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UMI
Characterization of the Post-translational Modifications of the Secreted Acid Phosphatase of *Leishmania donovani*

by

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B.Sc., University of Alberta, 1993

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biochemistry and Microbiology

_We accept this dissertation as conforming to the required standard_

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The secreted acid phosphatase (SACp) of *Leishmania donovani* is a secreted glycoprotein modified with unique glycoforms which share a structural heterogeneity and immunological similarity with the dominant, cell surface phosphoglycolipid produced by all species of leishmania parasites. The post-translational modifications of this enzyme are structurally diverse and include standard high-mannose type N-linked glycosylations as well as a novel O-linked phosphoglycan. This dissertation encompasses the analysis of these structures including assessment of their size, hexose composition and sites of attachment to the protein. These analyses have employed both carbohydrate and protein chemistry techniques, as well as physical methods such as mass spectrometry. The N-linked glycosylations have been compared with those previously characterized on other *Leishmania* proteins and show substantial structural similarity. The O-linked phosphoglycans are unique to *L. donovani*, and are composed of phosphodisaccharides with the structure 4-O-(beta-D-galactopyranosyl)-alpha-D-mannopyranosyl-1-phosphate. These phosphodisaccharides are arranged in linear polymers by way of a phosphodiester linkage between the C1 hydroxyl of mannose and the C6 hydroxyl of galactose. Linkage of this structure to the protein is novel and proceeds via a phosphodiester to selected serine residues that are contained within a consensus protein sequence. This sequence occurs in excess of 20 times within the SACp, which results in abundant glycan modification and contributes to the heterogeneity displayed by this enzyme. The biosynthetic machinery used to produce these structures was also investigated. The addition of phosphoglycan to the SACp is initiated by the transfer of alpha-D-mannopyranosyl-1-phosphate from GDP-Man to the protein catalysed by a mannosyl phosphate transferase (MPT). An assay for this enzyme is described using a synthetic peptide substrate to which radiolabeled mannose can be transferred from GDP-[¹⁴C] Man. This assay has been used to partially characteriz the MPT and has assisted in the isolation of the enzyme using an affinity chromatography approach. Sequence analysis and amino acid analysis of the enzyme isolated in this way has shown that the MPT is a novel molecule that does not presently exist in the public database.
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1. Introduction

A. Description of Leishmaniasis

General Statistics and Geographical Distribution

Leishmaniasis is the name given to a collection of diseases affecting human and other mammalian populations caused by infection with protozoan parasites of the genus *Leishmania*. While not as common or as well known as malaria or tuberculosis, it is still assessed by the World Health Organization (WHO) as a significant international health concern. Current estimates set the global burden at 12 million cases with 2 million new cases occurring annually (World Health Organization, leishmaniasis fact sheet: http://www.who.int/inf-fs/en/fact116.html). It is endemic in 88 countries (Figure 1.1) throughout tropical and subtropical regions of the world, placing 350 million people at risk of contracting the disease. In recent years, endemic regions have seen a sharp rise in occurrence that is commonly associated with human development such as deforestation and rural-urban migration. This is punctuated by periodic epidemics such as that in Southern Sudan in the early 1990s, which saw 100,000 deaths and currently in Kabul, Afghanistan, where more than 200,000 cases are active (World Health Organization, Leishmaniasis control page: http://www.who.int/emc/diseases/leish/leisdis1.html). Finally, as will be mentioned below, Leishmaniasis is an opportunistic infection in immunosuppressed people and the AIDS epidemic has greatly enhanced the incidence of this parasitic disease.

Description of Disease and its Variation

There are six main *Leishmania* species that are recognized as being disease causing in humans, although there are many subspecies identified within this classification (Handman 2001). The organisms are morphologically similar, but are responsible for a wide variation in disease states. The Oriental Sore is a cutaneous form of leishmaniasis (CL) in which a small ulceration occurs at the site of infection. *L. major* and *L. tropica* are most commonly associated with this condition. These lesions heal spontaneously, leaving a noticeable scar, but recovery carries the added benefit of
providing significant immunity to repeat infections. It is important to note that sterile immunity is rare and that small numbers of parasites usually persist in patients after the clinical expression has faded, establishing a state of premunition where disease can be reinstated under appropriate circumstances. This type of disease represents roughly 75% of all new leishmania cases. A variation of the CL disease is known as diffuse cutaneous leishmaniasis (DCL) commonly caused by *L. mexicana amazonensis* and *L. aethiopica* infections. This condition results in the formation of multiple lesions (up to 200), which never heal spontaneously and usually relapse after chemotherapy. Espundia or mucocutaneous leishmaniasis (MCL) is a relatively rare condition caused by *L. braziliensis*. Lesions occur at the site of infection by this organism, which heal as in normal cutaneous disease, only to reappear in the mucous membranes of the nose and mouth. This condition causes extensive tissue damage, caused primarily by the host immune system and is frequently lethal if not treated. Although rare, MCL is the most disfiguring form of leishmaniasis and the intensity of its visible manifestation has caused
it to receive a disproportionate amount of media attention. Kala-azar (Black Disease) or visceral leishmaniasis (VL) is considered the most serious illness. Caused by \textit{L. donovani}, \textit{L. infantum}, and \textit{L. chagasi}, this disease is rarely noticed at the site of infection as it produces only a small ulcer. Instead, the parasite targets the reticuloendothelial system including the liver, spleen, and bone marrow. The symptoms associated with VL include fever, anaemia, hepatosplenomegaly, and hypergammaglobulinemia. Additionally, patients show a peculiar lack of responsiveness to \textit{Leishmania} antigens (Sacks, Lal et al. 1987). If afflicted individuals do not receive treatment, the fatality rate for VL is 100%. Finally, the risk of illness from any species of \textit{Leishmania} is severely heightened in immunocompromised hosts. \textit{Leishmania} resurgence has been identified in leukemia patients (Jewell and Giles 1996), transplant recipients (Fernandez-Guerrero, Aguado et al. 1987) and patients infected with Human Immunodeficiency Virus (HIV). The current AIDS epidemic, particularly, has had a serious impact on the global state of leishmaniasis. AIDS increases susceptibility to \textit{Leishmania} infection by 100-1000 times, and since both infections interfere with cell mediated immunity, the severity of disease is enhanced. Concurrent infections are easily transmitted by needle sharing, and this has led to a rapid spread in nonendemic regions. This has been initially observed in SW Europe, but the current spread of endemic regions predicts an overlap of \textit{Leishmania} and HIV that will reproduce this trend in Africa with devastating effects in the near future.

While it is generally assumed that the form of the disease is dependent solely on species of parasite, this may be an oversimplification. This is indicated by the occasional crossing of boundaries of disease symptomology, such as visceralization of normally cutaneous strains (Ozbel, Turgay et al. 1995). It is important to remember that neither host nor parasite genetics are homogeneous throughout their respective populations. This makes it difficult to ascertain the relative contribution of parasite and host to disease. The completion of sequence analysis of the human genome, and several \textit{Leishmania} genomes (Blackwell 1997; Ivens and Smith 1997) with the assistance of on-going proteomics investigations, will certainly assist identification of the molecular components that direct the course of these diseases.
B. Disease Control

Chemotherapy

Treatment of leishmaniasis is possible, but difficult due to the toxicity of currently available chemotherapeutic agents. The first line drug is the pentavalent antimonial Pentostam (Wellcome Foundation, London, UK). The mode of action of this compound is not well understood, but seems to involve disruption of glucose and fatty acid metabolism (Berman 1988). The drug itself is not toxic in the administered form, and requires reduction from Sb(V) to Sb(III) by the parasite to produce the toxic effect (Shaked-Mishan, Ulrich et al. 2001). Side effects include anorexia (30%), pancreatitis (90%), myalgias/arthralgias (50%), and thrombocytopenia (1-5%) but they are generally not severe (US Department of Defense Telemedicine Web Site: http://www.dod-telemedicine.org/gobook/pentost.html). Second line drugs for antimony resistant infections include Pentamidine (side effects include danger of pancreatitis, sudden low blood sugar and death) and Amphotericin B (kidney damage, diarrhea, and anemia among others). A promising experimental drug is Miltefosine (hexadecylphosphocholine), which affects cell signalling and membrane synthesis. It can be taken orally, has demonstrated a cure rate of >95% against VL in a small human trial (Jha, Sundar et al. 1999), and appears to cause few side effects. This compound is currently in phase III clinical trials.

Prophylaxis

Vaccination against leishmaniasis is currently unavailable. However, healing of cutaneous disease leaves an individual immune to further infection. This immunity is cell mediated and is reflected by a strong delayed type hypersensitivity (DTH). Historically, Bedouin and Kurdistani tribal societies have performed a process known as leishmanization, which involves exposing the buttocks of young children to leishmania infection (Handman 2001). This is to prevent infection and scarring of the face or other visible areas. ‘Vaccination’ by controlled infection has been attempted at a more organized level in the Soviet Union and Israel, but uncontrolled lesions, psoriasis and immune suppression have made this practice more of a risk than a benefit.
The subsequent search for a safe vaccine has proceeded by more scientific methods. The use of killed organisms has been explored more thoroughly than any other type of preparation. The results of these studies have been questionable. Several experiments have shown that heat killed or irradiated parasites are effective in preventing infections in mice (Alexander 1982; Howard, Nicklin et al. 1982). However, a recent human trial using a mixture of heat killed *L. major* promastigotes with bacille Calmette-Guérin (BCG) did not show a significant increase in protection when compared with BCG alone (Khalil, El Hassan et al. 2000). To date, this is the only type of preparation that has reached clinical trials, but there are several other strategies in various stages of development:

1) Live, attenuated strains have the advantage of mimicking natural infections and have shown reasonable success in immunizing mice against cutaneous disease (Titus, Gueiros-Filho et al. 1995; Alexander, Coombs et al. 1998).

2) Synthetic peptides based on immunogenic *Leishmania* proteins have also proven effective in mice when coupled with the correct adjuvant (Jardim, Alexander et al. 1990; Spitzer, Jardim et al. 1999).

3) *Leishmania* antigens can be expressed and delivered in attenuated bacterial or viral vectors. The antigen can be an intact protein (Abdelhak, Louzir et al. 1995), or more elegantly, an immunogenic peptide inserted and expressed within the sequence of a vector derived molecule (White, Collinson et al. 1999).

4) Finally, naked DNA vaccines encoding molecules such as *Leishmania* Activated C Kinase receptor (LACK) (Gurunathan, Sacks et al. 1997) are attractive because of their stability, ease of production, and stimulation of a cell mediated $T_h1$ response (Walker, Scharton-Kersten et al. 1999).

There has also been some interest in nonproteinaceous vaccines for *Leishmania*. Initially, the cell surface glycolipid lipophosphoglycan (LPG) was suggested as a vaccine candidate (McConville, Bacic et al. 1987). However, the immune response generated by this molecule was later ascribed to contaminating proteins within the preparation (Jardim, Tolson et al. 1991). In spite of this, synthetic LPG analogs were recently synthesized and
may help to reconcile the question of the utility of these carbohydrates as vaccine candidates (Routier, Nikolaev et al. 1999). Carbohydrate vaccines can be effective, but they tend to elicit Th2 type immune responses (antibody mediated), not the Th1 response that is typically required to combat *Leishmania* infection. This can be gleaned from the use of a synthetic hexasaccharide as a vaccine against prostate cancer, which resulted in high IgM and IgG antibody titers (Slovin, Ragupathi et al. 1999). Additionally, the carbohydrate portion of LPG has been identified as an effective inhibitor of IL-12 release (Piedrafita, Proudfoot et al. 1999). This cytokine is important in the amplification of Th1 T-cells. With this type of effect, it is likely that carbohydrates derived from LPG will only be capable of exacerbating the diseases.

C. *Leishmania* Life Cycle

*General Description*

*Leishmania* are digenetic protozoa, which means that they must pass through two hosts to complete their life cycle. The disease is generally zoonotic, the infection of humans is accidental and usually caused by close interaction with a natural animal reservoir. Efforts aimed at reducing the presence of *Leishmania* reservoirs in human settlements can drastically reduce the number of observed cases. The mammalian host range varies considerably, but rodents and dogs are most common. Exceptions occur in India and the Middle East, where humans are the primary vector for VL and CL respectively. There are two main stages in the *Leishmania* life cycle (Figure 1.2). In the mammalian host, the parasite survives intracellularly within macrophages. In this environment, the parasite persists as a small, round, nonmotile cell roughly 2-4 μm in diameter. This stage is known as the amastigote, which refers to the trypanosomatid life stage in which the cell is rounded and nonmotile, and the kinetoplast is immediately posterior to the nucleus. Transmission between mammalian hosts is accomplished by the arthropod host, the sandfly, while obtaining a blood meal. These are flies of the genera *Phlebotomus* and *Lutzomyia* which are generally responsible for transmitting Old World and New World strains respectively. During this portion of the life cycle, the parasite
exists as an elongated cell, roughly 20 μm in length. It is highly motile by means of a single flagellum that is of a similar length to the cell itself. This is known as the promastigote, which refers to a motile cell with the nucleus positioned at the center of the cell and the kinetoplast at the posterior end. During its passage through the sandfly, the parasite develops through two main stages. Procyclic promastigotes are actively dividing, non infective cells that are responsible for colonization of the gut, while metacyclic promastigotes are nondividing, infective cells that are responsible for continuing the life cycle.
cycle and infecting the next host. The interaction of the parasite with each of its hosts is extremely complex, and only partially understood. Some of the details of these interactions are outlined below.

**Host-Parasite Interactions in the Mammal**

*Leishmania* are relentlessly attacked by the mammalian immune system from the moment they are introduced into the bloodstream. Their survival depends on the outcome of numerous interactions with the host at all stages of infection. Disruptions in these processes frequently have drastic consequences for the invading microbe. It is hoped that a thorough understanding of these interactions will provide the necessary information for the development of new treatments and chemotherapeutic agents. In the mammal, the parasite exists primarily as the amastigote in an intracellular space, the phagolysosome of macrophages. However, promastigotes are also present transiently in the initial stages of infection. As such, each life stage has developed its own methods of avoiding clearance by the immune system. The interactions of the parasite with the host can be dissected into three stages, survival in the bloodstream, invasion of macrophages, and colonization of the phagolysosome.

When they first enter the bloodstream, *Leishmania* encounter the soluble factors that make up the complement system. These represent the first line of defense and the first challenge to *Leishmania* survival. The ability to resist killing by this system depends greatly on the life stage of the organism. Procyclic promastigotes of several species have been shown to activate complement very efficiently by the alternative pathway. Complement factor C3b is deposited on the surface of these cells, which directs the attachment of the C5b-9 complex (Mosser and Edelson 1984; Puentes, Sacks et al. 1988). This complex is capable of rupturing the procyclic cells, resulting in parasite death. In contrast, metacyclic promastigotes show resistance to killing in this fashion. While C3b and C5b-9 still collect at the parasite surface, the C5b-9 lytic complex is unable to compromise the parasite membrane and is eventually released (Puentes, Da Silva et al. 1990). The hypothesis extended to explain this observation is that an increase in the thickness of the parasite surface coat occurs as it converts to the metacyclic form (Sacks, Pimenta et al. 1995) and physically blocks the approach of the C5b-9 complex,
preventing it from inserting into the membrane. This is similar to the strategy used by certain strains of Salmonella. Smooth variants of Salmonella are serum resistant because their lipopolysaccharide (LPS) molecules contain long O-antigens. Rough variants, with smaller LPS, are complement sensitive (Joiner, Hammer et al. 1982; Joiner, Hammer et al. 1982). In the case of L. donovani, the means of avoidance is slightly altered. There is evidence that cells of this species accumulate C3bi instead of C3b (Puentes, Dwyer et al. 1989). C3bi does not trigger the complement cascade, and allows the parasite to avoid killing by this system altogether. The interaction of the amastigote with this process is somewhat different. They are strong activators of complement and readily take up C3 in vitro (Mosser, Wedgwood et al. 1985), irrespective of their resistance or susceptibility to lysis in vivo. How these cells are able to circumvent lysis is, therefore, less apparent. It has been noted that L. mexicana secrete large quantities of a proteoglycan (Ilg, Stierhof et al. 1995). This material can be identified in active lesions, and is a strong activator of complement. It is believed that this material may cause a localized depletion of serum complement in the region surrounding the lesion, protecting amastigotes in the area (Peters, Kawakami et al. 1997). This effect has yet to be proven, but is the only hypothesis presently available. Some species, such as L. donovani, do not secrete proteoglycan and cannot be protected in this way. However, amastigotes from this species have demonstrated a greater resistance to complement than is observed for other species (Hoover, Berger et al. 1984), suggesting that other resistance mechanisms are at work.

