix was zero filled to 4K x 2K and apodized as before prior to transformation. In the case of the HMQC and HMQC-TOCSY spectra, the data matrix of 1K (^{1}H, sweepwidth of 2000 Hz) by 256 (^{13}C, 8300 Hz) was zero-filled to 2K x 2K and apodized as before. Where necessary, spectra were baseline corrected using a third order polynomial function.

Quantification of mannose.

Parasites were counted and the wet weight of the resulting pellet determined. The protein concentration of the 100,000 x g cytoplasmic fraction was also determined by the BioRad protein assay. All samples were deproteinated by passing the samples over AG 50W-X4 (H^+) and AG 3-X4 (OH^-) until no protein was detected. The concentration of mannose in the cytoplasmic fraction was determined following chromatography of acid hydrolysates on the PA-1 column as described above. Mannose was quantified by comparing peak areas of unknown samples to that of standard mannose.

Preparation of *L. m. mexicana* crude cell homogenate for enzyme analysis.

Pellets of *L. m. mexicana* promastigotes were frozen at -90°C immediately after centrifugation and maintained at that temperature until use. Cells were resuspended in 20mM phosphate buffer, pH 7.4, containing 5 mM β-mercaptoethanol, 50% glycerol, 0.2% BSA, 0.5 mM EDTA and 20 $\mu g\ ml^{-1}$ aprotinin to a final concentration of 0.2 g wet

wt. per ml. The suspension was then sonicated as described above. Protein concentration was determined using the bicinchoninic acid protein assay kit (Sigma) (Smith et al., 1985).

Utilization of mannose 6-phosphate by *L. m. mexicana* promastigote cell homogenate.

The reaction buffer (NADPH buffer A) contained 50 mM phosphate, pH 7.4, 0.25 mM EDTA, 2 mM NADP, 0.5 Units ml^{-1} yeast glucose-6-phosphate dehydrogenase (G6P-DH) (EC 1.1.1.49) (Boehringer Mannheim). Two µl of the cell homogenate were added to 1 ml of reaction buffer and the sample was monitored at 340 nm with a Phillips Pye unicam SP6-550 UV/VIS spectrophotometer until a flat baseline was established. Phosphoglucose isomerase (PGI) (EC 5.3.1.9) (Boehringer Mannheim) (2 Units ml^{-1}) and mannose 6-phosphate were then added and production of NADPH monitored. The molar extinction coefficient of NADPH at 340 nm was determined as 6.22 $A.U._{340nm}$ per µmole.

Assay for mannan catabolism.

L. m. mexicana promastigote cell homogenate was incubated in 50 mM phosphate buffer, pH 7.4, containing 2 mM glutathione, 0.2 mM EDTA, 10 mM $MgSO_4$ and purified mannans (250 µg ml^{-1}) for 24 h. The digest was treated with perchloric acid (PCA), as described below, and chromatographed by anion exchange chromatography employing a sodium acetate linear gradient from 0 - 250 mM in 100 mM NaOH over 50 min (Gradient B). The gradient was initiated after a 10 min isocratic elution with 100 mM NaOH. The appearance of products, compared to a time zero control, was monitored by PAD.

The identity of the putative product of mannan catabolism was determined by comparing its retention time on anion exchange chromatography to various phosphorylated monosaccharide standards. To confirm the identity of the phosphorylated monosaccharides the time zero control and the 24 h digest (20 µl) were subjected to an alkaline phosphatase digest. This reaction was performed by addition of 30 µl of 100 mM Tris, pH 9.0, with 1 mM $MgCl_2$ and 0.1 mM zinc acetate containing 850 U ml^{-1} alkaline phosphatase to 20 µl samples followed by incubation for 3 h at room temperature. Half of each sample was desalted and monosaccharides were identified by anion exchange chromatography as previously described. The other half of the samples was

chromatographed using gradient B (above) to ensure that depletion of the product occurred.

Perchloric acid treatment.

To remove and denature all protein, perchloric acid was added to a final concentration of 4.5%. Samples were kept on ice for 5 min. and then centrifuged at 14 000 x g for 5 min. The supernatant was neutralized with 1.5 M K_2CO_3.

Determination of the intermediates of mannan catabolism.

L. m. mexicana crude cell homogenate (50 µl) was diluted to 200 µl with 50 mM PO_4 buffer, pH 7.4 and 10 mmoles of mannose 1-phosphate (M1P) were added. This sample and a control, containing no M1P, were incubated over night at room temperature. The samples were then treated with PCA and analyzed enzymatically for the presence of mannose 6-phosphate (M6P), fructose 6-phosphate (F6P) and glucose 6-phosphate (G6P). The amount of each intermediate was determined in triplicate.

Identification and quantification of phosphorylated monosaccharide intermediates.

The presence of phosphorylated monosaccharides was determined spectrophotometrically using a coupled enzyme assay and the sequential addition of enzymes. G6P-DH (0.5 U ml^{-1}) was added first to determine the concentration of G6P. After stabilization of the baseline, F6P was measured by addition of phosphoglucose isomerase (2 U ml^{-1}) (EC 5.3.1.9) (Boerhinger Mannheim) and finally, phosphomannose isomerase (1 U ml^{-1}) (EC 5.3.1.8) (Sigma) was added to quantify M6P. All samples were made to 200 µl with NADPH buffer A described above and the absorbance at 340 nm was measured using a UVmax kinetic plate reader (Molecular Devices Corp.). Internal standards were used for quantification of hexose phosphates. Internal and external standards were used for quantification.

Size fractionation of *L. m. mexicana* cytoplasmic proteins.

L. m. mexicana promastigotes (5.0×10^9 cells or 0.23 g) were frozen and the thawed sample sonicated in 0.92 ml buffer containing 20 mM phosphate, pH 7.4, 5 mM β-mercaptoethanol and 0.5 mM EDTA. The homogenate was centrifuged at 20,000 x g for 20 min and the supernatant was chromatographed at a flow rate of 0.5 ml min^{-1} on a Sepharose G75 column (1.5 x 25 cm) equilibrated with the homogenization buffer. One ml fractions were collected and the absorbance at 280 nm determined.

Assay for "mannan phosphorylase" activity.

Fractions from the G75 column were assayed for mannan phosphorylase activity by incubating 200 µl of the pooled fractions with purified mannans (200 µg ml^{-1}) for 48 h. The samples were then analyzed by anion exchange chromatography using gradient B to determine the identity and quantity of accumulated phosphorylated monosaccharides compared to time zero controls.

RESULTS

Structure of *L. m. mexicana* mannans.

L. m. mexicana promastigotes and amastigotes contain high cytoplasmic concentrations of mannose homopolysaccharide which range in size from 3.2 to approximately 23 glucose units (G.U.) (Figure 4-1). The mannans were not covalently linked to protein, since removal of protein prior to hydrolysis had no significant effect on the concentration of mannose present (Table 4-1). GC/MS analysis of the PMAA indicated that only terminal and C-2 linked residues were present in a ratio of 0.23 to 1.0. These linkages were believed to be in the β configuration because treatment of the polymers with the broad specificity exoglycosidase, Jack Bean α-mannosidase (JBAM), did not cause a reduction in the hydrodynamic volume of a purified polymer based upon Bio-Gel P4 chromatography, nor were mannose residues released as determined by Dionex PA-1 HPLC analysis. The structure, including anomericity of the linkages, was subsequently verified by one- and two-dimensional ^{1}H and ^{13}C NMR analysis (Fig. 4-2 and 4-3). Although the presence of small amounts of non-mannose cannot be ruled out, these spectra indicate that the polysaccharide was essentially homogeneous. The carbon-13 chemical shifts of the six major signals are consistent with the predicted β1-2 linked polymannose structure. The proton NMR spectrum was assigned with the aid of two-dimensional HMQC experiments (Fig. 4-3) and were confirmed by COSY and TOCSY experiments. Minor signals observed in the spectra arise from terminal reducing and non-reducing mannose groups. The anomeric configuration was confirmed from examination of the NOESY spectrum. In addition to the expected intense H1-H2 crosspeak in this spectrum, intraresidue H1-H3 and H1-H5 crosspeaks were prominent and a ss intense H1-H4 crosspeak was also observed. These observations confirmed that the anomeric configuration is beta. Proton and carbon-13 chemical shifts for the major signals are given in Table 4-2.

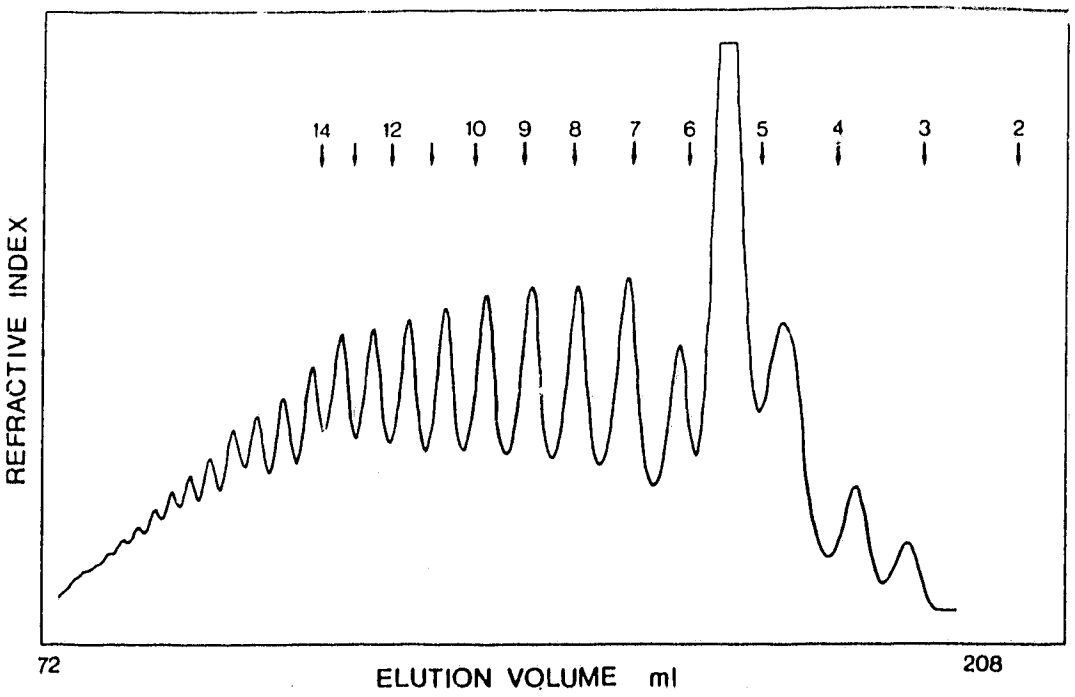

Figure 4-1: Gel permeation chromatography profile of cytoplasmic oligosaccharides from *L. m. mexicana* promastigotes and amastigotes. Oligosaccharides were separated on the basis of their hydrodynamic volume using a Bio-Gel P-4 gel permeation column eluted with water at a flow rate of 0.2 ml min^{-1} and were detected with a flow refractometer. The numbers and arrows along the top of the figure refer to the number of glucose residues in the eluting dextran standards and their elution positions.