Prior to cell replication, Leishmania must gain access to the intracellular space of host macrophages. The means by which the parasite gains entry is a point of much debate, and likely involves several distinct pathways. Many researchers have implicated complement components in the invasion process. Macrophages commonly take up particles that have been opsonized by complement and display a number of receptors at their surface, which mediate this process. As mentioned earlier, promastigotes bind the C3b component of complement. The macrophage receptor CR1 (complement receptor 1) is responsible for binding to this compound, and can mediate the phagocytosis of L. major (Da Silva, Hall et al. 1989). L. donovani can invade macrophages in a similar fashion using the receptor CR3, which is specific for C3bi (Blackwell, Ezekowitz et al.
Recent evidence suggests that this pathway may be more relevant, and has been demonstrated for *L. major* as well (Mosser and Rosenthal 1993), even though this species is not known for its binding to C3bi. This is supported by the observation that αCR3 antibodies are capable of blocking *Leishmania* invasion of macrophages (Blackwell, Ezekowitz et al. 1985; Mosser and Edelson 1985). There are, however, other means of access that are not complement mediated. The mannosyl-fucosyl receptor has been implicated in the uptake of *Leishmania* (Blackwell, Ezekowitz et al. 1985), although this is primarily true for avirulent lines of *Leishmania*, which are subsequently killed by macrophages (Chakraborty, Chakraborty et al. 1998). The latter suggests that the means of parasite entry is tied to its ultimate fate within the macrophage. Some pathways lead to survival and continued infection, while others result in parasite clearance. C reactive protein (CRP) is a soluble protein involved in inflammation. This molecule has been shown to bind to promastigotes, substantially increasing their invasion of human macrophages. The inflammation caused by bloodfeeding sandflies ensures the presence of CRP in the early stages of infection making this process a very plausible option for parasite invasion. Amastigotes on the other hand, are not involved in the initial infection but must invade macrophages once they have outgrown and ruptured their initial host cell. The processes they use appear to differ from those employed by promastigotes. Amastigotes bind significant quantities of heparin, which allow them to interact with heparan sulfate proteoglycans on the surface of many cell types, including macrophages (Love, Esko et al. 1993). The addition of soluble heparin or heparan sulfate can block this interaction with most cells, although it only partially blocks adherence to macrophages, suggesting the contribution of other processes. Another study has implicated surface glycosphingolipids (GSL) in amastigote invasion where antibodies to these molecules are capable of blocking 60-80% of *L. mexicana* amastigote invasion (Straus, Lavery et al. 1993). With so many different means of invasion, however, it becomes difficult to determine the relative contribution of each pathway. It is clear that having multiple means of entry ensures the success of infection across a heterogeneous host population, preventing disease resistance due to changes in individual receptors.
Once inside the macrophage, *Leishmania* live and proliferate within the phagolysosome – the compartment normally responsible for killing pathogens and the most inhospitable environment in the host. That *Leishmania* can thrive here is an indication of a remarkable adaptation. As with invasion, establishment and survival within the macrophage depend on several interactions occurring simultaneously. The initial stages of this process probably differ depending on whether promastigotes or amastigotes are responsible for infection, but this has not been directly studied (Antoine, Prina et al. 1998). Initially, the formation of a compartment known as the parasitophorous vacuole (PV) occurs. This involves the fusion of late endosomes and lysosomes to the phagosomal compartment (Alexander and Russell 1992). The recruitment of these organelles is indicated by the presence of a number of marker molecules in mature PV such as the lysosome associated membrane protein (LAMP1) (Lang, Hellio et al. 1994). Time course studies have shown that this process is delayed when metacyclic promastigotes infect (Desjardins and Descoteaux 1997). It is likely that this delay provides an opportunity for the parasite to convert to the much more resistant amastigote life stage. The morphology of the PV differs remarkably between species. *L. mexicana* and *L. amazonensis* cause the formation of an extremely large PV with many amastigotes, while *L. donovani* and *L. major* inhabit a much smaller PV containing only a few amastigotes. Also, some species, such as *L. amazonensis* and *L. donovani*, affix themselves to the PV membrane, while others remain free in the PV lumen (Benchimol and de Souza 1981). The significance of these differences remains unclear, but may be involved in virulence. The pH of this space remains acidic throughout parasite development (Antoine, Prina et al. 1990). Rather than being inhibited by this, *Leishmania* exhibit optimal metabolic performance under these conditions. This may be related to membrane bound proton pumps involved in the capture of metabolites, and the expression of a number of metabolite transporters that display a low pH optimum (Zilberstein and Shapira 1994). Along with low pH, amastigotes are able to coexist with the numerous hydrolytic enzymes that are normally found in lysosomal compartments (Prina, Antoine et al. 1990). These include a number of cathepsins (Lang, Hellio et al. 1994). The resistance to these molecules may be due to the expression of a reduced number of membrane proteins, as well as the presence of abundant...
glycosylinositolphospholipids (GIPLs), which provide a protective covering for the amastigote (McConville and Ralton 1997).

What is perhaps more remarkable is the ability of the parasite to avoid killing by normal immune processes. Macrophages are normally activated following phagocytosis by the concerted action of a panel of cytokines. Recent evidence suggests that under naturally occurring conditions, the parasite is able to invade the macrophage in a stealthy manner without triggering any of the normal cytokine mediated cell signalling processes (Racoosin and Beverley 1997). In fact, *Leishmania* seem to be capable of stimulating inappropriate signalling processes, one example being the stimulation of IL-10 release, which inhibits protein kinase C (PKC) and blocks the production of toxic oxygen metabolites that might otherwise be used to kill the organism (Bhattacharyya, Ghosh et al. 2001). The latter might even be staged prior to invasion to prepare the macrophage for colonization. The parasite is able to recruit host IgG, which can interact with Fcγ receptors on the macrophage surface thereby stimulating IL-10 release (Kane and Mosser 2001). Other cytokines are also affected. It is routinely accepted that IFNγ and IL-12 are critical components of a successful host response to *Leishmania* (Belosevic, Finbloom et al. 1989; Reiner, Zheng et al. 1994). The parasite is well known to prevent macrophage activation by IFNγ and also to prevent release of IL-12 (Piedrafita, Proudfoot et al. 1999).

Another important contributor to parasite clearance is the toxic compound nitric oxide (NO), which is produced by the inducible enzyme nitric oxide synthase (iNOS) (Liew, Millott et al. 1990; Stenger, Thuring et al. 1994). The production of this enzyme is inhibited by GIPLs that coat the surface of the amastigote (Proudfoot, O'Donell et al. 1995). Even antigen presentation, arguably the most important aspect of a response to intracellular pathogens, is disrupted by *Leishmania*. Partial impairment of this process can be observed in infected macrophages (Prina, Jouanne et al. 1993). This may be mediated by the observed collection of MHC class II molecules at sites of parasite attachment to the PV, which could be a means of preventing the MHC from reaching the cell surface and presenting antigen (Antoine, Lang et al. 1999). The parasite also appears to deplete MHC abundance by ingesting and degrading MHC molecules (De Souza Leao,
Lang et al. 1995), although these observations are based on immunofluorescence studies and have not been rigorously proven at a biochemical level.
Host-Parasite Interactions in the Sandfly

The interaction of *Leishmania* with its invertebrate host is equally fascinating. Although it has received far less attention from the scientific community, investigation into this portion of the life cycle has been steadily increasing over the last 15 years. The progression of *Leishmania* infection within the sandfly takes place over the course of one week (Walters, Modi et al. 1987). Initially the infection is confined to the blood bolus in the posterior midgut. Amastigotes convert to promastigotes and can be detected microscopically within 12-18 hrs. Movement forward through the digestive tract to the anterior midgut occurs 4-5 days following blood feeding. By day 7, the anterior midgut and cardia (adjacent to the mouth) are swollen and fully colonized by the parasite. At this point, the parasite is poised for transmission during the next bloodmeal. The parasite must overcome a number of obstacles during this progression. The first involves escape from the blood bolus. Initially, the bloodmeal is contained within a host-derived chitinous membrane called the peritrophic matrix (PM). The parasite must breach and pass through this membrane if it is to establish an infection. Failure in this regard results in parasite excretion during defecation. During a normal infection, the PM is degraded at 60 hrs postfeeding and is mediated by the production of a secreted chitinase (Schlein, Jacobson et al. 1991). This molecule was recently cloned and is conserved across all species of *Leishmania* (Shakarian and Dwyer 2000). The PM should not only be viewed as a barrier, however. There is also evidence for a protective role towards the parasite as it transforms from amastigote to promastigote. During this time the parasite is particularly sensitive to enzymatic degradation. Early removal of the PM exposes the parasite to the hydrolytic digestive enzymes of the sandfly gut, and leads to parasite mortality (Pimenta, Modi et al. 1997). Once it escapes the bloodmeal, the parasite must colonize the insect gut. To this end, the parasite embeds itself within the epithelium of the posterior midgut (Walters, Modi et al. 1987). Attachment to the gut lining allows the parasite to avoid being passed through the digestive tract. This attachment has been studied extensively in several species and appears to be stage specific. As demonstrated by studies with plant lectins (Wilson and Pearson 1984; Sacks, Hieny et al. 1985), the development from procyclic to metacyclic promastigotes is accompanied by changes in surface carbohydrates. It has
now been shown that changes in surface carbohydrates also result in a loss of affinity for the gut lining (Pimenta, Turco et al. 1992). This allows the parasite to bind transiently to the gut while it is multiplying. Once it converts to the infective form, it then detaches and migrates forward to the mouthparts to facilitate transmission. Sandfly gut lectins that are involved in this process have recently been identified in *Phlebotomus papatasi* (Dillon and Lane 1999). A further observation relating to this process is the formation of a gel like matrix which is present throughout the midgut lumen (Stierhof, Bates et al. 1999). This material appears to assist in the interaction of the parasite with the gut. Electron micrographs show that the parasite is embedded in this material during this portion of its development. Finally, transmission requires the parasite to pass through the sandfly feeding apparatus in a reverse direction. Since this is normally a one-way street, alterations must be made to facilitate transfer. The most dramatic of these is the degradation of the stomodeal valve found at the interface of the cardia and the pharyngeal pump (Schlein, Jacobson et al. 1992). This valve normally closes to prevent backflushing while the sandfly is withdrawing a meal. Once degraded, this check no longer functions, and parasites may be ‘backwashed’ into the proboscis during feeding. A plug of gel like material has also been observed in the region of this valve, and may assist in this process (Shortt and Swaminath 1928). This plug interferes with the taking of a bloodmeal by physically blocking the open valve. Presumably, this increases the occurrence of backflushing and causes the sandfly to probe repeatedly in an attempt to feed. This not only increases the rate of infection, but can result in multiple infections from a single fly. In one case, a single fly was responsible for producing 11 lesions (in the primary investigator) (Beach, Kiilu et al. 1985). This behavior has been observed in *Ph. argentipes/L. donovani* (Shortt and Swaminath 1928) and *Ph. dubosi/L. major* (Beach, Kiilu et al. 1985).

### D. *Leishmania* Derived Molecules that Bear Phosphoglycosylations

Phosphoglycosylations are rare post-translational modifications unique to *Leishmania* and a handful of other organisms. They are present in all species of *Leishmania* and are the most abundant structures produced by these parasites. They are
also the most well characterized. Their basic structure consists of a phosphoglycan (PG) polymer with a repeating galactosyl-mannosyl-phosphate subunit structure connected by phosphodiester linkages, but many variations on this theme have been described. Numerous functional roles pertaining to *Leishmania* parasite survival and virulence have been attributed to PG structures, and the final section of this introduction provides a description of the major PG molecules and their functions.

*Lipophosphoglycan (LPG)*

The lipophosphoglycan is easily the most well known molecule produced by *Leishmania*. It has been studied extensively, and a thorough review of the literature would eclipse the remainder of this thesis. Instead, a description of its structural variations will be presented here along with a much abbreviated discussion of its many functions. Further information can be obtained from the many published reviews on this molecule (Turco 1990; Turco and Descoteaux 1992; Mengeling, Beverley et al. 1997; Mengeling and Turco 1998; Descoteaux and Turco 1999).

LPG is expressed in high copy number (1-3 × 10⁶ copies/cell) on the surface of all *Leishmania* promastigotes. It is much reduced on the surface of amastigotes, with less than 1000 copies/cell (Bahr, Stierhof et al. 1993). The structure can be separated into four basic components, the lipid anchor, a hexasaccharide core, the phosphodisaccharide repeat, and a neutral oligosaccharide cap (Turco and Descoteaux 1992). The lipid moiety of LPG is a conserved lyso-alkylphosphatidylinositol in which the alkyl chain is either a C24 or C26 saturated, unbranched hydrocarbon (Orlandi and Turco 1987). The hexasaccharide core is also conserved, consisting of a glucosamine (GlcN), two Man, and three Gal, with one of the Gal in an unusual furanose configuration (Turco, Orlandi et al. 1989). The cap structures are mono-, di- or trisaccharides which contain only Man and Gal. The level of variation depends on the species; *L. major* LPG is always capped with dimannose (McConville, Thomas-Oates et al. 1990), while *L. donovani* displays many different structures (Thomas, McConville et al. 1992). The phosphodisaccharide repeats comprise the majority of the molecule, and, like the cap structures, vary to different extents in different species. In all species, the basic structure is a repeating chain of phosphate, Man, and Gal with the structure PO₄-6-Gal-β(1,4)-Manα-1- (McConville,
This represents the structure in its simplest form and is the mature structure found in *L. donovani*. In *L. mexicana*, this polymer is modified by Glc sidechains, which extend from the Gal residues. *L. major* shows the most complicated structure with numerous sidechains consisting of Gal, Glc, and arabinose (Ara). A peculiar observation of the LPG is that it undergoes structural modification as the parasite converts to the metacyclic form. The most obvious change is a doubling in the number of phosphodisaccharide units present. A more subtle difference can be observed in the composition of the PG chains. In *L. major*, the side chains that branch off of the main structure contain terminal β-Gal when the parasite is in its procyclic form. As it undergoes metacyclogenesis, the terminating residues change to α-Ara and occasionally β-Glc (McConville, Bacic et al. 1987).

The LPG is also a functional component of the parasite, and has been associated with most aspects of parasite survival. Immunoprecipitation of LPG that has been previously incubated with $^{125}$I-C3 shows that it is the main acceptor of C3b on the surface of the parasite (Puentes, Sacks et al. 1988). In this way, it appears to facilitate macrophage entry. It should be noted, however, that these experiments do not preclude the involvement of tightly associated protein contaminants such as the kinetoplastid membrane protein 11 (KMP-11). LPG also helps to prevent complement mediated lysis. The increase in length that accompanies metacyclogenesis is the cause of the surface coat thickening that has been observed (Sacks, Pimenta et al. 1995) and associated with complement resistance (Puentes, Da Silva et al. 1990). Its contribution to intracellular survival has been shown using LPG deficient mutant strains. These strains are normally killed by host macrophages, but resistance can be conferred by passive transfer of purified LPG from a virulent strain (Handman, Schnur et al. 1986). Recent studies have shown that the transient inhibition of phagosome / lysosome fusion observed following promastigote invasion is lost or reduced in LPG negative mutants (Dermine, Scianimanico et al. 2000). Evidence has been provided that LPG is able to interfere with killing via the oxidative burst (Frankenburg, Leibovici et al. 1990; Brandonisio, Panaro et al. 1994). It may be responsible for the inhibition of protein kinase C (PKC), which stimulates the oxidative burst, as this has been shown *in vitro* using purified PKC (Turco 1999). Additionally, the LPG structure is an efficient scavenger of the toxic metabolites
produced by the burst (Chan, Fujiwara et al. 1989). There is some indication of a role in blocking nitric oxide synthesis as well. Macrophages that are incubated with LPG prior to infection seem to be unable to produce iNOS in response to IFNγ stimulation. Interestingly, this is a time dependent process, since LPG shows a synergistic effect with IFNγ when added together (Proudfoot, Nikolaev et al. 1996). Another significant contribution to virulence may involve blocking the production of IL-12 by macrophages. This inhibition has been demonstrated using a synthetic form of LPG, which is capable of abrogating the release of IL-12 by stimulation with LPS (Piedrafita, Proudfoot et al. 1999). This is significant, because IL-12 is absolutely required for clearance of *Leishmania*.