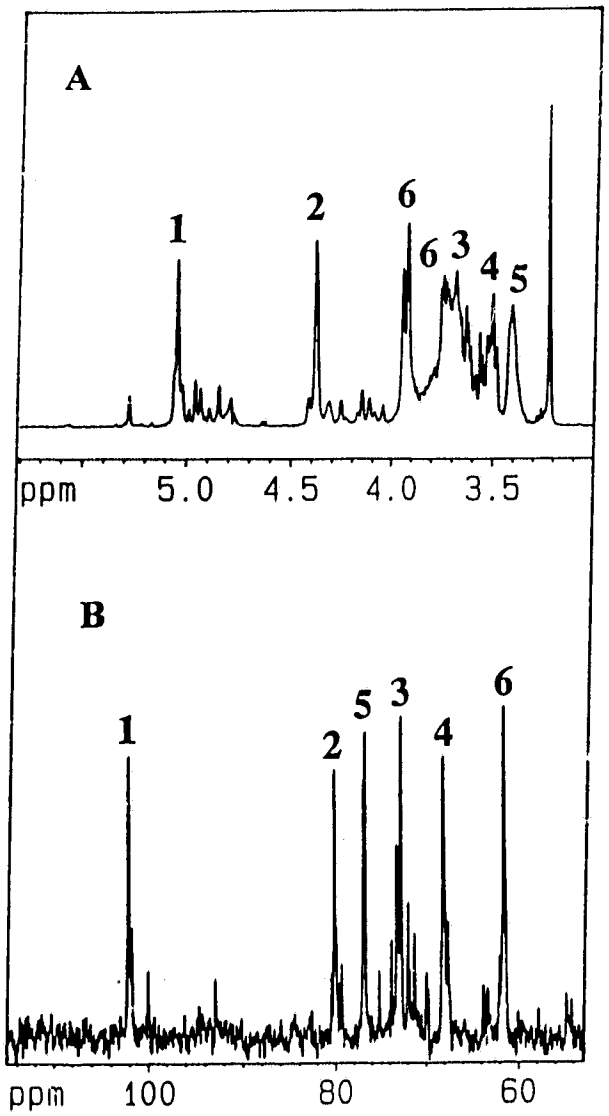

Figure 4-2: One dimensional NMR spectra of the cytoplasmic mannans, obtained at 25°C in D_2O solution. Small signals arise from terminal mannose rings. A) Proton NMR spectrum recorded at 500.13 MHz. Labelled peaks (1-6) indicate the mannose carbon to which the protons are bound. The large signal at ca. 3.2 ppm arises from a solvent impurity. B) Carbon-13 NMR spectrum recorded at 100.6 MHz. Numbers indicate the carbon giving rise to the signal.

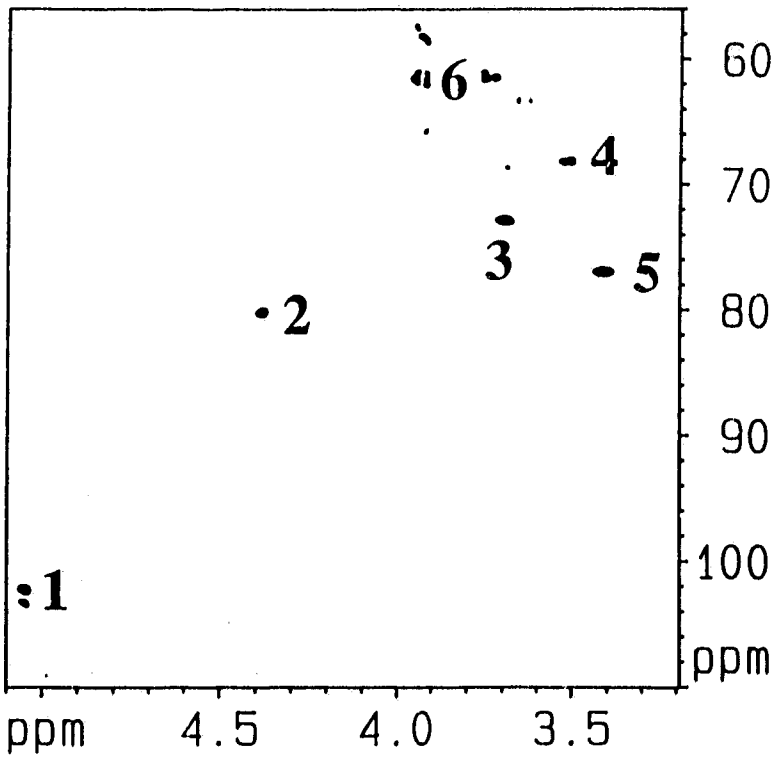

Figure 4-3: Two Dimensional HMQC spectrum of the cytoplasmic mannans. Proton chemical shifts are indicated on the horizontal axis, and carbon-13 shifts on the vertical axis.