Although LPG has been shown to have many potential functions in the mammalian host, it is likely that it is only involved in the early stages of infection. The amastigote produces very little LPG and is capable of maintaining a very successful infection. In the sandfly, this is not the case. LPG is required at all times and provides the means by which the parasite navigates the insect digestive tract. One example of the importance of phosphoglycan involves transfer of viability to an avirulent *Leishmania* strain. Under normal conditions, the avirulent strain is unable to maintain an infection within the sandfly. However, if a soluble form of LPG known as excreted factor is included in the feeding mixture, the survival rate of this strain improves markedly (Stierhof, Bates et al. 1999). LPG also represents the main parasite ligand involved in attachment to the insect gut lining. Like the LPG structure, the specifics of this interaction vary between species. This can be seen from comparative studies of *L. donovani* and *L. major*. In *L. major*, it is the side chain Gal residues that mediate binding to the sandfly gut. This interaction can be inhibited by oligosaccharides containing Gal at the nonreducing end (Pimenta, Turco et al. 1992). Also, mutants deficient in their ability to produce these side chain Gal residues are incapable of binding to the midgut of the *L. major* sandfly host (Butcher, Turco et al. 1996). As the parasite matures, and the terminal Gal is replaced by terminal Ara and Glc, *L. major* detach from the gut wall so that they can migrate to the fly mouthparts for transmission. This is the natural progression of metacyclogenesis. In *L. donovani*, there are no sidechains. Instead it is the cap structure which allows the parasite to bind to the gut. What is less obvious, is how this species
detaches during metacyclogenesis. The only apparent structural difference is the doubling in LPG length. The sugars available for binding are not altered. Nevertheless, *L. donovani* develops in the same fashion as other species. It has been hypothesized that a conformational change in the LPG structure as it lengthens may lead to the concealment of the cap structures, and thus facilitates detachment (Sacks, Pimenta et al. 1995). A final function for LPG in this host involves host specificity. Vector competence is based on LPG structure (Kamhawi, Modi et al. 2000). This has been shown for several species pairs such as *P. sergenti* : *L. tropica*, *P. argentipes* : *L. donovani*, and *P. papatasii* : *L. major*. In all cases, bloodmeal loss is correlated with parasite loss in improper species pairs.

**Secreted Acid Phosphatase (SACP)**

The demonstration of a protein covalently modified with PG occurred when a LPG specific antibody was used to immunoprecipitate LPG from spent *Leishmania* culture medium (Jaffe, Perez et al. 1990). This procedure resulted in the copurification of a high molecular weight compound which could be observed by SDS-PAGE. The material manifests as a broad, continuous smear in these gels, indicative of an extremely heterogeneous structure. The fact that LPG specific antibodies could detect it was exciting, because it suggested the presence of a novel post-translational modification. The protein was produced by promastigotes of all species of *Leishmania* (Loveland, Dwyer et al. 1986; Ilg, Stierhof et al. 1994). Initially, the protein was thought to be absent from *L. major*, but recent evidence has shown that these cells simply produce much lower amounts of SACP when compared with other species (Shakarian and Dwyer 2000). The enzyme is produced as a monomeric, highly soluble (>20 mg/ml) glycoprotein, and of the >40 secreted proteins produced by this parasite, it is among the most abundant (Bates, Gottlieb et al. 1988). The SACP of *L. mexicana* is produced in a substantially different form. It forms a filamentous proteophosphoglycan polymer that is readily observed by electron microscopy (Ilg, Stierhof et al. 1991). The purpose behind this variation, is unknown, as is the function of the glycoprotein. However, its similarity to LPG, as well as its abundance, implies that it does perform a function of significance to the well being of this parasite. The production of SACP by amastigotes is somewhat less certain, as there
are conflicting reports from different species. It has been observed in the phagolysosome in *L. donovani* infected macrophages (Bates and Dwyer 1987; Ellis, Shakarian et al. 1998), but has not been detected in *L. mexicana* infections (Ilg, Overath et al. 1994). The enzyme has been recently cloned from both *L. mexicana* (Wiese, Ilg et al. 1995) and *L. donovani* (Shakarian, Ellis et al. 1997). Deletion mutants constructed in *L. mexicana* seem to indicate that the enzyme is not strictly required for parasite virulence within the mammalian host (Wiese 1998). This is not entirely surprising, since amastigotes of this species do not normally produce SAcP. It does, however, reinforce the hypothesis that this molecule is important to the promastigote within the sandfly. It is likely that studies performed within this host will lead to a better understanding of SAcP function.

**Proteophosphoglycan**

There is only one other macromolecule that bears the phosphoglycan epitope, this is the proteophosphoglycan or PPG. This material was originally identified in *L. mexicana* as a high molecular weight species on SDS-PAGE that was resistant to proteinase K digestion (Bahr, Stierhof et al. 1993). For this reason, it was initially thought to contain no protein. This has subsequently been disproven (Ilg, Stierhof et al. 1995; Ilg, Stierhof et al. 1996), although the molecule is estimated to be 70% carbohydrate by weight. The molecule has now undergone detailed structural characterization in this species (Ilg, Craik et al. 1998). The glycans of the PPG appear to contain the phosphodisaccharide backbone that is common to LPG and SAcP. However, the observed branching patterns are much more complex in the PPG and contain novel di- and triphosphorylated subunits. A related molecule has been demonstrated in *L. major* by $^{32}$P labeling and SDS-PAGE (Stierhof, Ilg et al. 1994), but there does not appear to be a PPG produced by *L. donovani*. This is significant, because the PPG has been shown to form a gel-like matrix, and is at least partially responsible for the plug that is observed in the stomodeal valve of infected sandflies (Stierhof, Bates et al. 1999). That the plug is observed in *L. donovani* infections (Shortt and Swaminath 1928), suggests that there may be another component that remains unidentified. Unlike the LPG, the PPG is produced in abundance by amastigotes and is the only PG molecule produced by *L. mexicana* in the mammalian host. A putative function for the PPG in this host, is in the formation of the
large parasitophorous vacuoles observed in *L. mexicana* infected macrophages (Ilg, Craik et al. 1998). PPG is also a strong activator of complement and is the proteoglycan responsible for depleting complement (Peters, Kawakami et al. 1997), and protecting amastigotes in active *L. mexicana* lesions.
Objectives of this Dissertation

A. Isolate the Secreted Acid Phosphatase (SACP) of *Leishmania donovani* in quantities sufficient for characterisation of its post-translational modifications.

B. Determine the size and location of N-linked carbohydrates on the SACP.

C. Determine the effect of the N-linked carbohydrates on the activity of the SACP.

D. Structurally characterise the modifications leading to antigenic similarity of the SACP to the lipophosphoglycan by ascertaining their structure, their size and their means of attachment to the protein.

E. Identify and characterise the Mannosyl Phosphate Transferase (MPT) responsible for the initial step in the construction of the phosphoglycan post-translational modifications of the SACP.
2. Materials and Methods

Reagents and equipment

Reagents and protein standards were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated. SuperQ deionized water (Millipore, Bedford, MA, USA) was used in all procedures.

Parasite strains and tissue culture

Promastigotes were cultured at 26°C in M199 medium supplemented with hemin (5 mg/l) and penicillin/streptomycin (1:100 v/v). L. donovani strain LD3, used throughout this work, is a subclone of strain 1S from Sudan (WHO designation MHOM/SD/00/1S-2D). The strain C3P0 (generously supplied by Dr. S. Turco) is a mutant form of L. donovani generated by chemical mutagenesis of the 1S strain; C3P0 is deficient in phosphoglycan synthesis. This strain was grown in similar medium, but required the addition of 5% fetal bovine serum for efficient growth. Parasites were harvested by centrifugation at 5000 x g for 15 min. In general, harvesting took place 2-3 days following stabilization in cell density, normally at 1 x 10^7 cells/ml. In some cases, log phase promastigotes were used by harvesting at a density of 5 x 10^6 cells/ml. Sodium Azide (0.1% w/v) was added to cell free culture supernatants which were stored at 4°C. This material was retained for the purpose of SAcP isolation.

Purification of the L. donovani SAcP

Leishmania donovani (MHOM/SD/00/1S-2D/LD3) promastigotes were grown to a density of 1.0x10^7 in M199 medium as previously described (Jardim et al., 1995). In most experiments, 8-10 liters of spent Leishmania culture supernatant were used for isolation of the SAcP. In some cases, ultrafiltration was used to reduce the volume of the supernatant to 75-100 ml prior to chromatographic separation. The concentrated supernatant was either filtered (0.4 μm filter, Millipore, Bedford, MA, USA) or centrifuged at 2000xg for 10 min to remove particulate material. Alternatively, the culture supernatant was subjected to chromatography without prior manipulation. Spent culture supernatant was passed over a 2.5 X 30 cm octyl Sepharose column from which
phosphatase activity was collected in the void fraction. The octyl Sepharose void was then subjected to anion exchange chromatography. Ultrafiltered samples were chromatographed on a MonoQ SAX column (5 mm x 50 mm, Amersham Biosciences Corp., Piscataway, NJ, USA) equilibrated in 20mM Tris pH 8.0 and eluted with a linear gradient of NaCl (0-500mM in 50 min) in the same buffer. Fractions were collected automatically at one minute intervals and screened for phosphatase activity as described below. SAcP containing fractions were pooled, dialyzed overnight against water in 6-8000 molecular weight cutoff dialysis tubing (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) and lyophilized. Samples that were not ultrafiltered were applied to a 2.5 X 45 cm DEAE cellulose column equilibrated in 20 mM Tris/HCl, pH 8.0. For these samples the octyl Sepharose and DEAE columns were serially linked to minimize sample manipulation and simplify the process. Since the SAcP did not bind to octyl Sepharose, it passed through this column and collected on the DEAE column. This resulted in concentration as well as purification in a single step. The DEAE column was subsequently separated from the octyl Sepharose and developed using a two step NaCl gradient consisting of 0.2 M and 0.4 M NaCl in 20 mM Tris/HCl pH 8.0 with the SAcP eluting in the 0.4 M NaCl eluant. The enzymatically active fraction was concentrated using an Amicon ultrafiltration cell fitted with a 30,000 molecular weight cut-off membrane, washed three times with 10 ml of 0.15 M NaCl in Tris/HCl, pH 8.0 and reduced in volume to 0.5 ml. In order to separate the enzyme from the lower Mr released phosphoglycan contaminant, the preparation was finally passed over a 1 x 30 cm Superdex 200 column (Amersham Biosciences Corp., Piscataway, NJ, USA) equilibrated with 0.15 M NaCl in Tris/HCl, pH 8.0 where the SAcP eluted in the void volume. Active fractions were either stored at −20°C in this buffer, or dialyzed against water and lyophilized prior to storage at −20°C.

**Protein quantitation**

Protein content was determined using the BioRad Protein Assay (BioRad, Richmond, CA, USA). The assay was performed according to the manufacturer’s instructions for the micro assay. Ovalbumin was generally used to prepare a standard curve ranging from 0-
25 μg of protein rather than bovine serum albumin which gives an artificially high reading with this procedure.

**Radiolabeling of the SAcP**

Mid-log phase cells were resuspended in 25 ml of phosphate deficient M199 medium at a concentration of 1.0 x 10⁶ /ml. Two mCi of [³²P]-NaH₂PO₄ (Amersham Biosciences Corp., Piscataway, NJ, USA) were added and the medium was supplemented with sufficient cold phosphate to bring the concentration to 10% of that found in normal M199. This was found to be the minimum level of phosphate required to allow normal cell growth while still providing maximum incorporation of the radiolabel. The ³²P-labeled enzyme was purified as described above.

**Acid phosphatase activity assay**

Phosphatase activity was measured by the hydrolysis of p-nitrophenyl phosphate (pNPP) to p-nitrophenol (pNP). Assays were typically performed in 100mM sodium acetate buffer pH 5.0 supplemented with 50mM NaCl and 25mM pNPP. This concentration of substrate is >10 times the Kₘ of the SAcP. Reactions were routinely performed in triplicate and were incubated at 37°C for 5 min. The reaction was stopped by the addition of 50mM NaOH to a final volume of 1ml. The production of pNP was then measured by measuring the absorbance at 405 nm. This compound has an extinction coefficient of 17000 M⁻¹cm⁻¹ in alkaline solution at this wavelength, and could be used to calculate units of phosphatase activity. One unit of phosphatase activity was defined as the amount of enzyme needed to hydrolyze 1nmol of pNPP per minute at 37°C.

**SDS-PAGE**

SDS-PAGE was carried out as previously described (Laemmli 1970). Gels were 10% acrylamide with a 5% stack, and were performed in an upright BioRad mini-gel apparatus (BioRad, Richmond, CA, USA). Samples were prepared by resuspension in 1X Laemmli sample buffer (dried samples) or diluted with an equal volume of 2X sample buffer (liquid samples) followed by boiling for 5 min and centrifugation for 5 min at 13,000 RPM to remove particulate material.
Tricine SDS-PAGE for small peptide separation

The tricine gel system (Schagger and von Jagow 1987) was used to separate peptides of low molecular mass (<15 kDa). Acrylamide gels (16.5%T / 6%C; T represents the total acrylamide percentage, C is the percentage of T that was crosslinker) containing 13.33% (w/v) glycerol (4ml glycerol / 30ml gel solution) were used as described. A 4%T/3%C stacking gel was used along with a 10%T/3%C spacer gel. All buffers were as described in the literature. Gels were electrophoresed at 90V (constant voltage) for 12-16 hrs depending on the sample and migration of the dye front.

Staining of acrylamide gels

A variety of protein stains were used. For Coomassie Brilliant Blue staining, gels were placed in 50% propanol/10% acetic acid containing 0.1% Coomassie Brilliant Blue R250 (CBB). Staining was performed from 1hr to overnight, with shaking, at room temperature. Gels were destained in 10% acetic acid until good contrast was achieved. For PVDF membranes, 0.025% CBB in 40% MeOH was used with destaining in 50% MeOH.

Silver staining was performed by a variety of methods. A current protocol that is compatible with mass spectrometry is as follows (Shevchenko, Wilm et al. 1996). Fixation in 50% MeOH / 5% acetic acid for 20 min, 50% MeOH for 20 min, and water for 20 min. Sensitization with 0.02% sodium thiosulfate for 1 min. Wash in water for 2 x 1 min. Exposure in 0.1% AgNO₃ for 20 min at 4°C. Wash in water for 2 x 1 min. Develop in 0.04% formalin / 2% sodium carbonate until desired contrast is reached. Stop development with 5% acetic acid.

SACP staining was frequently achieved using the cationic dye Stains-All (Green, Pastewka et al. 1973), for which it shows a much greater affinity. Gels were fixed in 50% isopropanol 3 times for 10 min at high temperature (50°C). SDS interferes with staining and must be completely removed prior to staining. Gels were then incubated in staining solution which contains 20% formamide / 25% 2-PrOH / 0.005% Stains-All and is buffered with Tris to pH 8.8. In a properly stained gel, the SACP would appear within seconds as a dark blue smearing band. Gels were preserved by drying between sheets of
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cellophane. CBB, silver, and Stains-All remain visible when the gels are stored this way, although Stains-All is light sensitive and must be protected from light while drying.

Sypro orange is a fluorescent stain that is specific for protein. Commercially prepared stain is diluted in water to a concentration of 0.0002% (v/v). Destaining is not necessary, but the gel is rinsed in water prior to visualizing/photography. Fluorescence is induced by exposure to long wave UV light. An orange filter, specific for the stain, is used for documenting the gel by photography.

Carbohydrate staining was achieved using the periodic acid schiff (PAS) protocol. The gel was washed in 10% acetic acid / 15% 2-PrOH and fixed overnight in 10% acetic acid / 25% 2-PrOH. On the following day, the gel was treated with 0.5% periodic acid (2 hrs), 0.5% sodium arsenite / 5% acetic acid (30 min), 0.1% sodium arsenite / 5% acetic acid (2 x 20 min), and 5% acetic acid (10 min). The gel was then left in Schiff reagent (1% pararosaniline, 4% sodium bisulfite, 0.25N HCl) overnight. Finally, the gel was rinsed in 0.6% sodium metabisulfite in 0.01N HCl until rinse solutions failed to turn pink with the addition of formaldehyde. Glycoproteins were visualized as dark pink bands against a pink background.