Table 4-1: Quantification of mannose present in the cytoplasmic fraction of *L. mexicana* and *L. major* promastigotes before and after deproteinization. Mannose polymers were hydrolyzed and the resulting monosaccharides were identified by anion exchange chromatography with pulsed amperometric detection. Peak areas were compared to standards for quantification.

Description of cytoplasmic fraction hydrolyzed	μmoles of mannose per 5×10^9 parasites
L. mexicana pros. - before deproteinization	2.45 ± 0.06 (n=3)
L. mexicana pros. - after deproteinization	2.3 ± 0.1 (n=3)
L. major pros. - before deproteinization	0.57 ± 0.07 (n=3)
L. major pros. - after deproteinization	0.47 ± 0.07 (n=3)

Table 4-2: One-dimensional proton and carbon-13 chemical shifts for the major signals from *L. m. mexicana* cytoplasmic mannans.

Position	Proton shift	Carbon shift
1	5.04	102.14
2	4.38	80.15
3	3.68	72.75
4	3.5	68.15
5	3.4	76.79
6	3.73, 3.92	61.51

Evidence for a storage polysaccharide and species distribution.

Because these polymers were present free in the cytoplasm and appeared not to be glycoprotein precursors, we investigated the possibility that they may function as an energy store. A 6 h incubation of *L. m. mexicana* promastigotes in the absence of glucose decreased the mannose concentration by 50% (Table 4-3), indicating that the mannans of *L. m. mexicana* may function in this capacity. These findings were similar to those reported earlier for *L. donovani* promastigotes (Keegan and Blum, 1992).

Table 4-3: Comparison of mannose concentrations in the cytoplasm of various *Leishmania spp.* and one trypanosome, following acid hydrolysis.

Source of cytoplasmic supernatant	µmoles mannose per 5 x 10^9 parasites	µmoles mannose per g wet weight	µmoles mannose per mg protein
L. m. mexicana pros.	3.6	15.7	0.62
	2.4	10.4	0.42
L. m. mexicana pros. - after 6 h starvation	1.3	5.6	N/D[1]
L. m. mexicana amastigotes	4.5	30.5	2.25
L. d. donovani LD3	5.2	15.6	0.67
L. major Neals pros.	2.1	6.9	0.23
L. major A2 pros.	0.24	0.75	0.05
	0.57	1.78	0.08
	0.17	0.53	0.02
T. b. rhodesiense	0.05	0.07	2.0 nmoles
	0.03	0.08	1.6 nmoles

[1]N/D, no data.

Having established the structural nature of the mannans in *L. m. mexicana* and provided evidence that the mannans may be energy storage molecules, we then investigated the species distribution of these unique polymers. Compositional analysis indicated that mannose was the only detectable monosaccharide in the deproteinated cytoplasmic fraction of all species tested: *L. d. donovani* LV9, LD3 and NLB-065, *L. m. amazonensis, L. tropica, L. major* A2 and *L. major* Neals promastigotes as well as *T. brucei rhodesiense*. However, not all species contained mannans that were resistant to digestion by JBAM. Up to 50% of the mannans from *L. donovani* NLB-065 and *L. major* A2 were sensitive to JBAM and *T. b. rhodesiense* contained mannans that were completely susceptible to cleavage by JBAM (Table 4-4). Other *Leishmania spp.* tested contained only a small pool of JBAM sensitive mannans relative to the total amount of mannose present following acid hydrolysis (Table 4-4). Control samples were also

analyzed to ensure that there was no free mannose present prior to either acid hydrolysis or digestion with JBAM.

Table 4-4: Percent of total mannose released following treatment with JBAM. No mannose was detectable prior to either treatment.

Source of cytoplasmic supernatant	Per cent of total mannose susceptable to cleavage by JBAM
L. m. amazonensis	4%
L. d. donovani LD3	8%
L. tropica	10%
L. major Neals	2%
L. major A2	32%
L. d. donovani NLB-065	52%
T. b. rhodesiense	100+%

Quantification of total cytoplasmic mannose revealed that some parasites contained at least 10-fold more mannose than others (Table 4-3). It was also interesting to note that because all the cytoplasmic fractions had been passed over both anion and cation exchangers prior to analysis, the small pool of neutral α-linked mannans present in all species was unlikely to be linked to protein.

Investigations into the intermediary metabolism of cytoplasmic mannans.

Incubation of *L. m. mexicana* crude cell homogenate with purified mannans for 24 h resulted in the accumulation of a product which was identified as glucose 6-phosphate (G6P) (Figure 4-4A and B). Anion exchange chromatography demonstrated coelution of the product with mannose 6-phosphate (M6P) and G6P. Treatment of the product with alkaline phosphatase resulted in depletion of the phosphorylated product and generation of glucose (Figure 4-5A and B). No glucose was detected prior to treatment with alkaline phosphatase (results not shown). Finally, a G6P-dehydrogenase (G6P-DH)

coupled assay revealed that the 24 h mannan digest contained 0.4 mM G6P (6 nmoles G6P in 15 µl) while the time zero control had no detectable G6P.