**Capillary electrophoresis (CE)**

CE was performed on a model 270A-HT CE System (Applied Biosystems, Foster City, CA, USA) using a 49 cm capillary. Samples were injected hydrodynamically for 1-3 s with 5 inches of pressure and electrophoresed in 50 mM NaPO₄, pH 6.8 towards the cathode at 30 kV for 20 min. Protein/peptide migration was monitored at 210 nm.

**Thin layer chromatographic separation of phosphorylated amino acids**

Protein samples were radiolabeled with [³²P]-PO₄ as indicated above and approximately 10,000 cpm of radiolabeled protein was hydrolyzed in the gas phase at 100°C using 6 N HCl for 16 h. HCl was removed under vacuum and the samples were dissolved in water and spotted onto pre-coated cellulose TLC plates (20 x 20 cm) with phosphoserine, phosphothreonine and phosphotyrosine standards. Plates were developed using 7:2:1 ethyl acetate:formic acid:water. Detection of standards was accomplished using ninhydrin spray while ³²P labeled amino acids were detected by autoradiography.
Standards were marked with $[^{32}P]$-NaPO$_4$ so that they would also appear on the autoradiogram.

**Endoglycosidase digestions**

Endoglycosidases were obtained from Oxford Glycosystems (Oxford, UK) and used according to the manufacturer's instructions. Endoglycosidase H digestion was performed in 100 mM sodium acetate pH 5.5 at 26°C. In some cases, 0.2% SDS was added to achieve more efficient digestion, but this treatment was incompatible with determination of SAcP activity. Digestion with Endoglycosidase F/N-glycanase was similar. In this case, 20mM sodium maleate buffer pH 6.0 was used, and the digestion was performed at 26°C. No SDS was required for this reaction.

**Concanavalin A Sepharose selection of glycosylated molecules**

Protein samples were applied to a small (1ml total volume) ConA Sepharose column in 50 μl of ConA buffer (20 mM Tris pH 7.5, 1 mM each CaCl$_2$, MgCl$_2$, and MnCl$_2$) and the column was washed with >10 column volumes to remove nonglycosylated material. Bound glycoproteins were eluted with three bed volumes of the preceding buffer with addition of 500 mM methylmannopyranoside. Samples were dialyzed against distilled water overnight at 4°C to remove salts and monosaccharides. Glycopeptides from tryptic digests of the SAcP were isolated in the same fashion on a smaller column (~0.1ml total volume). Desalting was performed by repeated (2x) gel filtration on a 3 ml Sephadex G-10 column equilibrated and run in water.

**Mild acid deglycosylation of the SAcP**

The SAcP was deglycosylated using the method of Bates et. al. (1990). Briefly, 0.5 ml of 20 mM HCl was added to lyophilized SAcP followed by incubation at 60°C for 60 min. Released sugars were separated from the protein by filtration with a Centricon microconcentrator (Millipore, Bedford, MA, USA) with a 10,000 M$_r$ cut off.
Amino acid and monosaccharide analyses

Lyophilized protein samples were hydrolyzed using gaseous HCl at 160°C for 1 h. Free amino acids were subsequently converted to their PTC derivatives in a model 420A derivatizer and analyzed on an Applied Biosystems model 130A amino acid analyzer (Applied Biosystems, Foster City, CA, USA). Sugars, released by the mild acid treatment outlined above, were further hydrolyzed in 2 N HCl at 100°C for 3 h to release monosaccharides. The HCl was removed at reduced pressure and the hydrolyzed sugars were treated with alkaline phosphatase (Sigma, St. Louis, MO, USA) at 37°C in 25 mM Tris/HCl (pH 9.5) with 1 mM MgCl₂. This digest was applied to a mixed bed column of Ag-50 (H⁺) and Ag-3 (OH⁻) ion exchange resins (1 ml of each resin). The columns were washed with 3 bed volumes (6 ml) of water and the effluent was pooled and dried on an Eyela Rotary Evaporator (Tokyo Rikakikai Co. Ltd., Tokyo, Japan). Sugars were analyzed on a CarboPac PA-1 anion exchange column (4 x 250 mm) developed isocratically with 16 mM NaOH and monitored with pulsed amperometric detection (Dionex Corporation, Mississauga, Ont.). Standard curves were prepared using stock solutions of mannose and galactose, which were hydrolyzed in parallel to correct for hydrolytic losses.

Protein sequence analysis

Protein samples were desalted prior to lyophilization and stored dry at -20°C until required for analysis. N-terminal sequencing was performed on a model 473A gas phase protein sequencer (Applied Biosystems Inc., Foster City, CA, USA) using standard Edman chemistry. In some cases, liquid samples were adsorbed to PVDF prior to analysis.

N-terminal sequencing of the SAcP was performed by first running the sample on SDS-PAGE and then blotting to PVDF. Protein bands were visualized by staining with CBB as indicated above. Individual protein bands were excised with a sterile scalpel and submitted for sequencing.
Reduction and alkylation of protein samples

Lyophilized protein samples were reduced in 0.5 ml of 6 M guanidinium HCl, 0.2 M Tris pH 8.0 containing 0.1 M DTT. Reduction was carried out for 4 hrs at room temperature, whereupon 75 μmoles of iodoacetic acid was added and reaction was allowed to continue for a further 20 min in the dark. The reduced and alkylated SAcP was desalted on a 1 X 30 cm Superdex 200 (Pharmacia LKB, Uppsala, Sweden) gel permeation column equilibrated in 20 mM Tris, pH 8.0, 0.15 M NaCl, and finally dialyzed and lyophilized.

Chemical digestion of the SAcP

CNBr digestion of the SAcP was carried out by dissolving 2 mg of reduced and alkylated SAcP in 500 μl of 70% formic acid to which was added a 50 fold molar excess of CNBr in the presence of 35 mg of glycine added to scavenge free sugar aldehydes. The reaction was sparged with nitrogen and allowed to proceed for 16 h at room temperature whereupon it was stopped by dilution and lyophilized.

Proteolytic digestion

Proteolytic digestion with trypsin was as follows. Lyophilized protein was dissolved in a minimal quantity of digest buffer (0.1 M Tris, pH 8.0), to which trypsin was added to make a 50:1 substrate:protease (w/w) ratio. Digests were allowed to proceed at 37°C for 4-16 hrs, while monitoring progress by capillary electrophoresis. Digestions were also performed using endoproteinase Glu-C and endoproteinase Asp-N using 0.1 M NaPO₄, pH 7.8 and 0.1 M Tris, pH 8.5 buffers respectively. Digestion protocols were otherwise identical to that of trypsin, although monitored by RP-HPLC.

Isolation of phosphorylated peptides

Two ml of Macroprep ceramic hydroxylapatite resin (BioRad, Richmond, CA, USA) was packed into a 5 x 50 mm column and equilibrated in 10 mM NaPO₄, pH 6.8. Samples were eluted from the column with 1 M NaPO₄ at a flow rate of 1 ml/min delivered by a Beckman HPLC system. Half ml fractions were collected and monitored by ³²P scintillation counting of 5 μl aliquots. Radiolabeled phosphopeptides were pooled
and subsequently desalted on a 10 x 100 mm Sephadex G-10 column and dried in a Speed Vac concentrator (Savant/EC Instruments Inc., Holbrook, NY, USA). Radioactive peptides were also purified using a MonoQ HR 5/5 anion exchange column (Amersham Biosciences Corp., Piscataway, NJ, USA) equilibrated with 0.02 M Tris/HCl, pH 8.0 and operated at a flow rate of 1 ml/min. The column was developed with a gradient from 0-500 mM NaCl over 50 min and monitored at 229 nm. Scintillation counting was carried out as above.

**High performance liquid chromatography (HPLC)**

HPLC was carried out on a range of Beckman instruments including Model 112 pumps, Beckman Gold and microbore systems. The chromatographic methods employed varied with individual experiments and are outlined below. All solvents and buffers were filtered through 0.45 µm pore size filters and degassed under vacuum prior to use.

Reverse phase (RP) chromatography was performed on BrownLee octadecylsilane (ODS), octyl (C8) and butyl (C4) cartridges. ODS columns were 4.6 x 100 mm and operated at flow rates of 0.5-1.0 ml/min, C8 and C4 columns were 2.1 x 100 mm and operated at flow rates of 0.2-0.3 ml/min. Columns were generally equilibrated in 0.1% trifluoroacetic acid (TFA) prior to sample application and developed with a linear gradient of acetonitrile (ACN) at a rate of 0.5-2% B per minute depending on the application. In some cases, other elution solvents were used, including isopropanol and methanol, particularly for direct MS analysis. Samples were usually lyophilized following chromatography.

Anion exchange chromatography was performed using either MonoQ or DEAE column packings. Flow rates of 1 ml/min were routine. Typically these columns were operated in Tris buffer (25-50 mM, pH 8.0) using a linear gradient of sodium chloride (0-500 mM) for elution. In all cases, samples were desalted prior to chromatography.

Gel filtration was performed using primarily Superdex 200 (10 x 300 mm) and Superdex peptide (10 x 300 mm) columns at a flow rate of 1 ml/min. For the purpose of desalting, columns were equilibrated and operated in water. For the purpose of separation, 20 mM Tris (pH 8.0) containing 150 mM NaCl was used as a buffer to prevent charge interactions between solutes and resin materials.
Mass spectral analysis of mild acid released glycans

Most samples were analyzed on a VG Quattro triple quadrupole mass spectrometer (Micromass, Manchester, UK) interfaced with an electrospray ionization (ESI) source. The spectrometer was operated in negative ion mode to facilitate the detection of phosphorylated species. Samples were dissolved in 1% NH₄OH/50% 2-propanol and 20 μl aliquots were introduced into a solvent stream of 50% 2-propanol and sprayed at a flow rate of 10 μl/min. For partial mild acid deglycosylation experiments, samples were diluted with an equal volume of 2-propanol or methanol and introduced into the mass spectrometer in an acidic solution. The capillary voltage was routinely set at -3 kV, the counterelectrode was set to -300 V and the declustering potential varied from -20 to -50 V depending on the experiment. Horse heart myoglobin (Mr 16,951) at a concentration of 20 pmol/μl and 5 mM NaI were used to calibrate the instrument. During MS/MS experiments, the gas in the collision cell (N₂) was maintained at a level of 5.5x10⁴ mBar. For some analyses, an API QSTAR™ Pulsar Hybrid LC/MS/MS System (Applied Biosystems, Foster City, CA, USA) interfaced with a nanospray source (Protana, Odense, Denmark) was used. This equipment is capable of much higher resolution and was operated using factory default settings. It was used to collect the high resolution data shown in figures 5.5, 5.6 and 6.13.

Peptide mapping by MALDI-TOF and ESI-quadrupole MS

Digested protein samples were desalted prior to analysis. Depending on the size of the sample, this could be accomplished by either reverse phase HPLC or using a C18 ZipTip. Samples were eluted in either case in 50% acetonitrile. Samples were mixed 1:1 with α-cyano 4-hydroxy cinnamic acid in 50% ACN and spotted onto a matrix-assisted laser desorption ionization (MALDI) target. After drying, they were analyzed on a Voyager DE-STR (Perseptive Biosystems, Foster City, CA, USA). In most cases, 75 laser shots were averaged per analysis.

Glycopeptides not observed by MALDI analysis were analyzed using electrospray ionization (ESI)-quadrupole MS. Lyophilized, desalted samples were resuspended in 50% MeOH/1% Acetic acid and introduced by continuous infusion to the mass
spectrometer at a flow rate of 5-7 µl/min. Scans were performed over a range of 200-2000 amu in 5 sec. A minimum of 10 scans were averaged for molecular weight determinations.

**Phenol/sulfuric acid assay for carbohydrate**

This procedure required concentrated sulfuric acid and 5% aqueous phenol (w/v). For sample volumes of 20 µl containing 0-20 µg of carbohydrate the procedure was performed in flat bottom well plastic assay plates. A 6 µl (300 µg) aliquot of 5% phenol was added to sample followed by mixing and rapid addition of 100 µl of concentrated sulfuric acid. The heat released drives the reaction. After 10 min at room temperature, color development was measured in an automatic plate reader at 490 nm. Unknowns were compared to a standard curve prepared with 0, 5, 10, 15, and 20 µg of a standard sugar. Most monosaccharides gave comparable results, but standards were chosen to match the sample. ACN interferes with color development, even in small amounts. Reverse phase fractions were lyophilized prior to analysis. Also, heating causes the sample wells in flexible plastic plates to shrink. A linear response was normally observed, but samples were occasionally transferred to unused wells prior to measurement. Plates were discarded after a single use.

**Isolation of Leishmania microsomal or Golgi membranes**

Microsomal membranes were prepared as follows. *L. donovani* LD3 promasigotes (1x10^10 cells) were suspended in 20 ml MPT buffer (100 mM HEPES, pH 7.4, 50 mM KCl, 1 mM MnCl₂, 1 mM MgCl₂, and the protease inhibitor cocktail described above) and lysed via N₂ cavitation (equilibrated at 2400 psi, 20 min, on ice). Cell membranes and debris were removed by centrifugation at 1500 x g and the supernatant was centrifuged at 100,000 x g to collect the microsomal membranes. The 100,000 x g pellet was resuspended in 0.5 ml of MPT buffer for use in subsequent assays.

Because pelleted membranes were difficult to resuspend following sedimentation, reproducible aliquoting of membranes was difficult. Therefore, a procedure was developed to isolate the Golgi fraction that avoided membrane sedimentation. This procedure was based on work done with rat liver cells (Hayes, Freeze et al. 1993). Cell
pellet resuspension, lysis, and removal of cell debris were performed as indicated above. The resulting lysate was layered on a cushion of 1.3 M sucrose and centrifuged in a Beckman SW-41 rotor at 24,000 RPM (~70,000 x g) for 1 hr at 4°C. The opaque membrane layer was harvested by Pasteur pipet from the sucrose:buffer interface and the sucrose concentration estimated by the phenol/sulfuric acid assay for carbohydrate. The concentration of sucrose was subsequently adjusted to 1.1 M by the addition of an appropriate amount of 2 M sucrose. A four step gradient was prepared with 1.3 M sucrose, sample in 1.1 M sucrose, 1.0 M sucrose and finally, MPT buffer. Centrifugation was repeated using identical conditions. During this step, Golgi membranes float from a layer of high density to a layer of lower density. The Golgi fraction collects at the interface between the 1.0 M sucrose and buffer layers. Membrane harvesting was performed by puncturing the base of the tube and collecting the 1.0 M sucrose:buffer interface as it eluted from the tube. This was found to be the most efficient way of obtaining the membrane enriched fraction in a small volume. Membranes were stored at 4°C for up to a week with no apparent loss in activity. For long term storage, the membranes were aliquoted in 50 μl volumes and flash frozen in liquid N₂ before storing at −80°C.

In vitro glycosylation of synthetic peptides

A synthetic peptide with the sequence acetyl-WSSEGTTASSS-amide (sacp-1) was synthesized using a Model 430A peptide synthesizer (Applied Biosystems Inc., Foster City, CA) using optimized FastMoc chemistry. An additional peptide with the sequence SSSEGTTASSSEGTTASSS-amide (sacp-2) was synthesized on special, low substitution resin. The in vitro glycosylation of these molecules was performed as previously described (Carver and Turco 1991). This involved addition of 100 μl of the membrane preparation to 250 μl of the above lysis buffer containing 30 mmol GDP-Man, 30 mmol UDP-Gal, 5 mmol ATP, 1 mmol DTT and 1 mmol of the synthetic peptide substrate. A protease inhibitor cocktail was also included, the final inhibitor concentrations were 50 μM TLCK, 50 μM TPCK, 1 μM E64, 1 μM leupeptin, and 2 μM pepstatin. Sonication or detergent disruption of membrane vesicles was required for enzyme activity to be detected. Sonication was applied on ice in three 10 s bursts spaced
30 s apart. CHAPS, octyl-thioglucoside, Triton X-100, and n-dodecyl β-D-maltoside were all tested at either 0.1% or 1.0% and were found to be compatible with enzyme activity to varying degrees. 1.0% Triton X-100 was found to be most effective at solubilizing the MPT and was used in most cases. The reaction mixture was allowed to incubate for 16 h at 26°C. After centrifugation at 1500 x g, the supernatant was passed through a J&W Scientific Accubond C18 Solid Phase Extraction column (Fisher Scientific, Nepean, ON, Canada). Sacp-1 was eluted with 50% ACN, while sacp-2 was collected in the void. Sacp-2 was further treated with fluorescamine as described above. Modified peptides were then applied to a C8 reverse phase column (Brownlee, Foster City, CA, USA) equilibrated in 0.1% TFA and developed with a linear isopropanol ramp (0-60% in 40 min). The column effluent was monitored at 280 nm and peaks were either collected manually or 1 ml fractions were collected automatically. These samples were dried in a Speed-Vac concentrator and stored at -20°C until analyzed. In some cases, GDP-[\textsuperscript{14}C]Man was used as substrate and HPLC fractions were subjected to scintillation counting. Radiolabeled peptides were also separated on tricine SDS-PAGE gels, which were soaked in Amplify (Amersham Biosciences Corp., Piscataway, NJ, USA) prior to drying between cellophane sheets. Radioactivity was detected using a small Storage Phosphor Screen (20 cm x 25 cm). Exposure was performed up to a maximum of four days, after which no significant increase in signal was observed. Stored images were scanned using the STORM 820 Storage Phosphor Imaging system (Molecular Dynamics, Sunnyvale, CA, USA). Samples to be analyzed by MS were resuspended in an appropriate solvent and introduced into the mass spectrometer.

**Affinity column construction and usage in MPT purification**

Sacp-2 was coupled to cyanogen bromide activated Sepharose 4B (Amersham Biosciences Corp., Piscataway, NJ, USA) as follows. Sacp-2 (13.5 mg) was dissolved in 5 ml of coupling buffer (0.1 M NaH\textsubscript{2}CO\textsubscript{3}, pH 8.6, 0.5 M KCl). The coupling procedure was as described by the resin manufacturer (Pharmacia Fine Chemicals 1979). One gram of freeze-dried resin was swelled in 1 mM HCl on a sintered glass filter. It was then washed with copious amounts of coupling buffer and mixed with the peptide solution in a 50 ml polypropylene tube (Sarstedt, Newton, NC, USA). This was mixed by end-ove-
end rotation overnight at 4°C. Unreacted active groups on the resin were blocked by the addition of 0.2 M glycine (pH 8.0) and incubation for 2 h at room temperature. Excess peptide and blocking agent were washed from the resin using coupling buffer followed by acetate buffer (0.1 M, pH 4.0) containing NaCl (0.5 M) and finally coupling buffer. This resin was stored at 4°C until used. For long term storage, 0.1% NaN₃ was added as a preservative.

Sacp-2-sepharose was used for the affinity purification of the MPT using the following protocols. Purified *Leishmania* Golgi were solubilized using 1% Triton X-100 and centrifuged at 100,000 x g for 1 hr to pellet insoluble material. The MPT containing supernatant (2 ml) was mixed with 1 ml (0.33 g) of the affinity resin in MPT assay buffer containing 0.5 M KCl. High ionic strength is required to minimize nonspecific ionic interactions of solute with the charged glycine blocking groups on the resin. MPT binding was performed by end-over-end mixing overnight at 4°C. The MPT containing resin was washed with 10 column volumes (10 ml) of MPT assay buffer containing 0.5 M KCl to remove unbound material. All of this material was collected, pooled and reduced to its initial volume by ultrafiltration in an Amicon stirred ultrafiltration chamber (Millipore, Bedford, MO, USA) using a 10,000 molecular weight cutoff membrane. Elution of bound enzyme was attempted using low pH (0.1% TFA), high pH (10 mM NaOH), excess peptide substrate (3.3 mg/ml, which is 20 mM sacp-2), and denaturing conditions (8 M urea). In all cases, 3 column volumes (3 ml) of elution solvent was passed slowly over the resin (elution rate of 10 drops/min). Each of the elution conditions was tested in a separate experiment with the exception of 8 M urea, which was used at the end of each experiment to elute any remaining material. For elution with high and low pH, samples were collected into tubes containing 0.5 ml of 2x MPT assay buffer to minimize exposure to the extreme pH conditions. Eluted samples were washed in an ultrafiltration chamber by repeated dilution with MPT assay buffer and reduced to the volume of the initial sample so that they could be directly compared. MPT activity assays were performed on a 200 μl aliquot of each sample as outlined above. The remaining samples were further reduced in volume to 0.5 ml, chilled on ice and mixed with 5 ml of –20°C acetone to precipitate protein. After overnight storage at –20°C precipitated material was collected.
by centrifugation at 13,000 RPM in a microfuge, washed once with 1 ml of -20°C acetone and analyzed by SDS-PAGE.
3. Purification and general characterization of the SAcP of *L. donovani*

A. Introduction

A previous study of *Leishmania* parasites has shown that the organism actively secretes many proteins and glycoproteins into its environment (Bates, Gottlieb et al. 1988). There are predicted to be >40 macromolecular species in spent *Leishmania* culture medium, more than half of which are glycoproteins. The SAcP is an abundant component of this mixture, and is a heterogeneous, highly glycosylated molecule, which demonstrates poor chromatographic resolution and consequent analytical complications. These structural variations increased the complexity of the data collected, requiring larger quantities of protein than usual to adequately complete structural analyses. In addition, both this laboratory and others (Lovelace, Dwyer et al. 1986) have observed the occurrence of degradation during the standard isolation procedure, stimulating the development of alternative purification protocols for high volume samples of spent *Leishmania* culture medium. SDS-PAGE and other analytical techniques have been used to assess this procedure.

B. Results

*Purification of the SAcP Leads to Degradation and Significant Loss of Activity*

The SAcP was routinely purified from 8-10 litres of spent *Leishmania* culture medium. The medium was initially concentrated by ultrafiltration to reduce the volume of the sample more than 100-fold, to 50-75 ml, over the course of 5 to 6 days. Although the SAcP was completely retained by the ultrafiltration filter, as indicated by the lack of phosphatase activity in the filtrate, analysis of the concentrate showed a considerable loss of SAcP activity. More than 90% of the initial enzyme activity was lost during this process. Other researchers had not observed this, because enzyme yields had not been calculated at this step (Lovelace, Dwyer et al. 1986). The reason for this loss in activity was not clear, but may be caused by aggregation following the increase in protein
Western blot analysis of the purified SAcP seems to support the latter explanation. Two antibodies were routinely used for the analysis of the SAcP. MAb 16A1 (Sigurdson 1992) is specific for a protein determinant on the SAcP, and CA7AE is specific for the PG modifications common to the SAcP and LPG (Tolson, Turco et al. 1989). Comparison of the results obtained using these two antibodies to the SAcP showed a marked difference (Figure 3.1). Binding of CA7AE revealed a continuous smear at the top of the lane (Figure 3.1, lane A). This smear had a lower limit of approximately 100kDa and was consistent with the known behavior of the SAcP on SDS-PAGE (Lovelace, Dwyer et al. 1986; Bates, Hermes et al. 1990). However, 16A1 bound primarily to a lower molecular mass band (55kDa), while showing a weak interaction with the aforementioned smear (Figure 3.1, lane B). This 55kDa band was not observed prior to ultrafiltration, which suggests that it was a degradation product generated during filtration. Coomassie staining of this sample showed that the 55kDa band was actually composed of at least 3 separate bands, likely due to variable glycosylation (Figure 3.1, lane C). The 55 kDa band was not phosphoglycosylated as indicated by a total lack of recognition by CA7AE, but it was derived from the SAcP, as indicated by strong 16A1 binding. To prove that 16A1 was specific for the SAcP, an
antigen capture experiment was performed. In this experiment, we adsorbed a series of antibodies to a standard ELISA plate and incubated them with a sample containing active SAcP. After washing to remove unbound material, phosphatase substrate was added to each well to determine the presence or absence of SAcP. As indicated in Figure 3.2, the mAb 16A1 was the only Ab capable of capturing the phosphatase activity. Thus, 16A1 was specific for the enzyme and indicated that the 55kDa band was derived from the SAcP. The inability of CA7AE to perform the same function was surprising. Since it is well established that CA7AE can immunoprecipitate the SAcP (Jaffe, Perez et al. 1990), the most trivial explanation of this finding would be that the antibody bound to the plate via its variable region, making it unavailable for SAcP binding. The final piece of evidence proving the identity of the 55 kDa band, was obtained by N-terminal sequencing of PVDF blotted material. The N-terminal sequence of the SAcP (Arg-Leu-Val-Val-Arg) was obtained, conclusively demonstrating the degradation of the SAcP to a PG deficient form during ultrafiltration.
The difficulties presented above were eliminated by altering the purification protocol. Purification of the SAcP was achieved using three chromatography steps. Octyl Sepharose (OS) chromatography was performed first. This resin is normally used as a hydrophobic interaction resin, but can also serve as a reverse phase column because of its hydrophobic character. Most of the protein in the spent medium, save the SAcP, was adsorbed to this column, providing a useful initial isolation step. Presumably, the highly glycosylated nature of the native SAcP leads to the concealment of most hydrophobic surfaces and poor adsorption to the OS resin. The second stage of purification was anion exchange using diethyl amino ethyl (DEAE) cellulose. The SAcP is highly phosphorylated due to its PG modifications, which caused it to bind tightly to the ion exchange resin. This affinity was strong enough that prior desalting was not required and the SAcP was bound to the column directly from the OS flowthrough. Adsorption to the DEAE resin was quantitative, concentrating the SAcP from the large initial sample.

![Figure 3.1](image-url)  
*Figure 3.1 Elution profile of acid phosphatase activity when the SAcP was chromatographed on a DEAE anion exchange column.*
volume. Since the heterogeneity of the SAcP caused it to elute over a broad ionic strength range, a step gradient of NaCl was employed to collect all of the SAcP in one fraction (Figure 3.3). This reduced the efficiency of the separation, but an improvement in enzyme purity was still achieved. Furthermore, these two columns could be connected in series and were capable of processing up to 10 L of spent *Leishmania* culture medium in a single step. Analysis by SDS-PAGE (Figure 3.4, lanes 1-3) showed that the predominant *Leishmania* protein was the SAcP. Two major contaminants were routinely observed. The first was bovine serum albumin, which co-purified with the SAcP to this point (Figure 3.4, lane 3), but was only present when bovine serum was included in the growth medium. The second was only observed by staining with the cationic dye Stains-All (Figure 3.4, panel C). This material was determined to be excreted factor, a soluble form of PG that is secreted by promastigotes containing neither protein nor lipid. It was observed as a blue staining smear extending throughout the low molecular weight region of the gel (Figure 3.4, panel C, lane 3). Further purification of SAcP was accomplished with size exclusion chromatography (SEC). Because the enzyme has an extremely large hydrodynamic volume it is excluded from most SEC columns, providing a ready means of elimination of both the remaining protein contaminants and the excreted factor (Figure 3.4, lane 4).

![Figure 3.2](image)

**Figure 3.2** Assessment of SAcP purity by SDS-PAGE after each stage of purification. Four stains, Coomassie Brilliant Blue (Panel A), Sypro Orange (Panel B), Stains-All (Panel C) and Periodic Acid Schiff Stain (Panel D) were used. Individual lanes refer to different points during purification. Lane 1, crude medium; Lane 2, octyl Sepharose; Lane 3, DEAE cellulose; Lane 4, Superdex 200.
Table 3.1 shows the progression of SAcP purification in terms of yield and specific activity. This data was derived from a sample prepared in the absence of fetal bovine serum, and did not contain the BSA shown in Figure 3.4. There was an apparent drop in purity following the Superdex 200 stage of purification. The purification at this step primarily involved the removal of excreted factor, which is composed solely of carbohydrate. Since the specific activity was dependent on the amount of protein remaining, the removal of excreted factor was not reflected in the specific activity calculation. Also, the heavily glycosylated SAcP gave a poor colorimetric response when assayed using most protein analytical methods, resulting in no detectable protein after the DEAE stage of purification, even though the SAcP was the predominant feature by PAGE analysis (Figure 3.4). Therefore, only an upper limit of remaining protein was provided based on the detection limits of the protein assay. Recovery of the SAcP has ranged from 650 to 800 μg by dry weight from 10 litres of spent culture media using this procedure.

Table 3.1 Purification of the secreted acid phosphatase of *L. donovani* LD3.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>SAcP activity</th>
<th>protein (mg)</th>
<th>yield (%)</th>
<th>specific activity (U/mg)</th>
<th>fold purification</th>
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<tr>
<td>crude medium</td>
<td>243</td>
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</tr>
<tr>
<td>octyl Sepharose</td>
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<td>96.7</td>
<td>53.4</td>
<td>19.1</td>
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<tr>
<td>DEAE cellulose</td>
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<td>&lt;0.5</td>
<td>&gt;120.2</td>
<td>&gt;100.4</td>
<td>42.9</td>
</tr>
<tr>
<td>Superdex 200</td>
<td>122</td>
<td>&lt;0.5</td>
<td>50.2</td>
<td>&gt;100.4</td>
<td>35.8</td>
</tr>
</tbody>
</table>

**Radiolabeling of the SAcP with $^{32}$P**

The abundance of PG structures produced by *Leishmania* contributes to a significant requirement for phosphate when grown in tissue culture. The phosphorylated state of the SAcP was shown by labeling with radioactive phosphate and this was used to facilitate structural studies of the PG post-translational modifications. The minimum level of phosphate required to support a normal parasite growth rate was assessed in order to maximize the incorporation of labeled phosphate into the SAcP. Phosphate deficient medium M199 was prepared and reconstituted with varying levels of unlabeled phosphate. The cell density was determined daily and Figure 3.5 shows that growth rates were not significantly affected until the phosphate content of the media was reduced below 10% of its normal level. Also, the production and release of the SAcP was not
Figure 3.1 Growth of LD3 in medium containing varying levels of inorganic phosphate. Cells were grown in normal medium or phosphate free medium supplemented with the indicated percentages of the normal phosphate level.
altered in response to changes in phosphate availability (Figure 3.6). Radiolabeling of the SAcP was accomplished by culturing the parasite in phosphate free medium reconstituted with radioactive phosphate. Two millicuries of commercially available Na\(^{32}P\)PO\(_4\) contained 138 $\mu$g of phosphate, which was sufficient to supply the requirements (122 $\mu$g) of a 20ml culture grown from a density of $1 \times 10^6$ cells/ml to stationary phase ($1.5 \times 10^7$ cells/ml). Purification of radiolabeled SAcP from the culture supernatant, followed by SDS-PAGE and autoradiography, showed the incorporation of radiolabel and a banding pattern matching that previously observed by protein staining (Figure 5.7, Lane A). Scintillation counting of the purified enzyme demonstrated that 0.4% (440 ng) of the total phosphate present in the growth medium was incorporated into the SAcP. A separate culture was prepared with unlabeled phosphate and was used to measure the amount of SAcP produced by a culture of this size. An estimated 2.4 $\mu$g of SAcP was produced as assessed by amino acid analysis, indicating the weight ratio of protein:phosphate within the SAcP is approximately 5.5:1. This is a rough approximation, as the quantitation was performed only once.
C. Discussion

Purification of the SAcP in the past has been hampered by poor recovery of active enzyme and degradation of the protein during the purification process. Most of these problems arose while concentrating spent Leishmania culture via ultrafiltration. Interestingly, this phenomenon has not been highlighted as a problem by other researchers. Indeed Gottlieb and Dwyer (1982) reported a recovery of 80% following ultrafiltration, with negligible losses due to degradation. These researchers used a filtration membrane with a significantly higher molecular mass cutoff (300 kDa) and may have separated the SAcP from smaller proteins, such as proteases, present in the culture supernatant. The losses they report were likely due to a preferential loss of low molecular weight forms of the SAcP, which presumably contain a lower level of PG modification. This would obviously skew any subsequent quantitative studies designed to estimate the average level of glycosylation. We have presented an alternative approach to SAcP purification which eliminated ultrafiltration and the problems associated with this method. The procedure incorporated three chromatographic steps resulting in a 35-fold increase in enzyme purity from growth media, eliminating major non-protein sources of contamination, such as the substance known as excreted factor.