Incubation of crude cell homogenate for 24 h with 10 mM mannose 1-phosphate (M1P) resulted in production of 36 ± 4 nmoles G6P per mg wet wt., 7.8 ± 0.6 nmoles F6P and 11.8 ± 0.8 nmoles M6P per mg wet wt. Apparently the crude cell homogenate has the enzymatic capacity to process M1P to M6P and then to fructose 6-phosphate (F6P) and finally to G6P (Figure 4-8). The equilibrium ratio among these intermediates was 1.5 : 1 : 4.7. In the absence of exogenous M1P, only 0.7 nmoles G6P per mg wet wt. was produced in 24 h, during which time F6P and M6P remained undetectable. These findings support the contention that the immediate precursor of the above mentioned products was M1P.

The activity of endogenous phosphomannose mutase and phosphoglucose isomerase was too low to detect product formation during a 10 min incubation period. However, the activity of phosphomannose isomerase (Figure 4-8, step 3) in the *L. m. mexicana* promastigote crude cell homogenate was estimated to be equivalent to 6 µmoles NADPH/min/g wet weight of cells (85 nmoles/min/mg protein) under saturating substrate concentrations. Product formation was linear with time and cell homogenate concentration. Coupling enzymes (phosphoglucose isomerase and G6P-DH) were in excess and control experiments confirmed that both the cell homogenate and the coupling enzymes were necessary for production of a detectable level of NADPH within a 10 min incubation period.

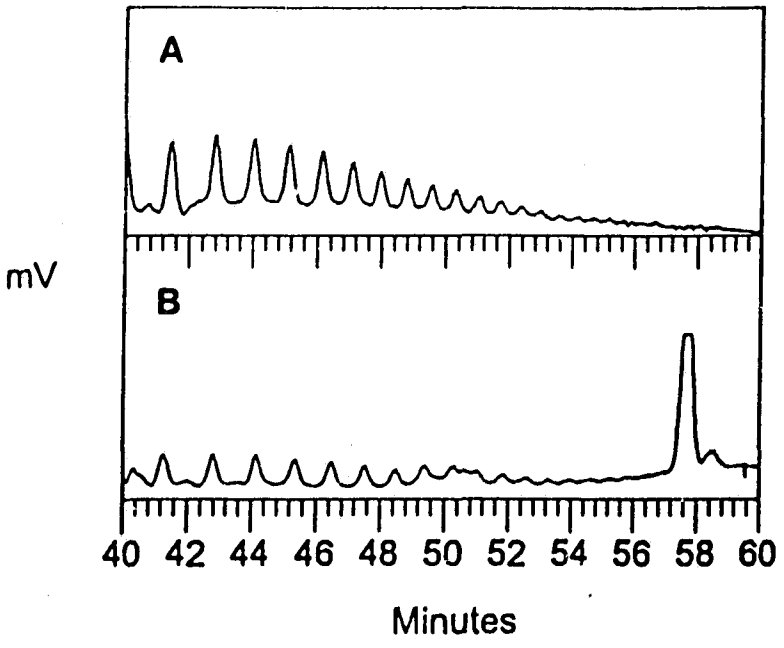

Figure 4-4: Anion-exchange chromatographic separation of purified mannans before and after incubation with crude cell homogenate from *L. m. mexicana* promastigotes. A) Time zero control illustrating the elution of purified mannans. B) Generation of a hexose 6-phosphate product peak following 24 h incubation of mannans with *L. m. mexicana* crude cell homogenate. Following treatment with alkaline phosphatase, the elution profile was the same as part (A).

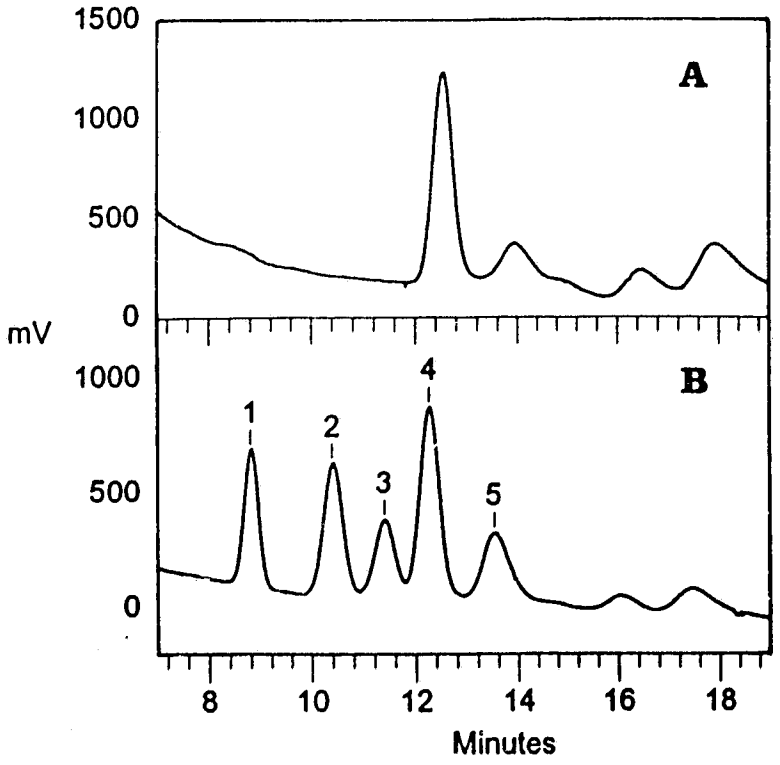

Figure 4-5: Anion-exchange chromatographic separation of glucose following treatment of the 24 h mannan digest with alkaline phosphatase. A) Generation of a product peak following treatment of the phosphorylated sugar with alkaline phosphatase. B) Chromatography of the unknown with internal standards showing coelution with the glucose standard: peak 1, galactosamine; peak 2, glucosamine; peak 3, galactose; peak 4, glucose; peak 5, mannose.