The purified SAcP is a heterogeneous glycoprotein that appears as a high molecular weight polydisperse band by SDS-PAGE. The estimated molecular mass by this procedure was in excess of 100 kDa. The molecular mass estimated by SEC was somewhat larger than this. When chromatographed on a Superdex 200 column (Amersham Biosciences Corp, Piscataway, NJ, USA) the enzyme was found to be completely excluded, a result consistent with earlier findings (Lovelace, Dwyer et al. 1986) where the SAcP was reported to elute prior to thyroglobulin (Mr = 669 kDa) on a size exclusion column. The published exclusion limit of the Superdex 200 resin is 700 kDa, showing that the SAcP has an extremely large hydrodynamic volume. This physical characteristic can be ascribed to the degree of glycosylation of the SAcP which is estimated to be at least 30% of the enzyme by weight. Early evidence for glycosylation of the enzyme was obtained from reaction with periodic acid Schiff reagent (Figure 3.4, panel D). The blue color reaction observed with the Stains-All reagent indicated the
presence of phosphorylation (Green, Pastewka et al. 1973) which was subsequently confirmed by metabolic incorporation of $^{32}$P. These initial observations were consistent with the presence of PG modifications on this protein.

One of the putative functions of the SACp is nutritional; it may supply the growing parasite with free inorganic phosphate not present in sufficient quantities in the growth environment. Although production of SACp did not appear to be regulated by phosphate availability, since the amount of SACp produced was not altered in response to limiting phosphate (Figure 3.6), this does not discount the possibility of a constitutive nutritional function.
4. Study of SAcP N-glycosylation and its effect on enzyme stability

A. Introduction

Protein glycosylation most commonly occurs in one of two ways. It can be linked to the nitrogen of asparagine residues, or through the oxygen of serine or threonine. It was reasonable to expect that the phosphoglycan chains of the SAcP be attached to the protein by one of these mechanisms. While O-linked glycans vary in terms of their linkage and site of attachment, N-linked structures are much more regular and therefore easier to study. They are added to the consensus sequence (Asn-X-Ser/Thr) which can be used to predict putative sites of modification (Burda and Aebi 1999). These modifications also have a common structural motif, known as the core oligosaccharide. It consists of two N-acetylglucosamine residues and three mannose residues combined via conserved linkages. This core structure is recognized by a number of endoglycosidases that can usually remove the glycans from the protein molecule providing useful tools in the analysis of N-linked sugars.

The functional significance of N-linked glycosylation is frequently related to the structural integrity of the modified protein (Burda and Aebi 1999). These modifications are usually added to growing protein chains on the inner surface of the endoplasmic reticulum. Since this occurs during protein synthesis, these structures can have significant effects on protein folding. Often, correct assembly of glycans is required to ensure that the proper protein conformation is achieved. For the SAcP this has been demonstrated with the use of tunicamycin, a UDP-GlcNAc analog that interferes with the biosynthesis of N-linked glycosylations by preventing the addition of GlcNAc to dolichol phosphate, the first step in the formation of the core oligosaccharide (Stryer, 2001). This antibiotic causes *Leishmania* to secrete inactive enzyme, showing that these glycosylations are in fact required for protein folding (Lovelace and Gottlieb 1987). However, glycosylation is also known to effect both protein function and stability post-synthesis. No information of this type was available for the SAcP. I used a number of endoglycosidases to probe the significance of the N-linked glycosylations of the SAcP. This experiment was designed to
determine whether the PG modifications of the SAcP were connected to the protein via conventional N-linkages, as well as to ascertain the effect, if any, of N-linked sugars on enzyme activity of the mature, secreted molecule.

B. Results

Endoglycosidase H Digestion

Endo-β-N-acetylglucosaminidase (Endo H) is an endoglycosidase that recognizes the core oligosaccharide common to all N-linked glycosylations (Tarentino and Maley 1974). It cleaves between the two GlcNAc residues found in the core structure, resulting in removal of the glycan chain, leaving a single GlcNAc residue on the protein. The SAcP was treated with this enzyme, and analyzed by SDS-PAGE. It was found that the SAcP was resistant to digestion under standard, non-denaturing conditions (Figure 4.1, lane C). However, because Endo H retains its activity in the presence of small quantities of detergent, the addition of 0.2% SDS to the reaction was sufficient to allow access of endoglycosidase by partially denaturing the SAcP. Following this treatment, a decrease in the apparent molecular weight of the protein was observed (Figure 4.1, lane B). However, this decrease was not accompanied by a reduction in heterogeneity. The protein was still observed as a broad, high molecular weight band on SDS-PAGE with the only apparent difference being a further broadening of the band to a lower molecular weight. In addition, this treatment did not affect the binding of mAb CA7AE (Figure 4.1, lanes D and E), which indicates that the PG chains were still associated with the SAcP. Two conclusions could be drawn from these results. First, the PG changes.

Figure 4.1 Treatment of secreted acid phosphatase with Endoglycosidase H. Purified SAcP was left untreated (lane A), and treated in the presence (lane B) and absence (lane C) of 0.2% SDS. Western blotting with phosphoglycan specific mAb CA7AE was also performed on untreated (lane D) and deglycosylated (lane E) SAcP. Molecular masses are indicated in kDa.
chains of the SAcP were not connected to the protein by standard N-linkages since they cannot be removed by N-glycosylation specific enzymes. Secondly, the SAcP is decorated by more than one type of glycosylation as endoglycosidase digestion reduced the apparent molecular weight of the protein as determined by SDS-PAGE, indicating the presence of standard N-linked glycosylations. Similar results were obtained using Endo F and PNGase F, two other enzymes with specificity for N-linked glycosylations (Figure 4.4).

**Peptide mass mapping by MALDI-TOF and ESI-quadrupole mass spectrometry**

The N-linked carbohydrates from the SAcP of *Leishmania donovani* had not been previously characterized. Generally, N-linked glycans occur as either high mannose type structures, consisting of polymannose chains, or complex type structures, which vary in their linkage and monosaccharide composition. Past studies have examined the glycans of the *Leishmania* surface protease, GP63, (Olafson, Thomas et al. 1990) as well as the total cell-associated oligosaccharide pool (Parodi, Martin-Barrientos et al. 1984; Funk, Thomas-Oates et al. 1997). These analyses have indicated that *Leishmania* produce mainly high mannose type structures with the exception of a novel terminal glucose oligosaccharide (Olafson, Thomas et al. 1990). A mass spectrometry based approach was used to determine the level of consistency between the glycans of the SAcP and these previously published structures. In this study, proteolytic digestion was employed to generate a panel of SAcP peptides which were subsequently analyzed by MALDI-TOF mass spectrometry. With the benefit of the SAcP gene sequence (Shakarian, Ellis et al. 1997) it was possible to generate a theoretical peptide mass map for comparison with the experimentally derived peptide masses. Differences between these two data sets would indicate the presence of modifications and possible structures through the masses of these adducts.

The raw MALDI data are shown (Figure 4.2) and the complete protein sequence is included for reference (Figure 4.3). The data are summarized in tabular format (Table 4.1) with the peptides organized in a consecutive fashion based on their occurrence in the gene sequence. A few small peptides were absent from these data, possibly obscured by matrix peaks that are routinely observed in the low mass region of MALDI data. They
Figure 4.1 MALDI analysis of a SAcP tryptic digest.

Figure 4.2 Amino acid sequence of the SAcP as predicted by the gene SAcP-1 (Shakarian, 1997). The signal sequence is indicated in italics and is not present in the mature enzyme. Additionally, the C-terminal region that was not observed by MS is underlined.
Table 4.1 Predicted and observed masses for tryptic peptides derived from the SAcp.

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<tr>
<th>Amino acids</th>
<th>Expected mass (M+H)^+ (Da)</th>
<th>Observed mass (M+H)^+ (Da)</th>
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<td>-</td>
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^1 Abbreviations: Hex (hexose), HexNAc (N-acetylhexosamine), cmCys (carboxymethyl cysteine)

represented <1% of the total sequence. Additionally, a very significant large segment representing approximately one third of the protein could not be represented in this experiment. As will be shown in the following chapter, this region consisted of a Ser/Thr rich section that was resistant to protease digestion and was not amenable to this type of analysis (Figure 4.3). Only one of eight N-glycosylation consensus sites (Asn-45) was
missed due to this omission. Of the remaining protein, only peptides spanning putative N-glycosylation sites were not detected. These peptides were modified, most likely by glycosylation. An exception was the peptide containing Asn$_{341}$ that appeared at 1041.20 amu (corresponding to residues 339-347) and 1536.78 amu (corresponding to residues 334-347, due to a missed cleavage at 338). Further inspection of the data uncovered the presence of a set of higher mass signals that corresponded to the missing peptides with an addition of 1379 amu. This mass shift was consistent with the presence of a glycan chain with the generic composition Hex$_3$HexNAc$_2$. Glycosylated versions of the peptides spanning Asn$_{341}$ were also detected, indicating that this site was inconsistently modified.

Studies performed on *Leishmania* have shown that a predominant form of protein glycosylation is a structure with the composition Man$_6$GlcNAc$_2$ (Parodi, Martin-Barrientos et al. 1984; Olafson, Thomas et al. 1990; Funk, Thomas-Oates et al. 1997). These data obviously conformed to this finding, suggesting that the N-glycans of the SAcP were similar to those found on other *Leishmania* proteins. Studies of the major surface protease (GP63) have identified a glycosylation variant that contains an additional terminal glucose residue (Olafson, Thomas et al. 1990; Funk, Thomas-Oates et al. 1997). This modification would have shown a mass difference of 1541 amu over the unmodified peptide mass. There was no indication of this type of modification on the SAcP as analyzed by MS.

*Effect of N-glycosylation on SAcP enzyme activity*

It is well known that protein glycosylation can play a role in protein folding and stability (Bhatia and Mukhopadhyay 1999; Imperiali and O'Connor 1999; Wormald and Dwek 1999). The production of inactive SAcP in the presence of tunicamycin indicated that the modifications on this protein were important for enzyme activity (Lovelace and Gottlieb 1987). However, this experiment investigated the involvement of N-linked sugars in protein folding during synthesis, and did not assess the requirement of glycosylation for activity in the mature enzyme. A number of endoglycosidases were used to address this question. Endoglycosidase H could not be used, because it required the presence of detergent to ensure complete glycan removal, which inactivated the
Treatment of secreted acid phosphatase with a mixture of Endoglycosidase F and N-glycanase. Untreated enzyme is shown in lane A. Deglycosylation causes a shift to lower molecular weight as shown in lane B. This gel was stained with Coomassie, Brilliant Blue which stains the SAcP poorly. The two main bands that are routinely observed at the lower edge of the SAcP smear are highlighted with arrows.

SAcP, and it also left a single GlcNAc bound to the protein, which could be as effective in protein stabilization as an intact glycan chain (Imperiali and O'Connor 1999). However, Endo F and N-glycanase are endoglycosidases that remove the entire glycan from a glycoprotein in the absence of detergent.

Treatment of the SAcP with a mixture of N glycanase and Endo F resulted in a molecular weight decrease similar to that observed with Endo H under denaturing conditions (Figure 4.4). To assess the effectiveness of the N glycanase / Endo F treatment, we applied the deglycosylated enzyme to a Concanavalin A (Con A) affinity column. Con A is a mannose specific lectin that is commonly used to purify

![Figure 4.2](image_url) **Figure 4.2** Effect of deglycosylation on the binding of the SAcP to a Concanavalin A sepharose affinity column. Each bar represents the mean of three replicates.
glycoproteins, because it demonstrates an affinity for N-linked carbohydrates. Unexpectedly, the deglycosylated SAcP retained its ability to bind to this lectin (Figure 4.5). An explanation for this is that the PG chains of the SAcP, which are not removed by these enzymes, were binding to the affinity resin. To test this hypothesis, we used the SAcP produced by a mutant strain of *L. donovani* with the designation C3P0. This strain is deficient for a GDP-Man transporter activity (Descoteaux, Luo et al. 1995; Ma, Russell et al. 1997), resulting in an inability to synthesize PG structures including those on the SAcP. As a result, the SAcP from C3P0 should display only the N-linked glycosylations. The SAcP from C3P0 was subjected to an identical deglycosylation procedure and applied to a Con A sepharose column. In this case the enzyme was predominantly recovered in the column void (Figure 4.5). The lack of Con A binding indicated that deglycosylation under these conditions was complete. It was assumed that the deglycosylation of the native SAcP was equally efficient.

![Figure 4.3](image-url) The effect of exposure to acidic conditions on the SAcP enzyme activity. Reactions were performed at pH 6.0 (●), pH 5.0 (■), and pH 4.0 (▲). This data is representative of three separate experiments. Each data point represents the mean of two replicate assays.
The effect of deglycosylation with Endo F/N-glycanase on the enzyme activity of the SAcP. Digestion was performed at pH 6.0 overnight in the presence (■) or absence (▲) of Endo F and N-glycanase. This data is representative of three experiments. Each data point is the mean of two replicate assays.

The stability of the enzyme with respect to pH was then studied with both native and deglycosylated enzyme preparations (Figure 4.6). Storing the enzyme at various pH values at 26°C in 150mM KCl, resulted in a loss of enzyme activity that became more pronounced as the pH was decreased. At pH 6.0 there was a loss of 27% of the enzyme's activity, at pH 5.0 activity was completely lost during overnight incubation and at pH 4.0 inactivation occurred in less than one hour. This effect occurred independent of glycosylation state. However, after treatment with Endo F/N glycanase, a modest but reproducible loss of enzyme activity was observed in comparison to the undigested enzyme (Figure 4.7) at pH 6.0. This represented a further loss of 20% of the initial activity level and clearly demonstrated the contribution of glycosylation to the stability of the SAcP. Since the enzyme was unstable at lower pH, similar measurements could not be made below pH 6.0. One further experiment was performed to see if partial removal of the glycan chain could mediate this effect, or if the entire chain must be removed, as is
the case with Endo F / N glycanase. To accomplish this, we digested the SAcP with Jack Bean α-Mannosidase (JBAM), an exoglycosidase that removes terminal α mannose residues from proteins and glycan chains. This enzyme is capable of trimming N-linked glycans, but cannot remove the β mannose or the two GlcNAc residues that are adjacent to the protein. This reaction was performed at pH 5.5, and the effect of deglycosylation on SAcP activity is illustrated in Figure 4.8. Digestion with JBAM had no effect on the activity of the enzyme, indicating that only partial glycosylation was required to maintain enzyme stability at low pH. Specifically, the ManGlcNAc₂ of the N-glycan core structure was sufficient to mediate this effect.

![Figure 4.5](image)

Figure 4.5 Effect of deglycosylation with Jack Bean alpha Mannosidase (JBAM) on the enzyme activity of the SAcP. Digestion was performed at pH 5.5 overnight in the presence (●) and absence (■) of JBAM. This data is representative of two separate experiments. Each data point is the mean of two replicate assays.
C. Discussion

The SAcP of *Leishmania donovani* was heavily modified with N-linked carbohydrates. Six of the eight consensus sites within this protein were fully modified, while a seventh site was only partially occupied. The final site could not be detected due to its proximity to a protease resistant Ser/Thr rich region. These modifications did not contain the phosphoglycan determinants that have been identified on this protein. This was evident from their continued interaction with the LPG specific mAb, CA7AE, after enzymatic deglycosylation. Although these data did not eliminate the possibility that a completely novel linkage was being utilized, they suggested that the PG chains were O-linked glycosylations bound through Ser or Thr residues. To date, N- and O-linkages account for almost all examples of protein glycosylation. Other examples include glycolipid anchors, the linkage of glucose to lysine residues, which occurs chemically via Schiff base formation (Lapolla, Gerhardinger et al. 1993) or the modification of hydroxylysine residues in collagen (Rosenbloom, Blumenkrantz et al. 1968).

N-glycosylation in *Leishmania* has been studied in some detail. The process includes a few steps that are not observed in higher eukaryotic systems (Parodi 1993). This is evident from the structures observed on mature glycoproteins from these organisms. Higher eukaryotes produce larger high mannose glycosylations with nine mannose residues, while those observed on mature *Leishmania* glycoproteins contain only six (Parodi, Martin-Barrientos et al. 1984; Olafson, Thomas et al. 1990). In addition, complex structures that are abundant in other systems, have not been observed in *Leishmania*. MALDI-MS analysis of glycopeptides from the SAcP indicated that all of the N-linked oligosaccharides of this protein were of a single size. The observed mass was 1378.2 ± 0.9 Da, which corresponded to the mass of HexHexNAc2 (1378.5 Da). This suggested that the glycosylations of the SAcP were structurally consistent with the Man6GlcNAc2 structure displayed on other *Leishmania* proteins.

Glycosylations have been implicated in a variety of functions. It is commonly accepted that they play a role in folding during protein synthesis (Helenius 1994). Since N-glycosylations are added to the protein chain during translation, they occur before protein folding is completed, and can have a significant impact on the formation of local
folding patterns. Inhibition of glycosylation with inhibitors such as tunicamycin often leads to aggregation associated with incorrect or incomplete folding, as in the case of the SAcP (Lovelace and Gottlieb 1987). Similarly, the use of site directed mutagenesis to eliminate sites of glycosylation leads to loss of protein function. This has been shown for a number of proteins including an acid phosphatase of yeast (Riederer and Hinnen 1991), and cationic peanut peroxidase (Lige, Ma et al. 2001). The use of time resolved fluorescence energy transfer has suggested that glycosylation can lead to the formation of structural nucleation elements that are not otherwise accessible (Imperiali and Rickert 1995). These structural elements can direct the protein down alternative folding pathways. Throughout this process, glycosylation can prevent aggregation of partially folded intermediates by masking exposed hydrophobic motifs.

Glycan function in the mature glycoprotein is more varied and can frequently be involved in recognition processes. For example, blood type immunological specificity was found to depend on short glycan chains, and was among the first descriptions of the functional significance of glycosylation (Viitala, Karhula et al. 1981). Others include the clearance of glycoproteins following the loss of terminal sialic acid (Morell, Gregoriadis et al. 1971) and numerous cell-cell interactions (Kelm, Schauer et al. 1996). Receptor binding and signal transduction are often affected by glycosylation state, for example, chorionic gonadotropin activity can actually be altered by modifying the terminal residue of its glycans (Hoermann, Keutmann et al. 1991). N-glycosylation can also assist in the formation of higher order structures, such as in the dimerization of IFNγ (Sareneva, Pirhonen et al. 1994). Most commonly, however, N-links have been associated with protein stability and can influence this in a variety of ways. They can protect against proteolysis (Rudd, Joao et al. 1994), increase solubility (Wormald and Dwek 1999), and even assist in the refolding of denatured proteins (Kern, Kern et al. 1993), by preventing aggregation in the denatured state to allow a higher recovery of renatured protein after denaturing conditions are removed (Wang, Eufemi et al. 1996). The data presented above demonstrate two basic observations. In addition to the considerable pH sensitivity of the enzyme in its native state, we have shown that the loss of function was more pronounced once the protein had been enzymatically deglycosylated. This type of effect is rarely observed. Only cathepsin E has been shown to exhibit a similar phenomenon (Yasuda,
It is pH sensitive, but requires a lower pH (4.0) to produce a difference in the relative stabilities of native and deglycosylated molecules. However, cathepsin E does not naturally encounter extremes of pH such as this. In the case of the SAcP, the effect is more relevant. The SAcP is only functional at low pH, but shows limited stability under these conditions. This has obvious ramifications on the question of SAcP function. In the mammalian host, the phagolysosome is late endosomal in nature with a pH in the range of 4.7 to 5.2 (Antoine, Prina et al. 1990). The SAcP is unable to maintain enzymatic activity at this pH. At the same time, the deactivation is not so rapid as to make the enzyme completely ineffective within this compartment. Under these conditions, the action of the N-glycosylations in stabilizing enzyme activity could assist the SAcP in reaching a minimum threshold of specific activity, which might not otherwise be achieved. If the enzyme was not constitutively expressed, this gradual loss in activity would supply a naturally occurring ‘off switch’ for phosphatase activity once enzyme production had been halted. It is unclear how this sort of behavior would affect the development of the organism, as no phosphatase targets or function have been adequately identified within this space. It should be noted, however, that for some species of *Leishmania* amastigotes, these observations may be irrelevant as they do not appear to express this protein. The enzyme has been observed in *L. donovani* amastigotes (Bates, Hermes et al. 1989; Ellis, Shakarian et al. 1998), but not *L. mexicana* (Ilg, Stierhof et al. 1994). On the contrary, promastigotes of all *Leishmania* species produce the enzyme. For this stage of the parasite, the environment within the gut of the arthropod host is most germane. Measurement of insect gut pH is difficult, but several insects have been successfully analyzed and a very wide pH distribution has been found (Harrison 2001). Variation is also seen within different segments of an individual insect. These compartments also experience relatively large fluctuations due to CO$_2$ diffusion and meal content, but this is limited by exceptional buffering. Although microelectrodes have been designed to make these types of measurements (Biggs, Greenway et al. 1994), they have not been applied to the sandfly. An alternative approach involves feeding the insect a series of indicator dyes and microscopically estimating the pH by color. This was initially done in the mosquito (Walther, Couche et al. 1986), and more recently in *Lutzomyia*, the vector of New World *Leishmania* strains (Gontijo, Almeida-Silva et al. 1998). Use of
bromocresol purple and bromothymol blue indicated that the sandfly midgut had a pH of approximately 6, while the crop was slightly higher than 6. These are not ideal conditions for SAcP activity, which is maximal between 4.5-5.0, but they are ideal in terms of enzyme stability for the glycosylated protein. Enzyme stability is retained with reduced, but measurable, activity for several days (> 72hrs) at this pH, and it is likely to be continuously maintained throughout the course of infection, which lasts 7 days within this host (Walters, Modi et al. 1987). The stability provided by N-glycosylation is not as critical at this pH, as the enzyme will remain functional even after deglycosylation. However, the extra stability provided by glycosylation may help to offset the fact that the enzyme shows <50% of its potential activity at pH 6.

It is worth noting that the human seminal fluid acid phosphatase shows differences in response to inhibitors as a function of pH (Saha, Crans et al. 1991). This is due to alterations in the active site of the enzyme. The *Leishmania* SAcP shows sequence similarity to this class of phosphatase and may experience similar changes to its active site as a function of pH. It may be of value to test the substrate specificity of the enzyme as the pH is altered. This type of information could be useful in predicting likely targets for the enzyme within its natural environments.
5. Phosphoglycan structure on the SAcP of *L. donovani*

A. Introduction

It is well established that the SAcP of *Leishmania* is phosphoglycosylated. This has been shown repeatedly with Ab specific for the PG structure (Jaffe, Perez et al. 1990; Ilg, Harbecke et al. 1993). However, there is no explanation for the specific targeting of the SAcP for PG addition to protein. It is notable that there are numerous other secreted glycoproteins detected in spent *Leishmania* culture, but in spite of being exposed to the same biosynthetic machinery through a common secretory pathway, none acquire the PG modification (Bates, Gottlieb et al. 1988). This finding suggests the presence of a structural element unique to the SAcP, which allows targeting of the phosphoglycosylation process. Since PG was not conventionally N-linked, it was reasonable to expect the linkage to be made through serine or threonine residues. This explanation was especially attractive considering the presence of a Ser/Thr rich region near the C-terminal end of the protein (Figure 4.3). To date, the isolation and structural characterisation of protein bound PG has been technically hindered by the inherent heterogeneity of the modification. The following chapter enquires into the structural characterisation of this novel glycoform by a number of indirect, yet quantitative, means and specifically addressed the questions regarding the sites of modification, the type of protein linkage and the length of the PG chains to be found.

B. Results

*Effect of phosphoglycosylation on the physical characteristics of the SAcP*

The heterogeneous appearance of the SAcP by SDS-PAGE and chromatography has always been attributed to the PG modifications which it bears. This can be readily demonstrated by comparing the behavior of the protein during ion exchange and reverse phase chromatography. The PG chains of the SAcP are roughly 20% phosphate by weight. They are the dominant source of negative charge on this protein, and cause the
Figure 5.1 Comparison of SAcP elution behavior during anion exchange (panel A) and reverse phase (panel B) chromatography. SAcP elution position is indicated by corresponding phosphatase activity in panel A and is indicated by a star (*) in panel B as detected by SDS-PAGE.
SAcP to bind to anion exchange resins. Elution from a MonoQ anion exchange column required relatively high salt concentrations (0.3-0.4 M), and occurred over a broad range of the developing salt gradient (Figure 5.1, panel A). Poor resolution by anion exchange suggested the presence of considerable anionic heterogeneity. This was interpreted to mean that the size and possibly the number of PG chains varied considerably within the glycoprotein. The behavior observed on a reverse phase column provided other information. Elution from a C8 reverse phase column occurred as a narrow peak (Figure 5.1, panel B), indicating that the hydrophobic component of the SAcP was more homogeneous. Interaction with this type of column would be expected to be mediated by amino acid side chains of the protein with little or no contribution from the PG chains. The well resolved elution of the SAcP from the reverse phase column demonstrated that the protein component of the SAcP was homogeneous by this criterion and was not responsible for the polydisperse electrophoretic mobility on SDS-PAGE. This argument was further supported by chemical deglycosylation experiments. The phosphodiester linkages found in PG chains are sensitive to low pH. This was originally shown for LPG, which can be depolymerized by brief mild acid hydrolysis (McConville, Bacic et al. 1987). This treatment hydrolyzes Hexose-1-PO₄ linkages, cleaving between the Man and the phosphate of the PG structure. With a slight modification, the same procedure can be applied to the SAcP (Bates, Hermes et al. 1990). Incubation at 60°C for 1 hour in 20 mM HCl, results in complete depolymerization of the PG chains of the SAcP. This was seen on SDS-PAGE as a reduction in breadth of the high molecular mass band, and the appearance of two closely migrating but well resolved bands at 95 and 107 kDa (Figure 5.2) revealing that the heterogeneous nature of the SAcP was primarily

Figure 5.2 SDS-PAGE showing the effect of mild acid treatment on the SAcP. Lane A represents untreated SAcP, while lane B represents SAcP heated to 60°C at pH 2.0 for 60 min.
due to its PG chains. Two separate SAcP genes are present in the parasite genome (Shakarian, Ellis et al. 1997), explaining the two bands produced by mild acid.

Mass spectral analysis of PG subunit structure

Mild acid hydrolysis was used to study the structure of the PG found on the SAcP. As indicated previously, this treatment disrupts the phosphodiester linkages of the PG by specifically cleaving hexose-1-PO$_4$ bonds, resulting in PG depolymerization and the release of a population of PG subunits. These subunits represent the individual building blocks of the PG chain and could theoretically include both branched and linear components as observed in the analysis of related lipophosphoglycans (McConville, Thomas-Oates et al. 1990; Ilg, Etges et al. 1992; Thomas, McConville et al. 1992). Normally, carbohydrates are poorly ionized and require modification prior to MS analysis but in this case at least one phosphate is retained on each subunit, allowing direct analysis by negative ion mode mass spectrometry. Analysis of the material released from the

![Figure 5.1](image)

Figure 5.1 Negative mode ESI mass spectrum analysis of the mild acid released sugars from the SAcP. A single component with an (M-H)$^-$ of 421.1 was observed.
SAcP by mild acid revealed the presence of one major species (Figure 5.3). Its mass to charge ratio (m/z) of 421.1 corresponded to the deprotonated form of the structure. The molecular mass was 422.1 Da, corresponding well to the mass of a phosphodisaccharide (422.28 Da). This was consistent with the PO_4-Galβ-Man basic unit structure observed for all PG structures described to date. The absence of other signals within these data indicated that no branched structures were present. Collision induced dissociation (CID) of the 421.1 peak generated the fragmentation pattern shown in Figure 5.4. The fragments at 79 amu and 96 amu were representative of phosphate and demonstrated conclusively
the presence of this group in the 421.1 Da species. Fragmentation of carbohydrates is
generally more complex than that observed for peptide molecules. In addition to
cleavages of glycosidic linkages, there are numerous fragments that arise from cross-ring
cleavages (Reinhold, Reinhold et al. 1995). This results in a greater number of fragments
per residue, and a more complex spectrum. However, in these experiments, the phosphate
is required for ionization and only fragments containing this group were observed which
simplified the data. A schematic of the phosphodisaccharide is provided as a key to show
the cleavages consistent with the observed fragments. These data provide strong evidence
that the structure was a phosphodisaccharide. Fragments e and f in Figure 5.4 also
suggested that the phosphate was located on carbon six. Taken together, these data
proved that the PG structures of the SAcP of *L. donovani* were composed of linear chains
of phosphodisaccharides.

The acid hydrolysis reaction employed here to depolymerize the PG required an
hour to reach completion. Samples taken from this reaction prior to completion showed a
mixture of partially degraded PG chains and analysis of these mixtures provided
information regarding the length of the SAcP PG chains as well as insight into the
behavior of PG chains in a mass spectrometer. Mass spectrometers report the charge to
mass ratio of a molecule, not the mass. Under normal circumstances, large molecules like
peptides or nucleic acids are detected in a number of charge states seen in the MS
spectrum as an ion envelope. This is due to partial ionization of weakly acidic and basic
groups that are abundant in these molecules. Phosphoglycan chains do not behave in this
manner. In every analysis performed, each PG fragment appeared as a single species with
a negative charge equal to the number of phosphates in that structure. This was highly
irregular. Nucleic acids contain similar phosphodiester bonds, but these molecules are
routinely observed to contain numerous charge states (Doktycz, Hurst et al. 1995). It is
likely that the lack of other ionizable groups in the PG structures led to this result. An
advantage of this behavior was the simplification of the observed MS spectra for PG
fragments. The ability of modern mass spectrometers to resolve isotopes allows
determination of the charge of an ionic species and accurate measurement of the number
of phosphates in the molecule. The data that was obtained from partial PG
Figure 5.3 MS analysis of the phosphosugars released from the SACp by incomplete mild acid hydrolysis. Acid labile PG fragments have been analyzed on both low resolution (Panel A) and high resolution (Panel B) mass spectrometers. The sample depicted in panel A was the result of an 8 min hydrolysis, and shows a series of PG fragments of increasing length, but decreasing m/z. Panel B was the result of a 12 min hydrolysis, and shows baseline resolution of all observed fragments. See Table 5.1 for mass assignments.
depolymerization is shown in Figure 5.5 and the assignments of the masses are listed in Table 5.1. It can be seen that as the PG chains increase in length, their measured m/z decreases. This progression approaches a lower limit of 403 m/z. PG chains containing up to 8 phosphodisaccharide subunits were detected in this way. There was also an abundant signal at m/z 403 that was rapidly degraded. This represents a mixture of extremely long PG chains (>25 subunits), which are quantitatively degraded to the monomer by the mild acid depolymerization process. Additionally, other fragments were seen early in the depolymerization which were not composed solely of phosphodisaccharides (Figure 5.6). These fragments demonstrated a ratio of hexose:phosphate that was greater than 2:1 and could be interpreted as branching structures, but the fact that they are quantitatively lost once depolymerization is complete argues against this. These fragments very likely represent the terminal fragment of the PG

Figure 5.4 Negative ion nanospray MS of PG fragments released by a 12 min acid hydrolysis of the SAcP. A number of phosphosugars were observed with hexose:phosphate ratios in excess of 2:1. These signals have been labeled and their structural assignments are presented in Table 5.1.
chain and suggest the presence of a neutral cap structure as observed in LPG (Thomas, McConville et al. 1992). Fragments were observed containing from 2-4 hexose residues in excess of the normal hexose-phosphate ratio. A series of related masses were again observed showing chains of increasing length capped with di-, tri- and tetrasaccharides (Table 5.1). Even the relative abundance of the different sizes appeared to be consistent, although MS data is not reliably quantitative. This information suggests a common biosynthetic pathway in the construction of the PG chains from both LPG and SAcP.

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<td>746.2397</td>
<td>Hex₂(PO₄-Hex₂)</td>
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<td>988.2072</td>
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<td>574.1581</td>
<td>-2</td>
<td>1150.3310</td>
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<td>1150.2600</td>
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<td>655.1871</td>
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<tr>
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<td>2524.6825</td>
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<td>2524.5285</td>
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</tbody>
</table>
Identification of the amino acid involved in PG attachment

Analysis of $^{32}\text{P}$ labeled SAcP showed that the mild acid treatment used for depolymerization of PG chains released much of the incorporated phosphate from this protein. However, some of the radiolabel remained associated with the protein as shown by SDS-PAGE (Figure 5.7). Assuming that the residual phosphate was present as a monoester modification of specific amino acids, an attempt was made to identify phosphoamino acids from the mild acid treated protein. Conventional amino acid analyzers use reverse phase chromatography to separate individual amino acids. Phosphoamino acids bind poorly to these columns and cannot be resolved using standard techniques. Other methods for separating phosphoamino acids involve thin layer separations.