Evidence for the presence of a mannan phosphorylase.

The separation of *L. m. mexicana* cytoplasmic proteins by GPC is shown in Figure 4-6. Of the seven fractions assayed, only fraction II produced a product when incubated with purified mannans and the product coeluted with M1P and G1P (Figure 4-7A and B). An enzyme coupled assay, using phosphoglucomutase (2 U ml^{-1}) and G6P-DH, demonstrated that the product was not G1P. Treatment of the product with alkaline phosphatase resulted in depletion of the product peak and generation of mannose as determined by anion exchange chromatography (results not shown). Clearly, fraction II from the G75 column contained an enzyme with mannan phosphorylase activity and M1P appears to be the first intermediate in the catabolism of the β-linked mannans of *L. m. mexicana*.

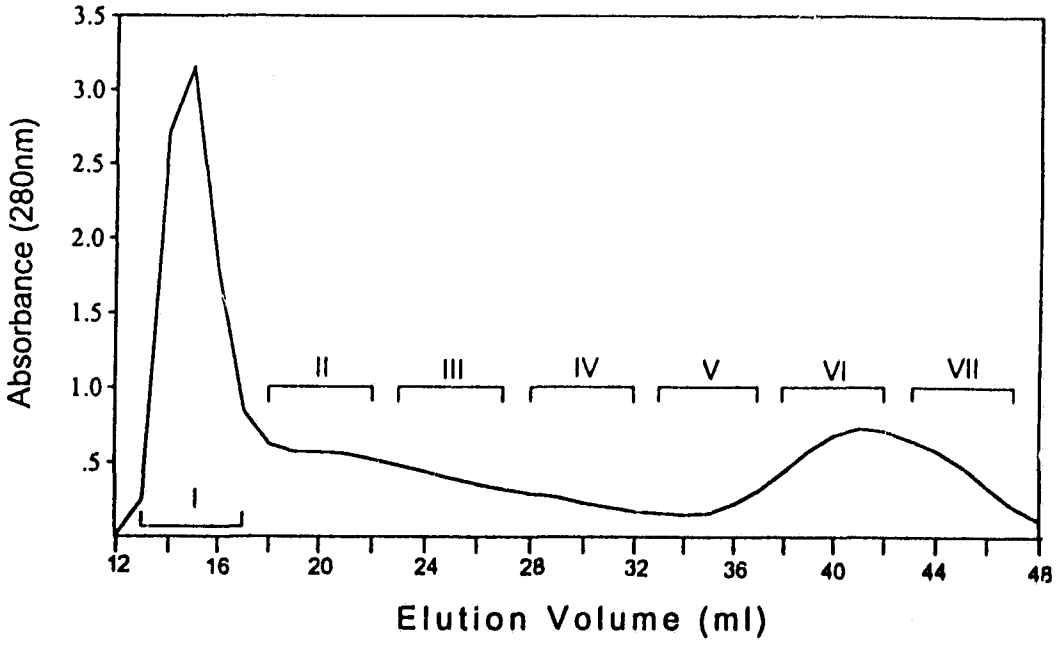

Figure 4-6: Absorbance profile (280 nm) of *L. m. mexicana* cytoplasmic proteins separated on a 1.5 x 25 cm Sepharose G75 column. I - VII represent pooled fractions that were assayed for mannan phosphorylase activity.

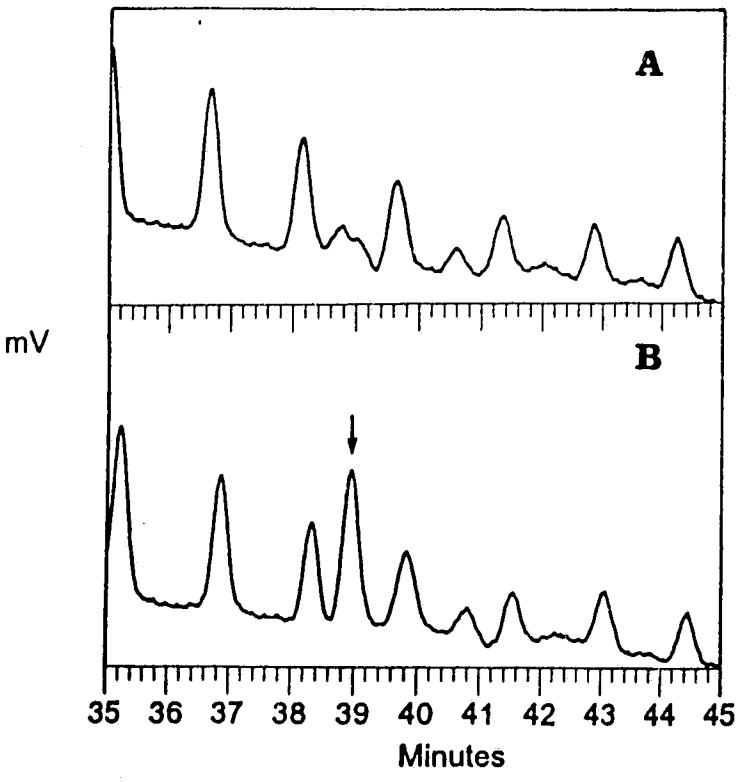

Figure 4-7: Anion-exchange chromatographic separation of mannans and hexose 1-phosphates following incubation with fraction II from the G75 column. A) Time zero control showing elution of mannans. B) Generation of a product peak eluting at 39 min following 48 h incubation of mannans with fraction II from the G75 column (indicated by an arrow). The peak coelutes with M1P and G1P and disappears following digestion with alkaline phosphatase.

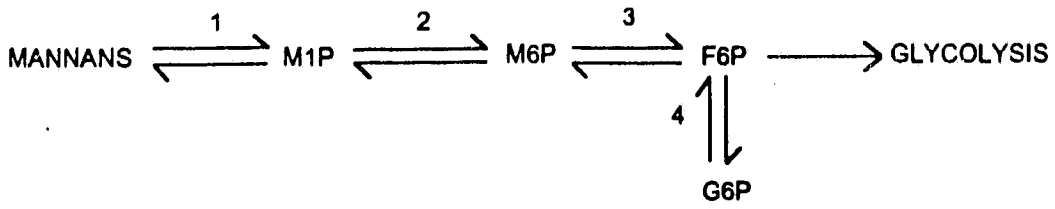

Figure 4-8: Intermediates of mannan metabolism and the enzymes involved: 1) mannan phosphorylase, 2) phosphomannomutase, 3) phosphomannose isomerase, 4) phosphoglucose isomerase.

Discussion

Although the protozoan mannan reported here appears to occupy a metabolic position analogous to glycogen of the mammalian host, there are some outstanding differences. Aside from the obvious difference in the monosaccharide component, the mannan is unique in having a 1-2 and not a 1-4 linkage as is found in glycogen. Moreover, the anomericity of the parasite polymer is β rather than α as found in glycogen. The mannans also have a much smaller average molecular weight than glycogen and are not branched. The largest detectable mannan was approximately 23 residues in length while glycogen typically consists of 14,000 to 28,000 residues. The mobilization of mannan by *Leishmania* was estimated as 5 - 10 μmoles per g wet wt over 6 h which is about 2 orders of magnitude slower than the depletion of mammalian liver glycogen.

A surprising finding was that high levels of β-linked mannans were not consistently found throughout *Leishmania spp.* The presence of β1-2 linked mannans in the closely related, monogenetic insect parasites, *Crithidia spp.* (Gottlieb, 1978; Previato et al., 1982) and their absence in the more distantly related *Trypanosoma spp.* (Previato et al., 1982; and this paper) would compel one to speculate that such metabolic capabilities evolved after the speciation of *Trypanosoma spp.*, but before the separation of *Leishmania* and *Crithidia* (Hernandez et al., 1990; Briones, et al., 1992; Fernandes et al., 1993; Landweber et al., 1994; Maslov et al., 1994). The reason and significance for the apparent reduction of this metabolic pathway within certain *Leishmania spp.* remains unknown. However, differences in the intermediary metabolism of *Leishmania spp.* have been previously noted. For example, *L. major* (Darling et al, 1989; Keegan and Blum, 1990; Walsh and Blum, 1992), *L. donovani* (Ghoshal et al., 1989; Walsh and Blum, 1992) and *L. braziliensis* (Darling et al., 1987, 1988A, 1988B) have a functional methylglyoxal pathway while there is no evidence of this pathway in *L. mexicana pifanoi* (Rainey and MacKenzie, 1991). The functional significance of a small pool of JBAM sensitive mannans in all species examined, which are free of any charged substituents and,

therefore, not dolichol intermediates in the synthesis of N-linked oligosaccharides, is unknown.

By analogy with the catabolism of mammalian glycogen, the first intermediate of mannan catabolism was expected to be M1P, the product of a mannan phosphorylase. However, the *Leishmania* mannan phosphorylase appears to be structurally distinct from mammalian glycogen phosphorylase as the former has an approximate molecular mass of 35-66 kDa while the latter is a homodimer of 195 kDa (Titani et al., 1977). Work is in progress to isolate and characterize this protein as well as to determine if the level of expression is decreased in those parasites which contain only small amounts of β-linked mannans. The other intermediates of mannan catabolism, M6P and F6P, were anticipated because they have been previously recognized in yeast as enzymes required for the synthesis of high mannose N-linked oligosaccharides from glycolytic intermediates (Payton et al., 1991). Phosphomannose isomerase is ubiquitous and has previously been identified in *Saccharomyces cerevisiae* (Smith et al., 1992), *Escherichia coli* (Miles et al., 1984), *Salmonella typhimurium* (Collins et al., 1991) and humans (Proudfoot et al., 1994) and is essential for the synthesis of N-linked cell wall mannans in yeast (Payton et al., 1991). Because all *Leishmania spp.* carry glycoproteins with high mannose oligosaccharides (Olafson et al., 1990; Funk et al., manuscript in prep.[2]; reviewed in Parodi, 1993) and lipophosphoglycans (Turco et al., 1987; McConville et al., 1990; Ilg et al., 1992) which also require the synthesis of mannose, they would be expected to express phosphomannomutase and phosphomannose isomerase.

Results presented in this paper show that G6P is rapidly produced from M1P relative to the control which contained only endogenous mannans and no exogenous M1P. The concentration of G6P present in the control was 50-fold less than when exogenous M1P was added, suggesting that mannan phosphorylase is the rate limiting enzyme in mannan catabolism. If mannan metabolism is regulated in a manner similar to mammalian glycogen, then one would expect that mannan phosphorylase would be a regulatory enzyme. An effort is being made to determine if the mannan phosphorylase is regulated by metabolic intermediates and/or phosphorylation/dephosphorylation. However, metabolic regulatory mechanisms of trypanosomatids appear to differ from

those identified in mammals. It is currently believed that regulation of glycolysis in trypanosomatids is primarily due to subcellular compartmentalization (Cazzulo, 1992). The only glycolytic enzyme of trypanosomatids recognized as being regulated to-date, is pyruvate kinase which is affected by metabolic intermediates (Van Schaftingen et al., 1985; Etges and Mukkada, 1988; Callens et al., 1991), but there is no data regarding the effect of phosphorylation/dephosphorylation as occurs for mammalian liver pyruvate kinase (Engstrom, 1978). In addition, inhibition of various *Leishmania* protein kinases did not affect the rate of glucose oxidation (Blum, 1994), suggesting that phosphorylation/dephosphorylation is not a regulatory mechanism for glycolysis in these organisms.

Since mannans are not present in mammals, the mannan phosphorylase appears to be a unique enzyme to the protozoa in the host-parasite relationship, perhaps providing a potential target for chemotherapeutic agents. The question that now must be addressed is whether metabolism of this storage material is important for the survival of *Leishmania spp.* within the mammalian host. The occurrence of the same putative storage material in *Crithidia*, a monogenetic parasite of insects, might suggest that the mannans are primarily important for the parasite while in the gut of the insect. It could also be argued that because amastigotes rely less on glycolysis than promastigotes and depend more on the oxidation of fatty acids for nutrition (Hart and Coombs, 1982; Blum, 1987; Rainey and MacKenzie, 1991), that the mannans would be less important for the amastigote stage. However, amastigotes still utilize glucose (Hart and Coombs, 1982; Rainey and MacKenzie, 1991). Furthermore, comparison of *L. m. mexicana* promastigotes and axenically grown amastigotes demonstrated that amastigotes contain a higher concentration of mannose per cell than promastigotes which suggests the mannans are important in both lifestages. It is conceivable that the cytoplasmic mannans of *Leishmania* are key energy molecules when the parasite encounters conditions less favourable for growth. In support of this hypothesis, it has been shown that under conditions of mild hypoxia (6% O_2/5% CO_2) the amount of carbon generated as products of intermediary metabolism is greater than can be accounted for by consumption of exogenous glucose implying the use of an endogenous carbon source (Keegan and Blum,

1990). Under such conditions, the flux through the glycolytic pathway increases, at least to the stage of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (Darling et al., 1987, 1989; Keegan and Blum, 1990). Such an increase in the glycolytic flux occurring at the same time as utilization of an endogenous carbon source suggests that the carbon source may be a hexose which feeds directly into this pathway. Further, it has been shown, both here for *L. m. mexicana* and by Keegan and Blum (1992), studying *L. donovani*, that the mannans are utilized under conditions of carbon limitation. It therefore seems reasonable to suggest that the cytoplasmic mannans may be utilized under hypoxic conditions. Assuming this is true, the mannans may be important for parasite survival in the mammalian host when transient hypoxic conditions occur (West, 1990). Keegan and Blum (1992) demonstrated that mannans are accumulated by *L. donovani* promastigotes during metacyclogenesis, supporting the theory that cytoplasmic mannans are important for promastigote survival following entry into the phagolysosome and for subsequent development of amastigotes. Work is presently underway to address these proposals.

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APPENDIX A

Abbreviations: ACN, acetonitrile; AMU, atomic mass units; BSA, bovine serum albumin; CID MS, collision induce dissociation mass spectrometry; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Con A, concanavalin A; COSY, J-correlated spectroscopy; CR!, type 1 complement receptor; CR3, type 3 complement receptor; D-MEM, Dulbecco's Modified Eagle Medium; ELISA, enzyme-linked immunosorbent assay; FAB+ MS, positive ion fast atom bombardment mass spectrometry; FBS, fetal bovine serum; F6P, fructose 6-phosphate; GC/MS, gas chromatography/ mass spectrometry; G1P, glucose 1-phospate; G6P, glucose 6-phosphate; G6P-DH, glucose 6-phosphate dehydrogenase; GPI, glycosyl-phosphatidylinositol; g.u., glucose units; HRP, horse radish peroxidase; JBAM, Jack Bean alpha-mannosidase; HMQC, homonuclear quantum correlated; HT, human transferrin; HVE, high voltage electrophoresis; JBAM, Jack Bean α-mannosidase; LPG, lipophosphoglycan; LPS, lipopolysaccharide; KLH, Keyhole Limpet hemocyanin; M1P, mannose 1-phosphate; M6P, mannose 6-phosphate; MFR, mannosyl-fucosy receptor; NMR, nuclear magnetic resonance; NOESY, Nuclear Overhauser effect spectroscopy; PAD, pulsed amperometric detection; PBS, phosphate buffered saline; PI-PLC, phosphatidylinositol phospholipase C; PMAA, partially permethylated alditol acetates; TFA, trifluoric acetic acid; TOCSY, total correlation spectroscopy; TOF, time of flight; TOF MS, time of flight mass spectrometry; TM, tunicamycin.

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