Most commonly this utilizes high performance thin layer electrophoresis, which requires special equipment capable of performing electrophoretic separations on thin layer cellulose sheets. A simpler technique, employed here, involves the use of standard thin layer chromatography (TLC) using more commonly available lab equipment. The $^{32}\text{P}$-labeled SAcP was subjected to mild acid treatment to remove carbohydrate, followed by total acid hydrolysis to reduce the protein to its component amino acids. Elimination of the mild acid step resulted in extensive carbohydrate degradation as shown by caramelisation of the PG chains during the protein acid hydrolysis step. These degradation products interfered with subsequent analyses. Thus the use of mild acid hydrolysis prior to complete hydrolysis allowed for direct spotting of amino acid mixtures onto thin layer cellulose plates along with standard phosphoamino acids. Figure 5.8 shows the autoradiogram of the developed TLC plate. One spot was detected co-migrating with the phosphoserine standard. A significant amount of free phosphate and a small amount of unhydrolyzed peptide were also observed. Although the hydrolysis conditions were
varied to allow detection of other phosphoamino acids (Martensen, et al. 1984), phosphoserine was the only radiolabeled amino acid observed. This data is consistent with the attachment of PG chains to serine residues through a phosphodiester linkage to a hexose.

![Figure 5.2 Thin layer chromatography of $^{32}$P labeled phosphoamino acids derived from the SAcP. Lyophilized SAcP was hydrolysed to its component amino acids by HCl vapor at 110°C for 16 hrs. Phosphoamino acid standards were detected by ninhydrin staining and their positions marked by the application of 3000 CPM of $[^{32}]P$-PO$_4$. Autoradiography was used to detect labeled amino acids for comparison to standards.](image)

**Identification of a consensus sequence for phosphoglycan modification of protein**

Identifying glycosylation sites usually requires protein fragmentation followed by glycopeptide purification and analysis. This approach was attempted repeatedly on the SAcP, with little success. Figure 5.9 shows the result of one such experiment. Tryptic digestion of the SAcP produced numerous fragments that were separated by reverse phase HPLC (Figure 5.9, panel A). The mAb CA7AE, which shows specificity for PG chains, was used to detect the presence of PG associated with these peptides (Figure 5.9, panel B). The antibody clearly detected PG elution throughout the separation and many of these peptides were sequenced. Surprisingly, none contained modified amino acids. These apparently contradictory observations were caused by the overlapping elution of nonglycosylated tryptic peptides and a large portion of the SAcP that was resistant to digestion. This region was heavily modified by PG and was therefore very heterogeneous, causing it to be poorly resolved chromatographically and resulting in elution across a broad portion of the developing gradient. Based upon a DNA sequence arising from a collaboration with Dr. Dennis Dwyer’s group at the NIH, this protease resistant sequence was predicted to comprise roughly one third of the SAcP protein.
sequence, and consist primarily of serine and threonine organized in a repeating octapeptide motif (Figure 4.3). There were no hydrophobic or basic residues, nor were there any methionine residues to allow cyanogen bromide cleavage. The only potential sites for cleavage were acidic residues, regularly spaced throughout the repeating sequence. These could be targeted by specific enzymes such as the endoproteinases Glu-C and Asp-N. Digestion was further hindered by the presence of glycosylations, which provided a physical barrier to the approach of potential proteases. These problems were overcome with the application of a procedure presented as a flowchart in Figure 5.10. There are several key steps that contribute to the success of this approach. First, the abundant PG of the SAcP was removed by mild acid treatment. This eliminates the physical barrier posed by these groups and increases the efficiency of subsequent proteolytic degradation. The phosphate retained by the protein following this treatment indicated the original sites of modification within the protein sequence. Secondly, the protein was radiolabeled with \( ^{32}\text{P}\)-PO\(_4\) to facilitate the detection of phosphorylated peptides during sample work-up. This provided high-level sensitivity when working with low level samples that may not be detectable by UV absorbance. Finally, a two stage digestion was used. The first stage involved chemical cleavage with cyanogen bromide.

**Figure 5.1** Chromatographic and ELISA analysis of a tryptic digest of the SAcP. Tryptic peptides were separated by reverse phase chromatography on a PRP-1 column (Panel A). Peaks were collected manually and tested for PG content by ELISA using the monoclonal antibody CA7AE (Panel B).
Figure 5.2 Flowchart detailing the protocol used to obtain sequence from the serine rich region of the SAcP.

(CNBr) providing quantitative cleavage at methionine residues without side reactions or nonspecific cleavages, and without hydrolysis within the Ser/Thr-rich region of the SAcP. By leaving this portion of the molecule intact, it was more easily separated from unmodified peptides. Once isolated, this large fragment could be proteolytically digested with Asp-N, an endoproteinase that cleaves specifically on the N terminal side of aspartic acid residues. The fragments generated in this way were amenable to protein sequencing, and the proximity of the cleavage sites to the potentially modified serines facilitated direct sequence analysis.

Figure 5.3 SDS-PAGE showing the effect of CNBr digestion on the SAcP. The native enzyme (Lane A) was treated with mild acid (Lane B), followed by CNBr (Lane C). Gels were stained with Stains-All, and the bands appear blue due to the presence of phosphate on the proteins and protein fragments.

The effects of the initial stages of this process on the SAcP were monitored using SDS-PAGE. Figure 5.11 shows the progression starting with the intact reduced and alkylated SAcP (Lane A) followed by successive treatments with mild acid (Lane B) and CNBr (Lane C). These treatments result in a stepwise reduction in the apparent molecular weight of the phosphorylated species. The final CNBr fragments were observed at 49 and 40 kDa which were substantially larger than the theoretical
molecular masses, which were predicted to be 25 and 23 kDa. These fragments are phosphorylated as indicated by Stains-All staining (Figure 5.11) and $^{32}$P incorporation (data not shown). It is likely that the abundant negative charge from the phosphate reduced the amount of bound SDS, via charge repulsion, resulting in an artificially high molecular weight estimate by SDS-PAGE. The peptides were sequenced in a separate experiment and both had the expected N-terminal sequence Gly-Cys-Pro-Arg-Thr. Hydroxyapatite resin was used to separate the peptide mixture into phosphorylated and nonphosphorylated components. Figure 5.12 demonstrates the binding and recovery of phosphorylated peptides from the hydroxyapatite column by scintillation counting. Only 47% of the radiolabeled peptides were recovered from this column, the remainder could not be eluted from the resin. The nonbound fraction contained many unlabeled peptides as demonstrated by reverse phase HPLC. Since the latter were not radiolabeled, they were not further characterized. In the final step, the phosphorylated peptides were digested with endoproteinase Asp-N. Because aspartic acid residues are irregularly placed throughout the serine rich region of the SAcP, cleavage at these sites was expected.

Figure 5.4 Hydroxyapatite chromatography of phosphopeptides from the CNBr digest of the SAcP. A low salt buffer was used for sample application, elution was via 1 M phosphate buffer. These values represent a single measurement, due to a limitation in available sample.
to produce a series of peptides which varied in length but had a common N-terminal sequence. Following the second digestion, the peptides were applied to a reverse phase column to purify them for subsequent protein sequencing. However, the hydrophilic, charged nature of the peptides prevented them from interacting with this column and they were quantitatively recovered in the void volume. The peptides were therefore purified by anion exchange chromatography. The chromatogram is presented in Figure 5.13 along with the associated radioactive counts. A single, broad peak of radiolabel eluted late in the developing salt gradient. The broad elution pattern demonstrates considerable variation in phosphopeptide structure. Following desalting, this material was submitted for N-terminal Edman sequencing. A single N-terminal sequence was obtained that is shown in Table 5.2 along with the associated amino acid recoveries per cycle. The data matched that of the serine rich region of the SAcP. In addition, no residue was observed in cycle 6, which should have contained the first of a triplet of serine residues. This lack of signal was due to modification of the amino acid at that position, in this case by phosphorylation and indicated that PG attachment occurs reliably at this position within the serine rich region of the

Table 5.1 Sequencing results for the [\(^{32}\text{P}\)]PO\(_4\) labeled SAcP peptides produced by CNBr and Asp-N digestion.

<table>
<thead>
<tr>
<th>Cycle #</th>
<th>aa expected</th>
<th>aa observed</th>
<th>yield (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Asp</td>
<td>Asp</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>Val</td>
<td>Val</td>
<td>33</td>
</tr>
<tr>
<td>3</td>
<td>Thr</td>
<td>Thr</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>Thr</td>
<td>Thr</td>
<td>31</td>
</tr>
<tr>
<td>5</td>
<td>Ala</td>
<td>Ala</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>Ser</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>Ser</td>
<td>Ser</td>
<td>4.2</td>
</tr>
<tr>
<td>8</td>
<td>Ser</td>
<td>Ser</td>
<td>6.4</td>
</tr>
<tr>
<td>9</td>
<td>Glu</td>
<td>Glu</td>
<td>5.8</td>
</tr>
<tr>
<td>10</td>
<td>Gly</td>
<td>Gly</td>
<td>4.7</td>
</tr>
</tbody>
</table>
SACP. Following this cycle, the sequence continued unbroken until the next serine triplet was reached, after which no further sequence was observed. The detection of the other serine residues in this sequence indicated that the modification was specific to this site and was the first indication that a consensus sequence might be involved in PG attachment.

This experiment was repeated using an alternative protocol to address two concerns raised by the initial experiment. Firstly, it was desirable to avoid the heavy losses incurred during hydroxyapatite chromatography and secondly, the sequence presented in Table 5.2 only represented one third of the potential PG sites. The others were too far removed from the aspartic acid residues to allow direct sequencing. As outlined in Figure 5.14, the protein was digested with trypsin to hydrolyze regions of the molecule not modified with PG. The reaction was monitored by capillary electrophoresis as shown in Figure 5.15. Significant digestion can be observed as early as 5 min following protease addition, and by 5 hrs the digest had gone to completion. A broad peak was observed throughout this analysis representing the Ser rich, trypsin resistant, PG modified region of the protein. The SACP was left in its glycosylated state to facilitate the separation of this species from the smaller, unmodified tryptic peptides. The presence of the PG chains increased the apparent mass of this fragment, causing it to be excluded from a Sephadex G-50 column while the much smaller tryptic peptides were included in the column and eluted late due to their small size. The analysis of the G-50 chromatography is shown in panel E of Figure 5.15. The PG

![Flowchart showing the protocol for obtaining complete sequence coverage of the SACP](image-url)
peptide was recovered leaving only a large tryptic peptide contaminant eluting from the CE column at 1.5 min, which was removed during subsequent purification and found not to be phosphorylated. The PG bearing fragment was subjected to mild acid deglycosylation followed by Endoproteinase Glu-C digestion. Under the conditions used, Glu-C was expected to cleave the peptide after acidic residues. Separation by anion exchange showed a broadly eluting peak of radioactivity, which did not correlate well with the UV absorbance trace (Figure 5.16). This suggested significant heterogeneity and possibly incomplete digestion. The small peaks observed were derived from the Glu-C enzyme, and were disregarded. Additionally, a large peak was observed that could not be identified, but was not radiolabeled. Despite the apparent lack of resolution, the radioactivity that eluted from this column was pooled with the exception of the large UV absorbing peak. The material was desalted, submitted for Edman sequencing and the data shown in Table 5.3 was obtained. As in the previous experiment, the sequence was strong until it reached the first serine residue. At this position no amino acid was observed. This was followed by partial recovery in the
next cycle and continuation of the sequence. Once again this suggested that the first of three serines was the site of modification. While the possibility remained that other serines were modified, these data indicated that this would be at a much lower frequency than the primary site. These findings have been corroborated by in vitro glycosylation experiments, which have never shown the ratio of phosphoglycan addition to exceed one per serine triplet (Figure 6.4). Also, small quantities of Val were observed in addition to Gly during cycle one of Edman sequencing. Since Val and Gly always follow Asp and Glu respectively in the region under scrutiny, enzymatic cleavage was occurring at all acidic amino acids. This indicates that all of the potential modification sites have been encompassed by sequencing (Figure 4.3).
Table 5.2 Sequencing results for the $[^{32}P]$-PO$_4$ labeled SAcP peptides produced by Trypsin and Glu-C digestion

<table>
<thead>
<tr>
<th>Cycle #</th>
<th>aa expected</th>
<th>aa observed</th>
<th>yield (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gly</td>
<td>Gly</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>Thr</td>
<td>Thr</td>
<td>37</td>
</tr>
<tr>
<td>3</td>
<td>Thr</td>
<td>Thr</td>
<td>56</td>
</tr>
<tr>
<td>4</td>
<td>Ala</td>
<td>Ala</td>
<td>20</td>
</tr>
<tr>
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<td>Ser</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>Ser</td>
<td>Ser</td>
<td>4</td>
</tr>
</tbody>
</table>

**Determination of phosphoglycan chain length**

There are no known chemical or enzymatic means of specifically removing intact PG chains from a protein molecule. Similarly, intact phosphoglycosylated peptides are too heterogeneous to permit isolation and structural characterisation. As a result, it has been impossible to directly measure the length of the PG chains produced by various Leishmania species. Addressing this question with respect to the SAcP was only possible following the characterisation of a specific, reliably modified sequence within the protein chain. Using this information, an estimate could be made of the number of PG attachment sites present in a sample. This was then compared to the amount of carbohydrate in the sample, which provided an estimate of length by way of a simple ratio.

Carbohydrate samples, free of N-linked glycosylations, were prepared by isolating the trypsin resistant portion of the SAcP as described above (Figure 5.14). This fragment was then treated with mild acid to release the phosphodisaccharide subunits, which could then be separated from the protein. Finally, standard acid hydrolysis was performed to reduce these structures to their component monosaccharides. The standard conditions for hydrolysis were 3N HCl for 3 hrs at 100°C. Under these conditions, partial degradation of the monosaccharides was observed resulting in a need to correct the results using a control hydrolysis of a known amount of monosaccharide standard. The latter was very reproducible, and could be reliably used for correction. Recoveries could be improved by changing the hydrolysis protocol to 2N TFA for 2 hrs at 100°C, but controls were still required to correct for losses. Analysis of the samples prepared in this
High performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) was used to separate monosaccharides and oligosaccharides. Figure 5.17 illustrates a typical HPAEC run showing the separation of Gal and Man in the presence of a small amount of contaminating Glc. The latter is a commonly observed contaminant during HPAEC. The other peaks present in this trace were also observed in the hydrolysis control and were discounted. Table 5.4 shows the result of carbohydrate quantitation. Although Gal and Man were expected to be present in roughly equal quantities, Gal recovery was lower than Man recovery in all cases. Because phosphosugars are not detected under normal HPAEC conditions, any Gal that retains its phosphate will not be observed and will contribute to low Gal recovery. An alkaline phosphatase step was added when repeating the experiment (trial 2), which led to an increase in Gal recovery from 56% to 80% (assuming Man to be 100%), and
Table 5.1 Monosaccharide composition of the phosphosugars released from the SAcP by mild acid hydrolysis.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Measured quantity (nmol)</th>
<th>Corrected quantity alkaline phosphatase</th>
<th>Molar ratio of Man to Gal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Man</td>
<td>Gal</td>
<td>Man</td>
</tr>
<tr>
<td>Trial 1</td>
<td>90.83</td>
<td>43.76</td>
<td>316.8</td>
</tr>
<tr>
<td>(3N HCl, 3hrs)</td>
<td>85.21</td>
<td>38.33</td>
<td>296.9</td>
</tr>
<tr>
<td>Trial 2</td>
<td>132.48</td>
<td>92.55</td>
<td>210.3</td>
</tr>
<tr>
<td>(2N TFA, 2hrs)</td>
<td>146.34</td>
<td>95.99</td>
<td>232.3</td>
</tr>
</tbody>
</table>

reduced the Man:Gal ratio to 1.2:1. This was closer to the theoretical value, although the possibility remained that the neutral caps of the PG chains or residual N-linked structures were inflating the Man estimate. Additionally, a control reaction with a Gal-6-PO4 standard indicated slightly lower than expected recoveries (93-95%) even with alkaline phosphatase treatment. With these considerations, the ratio of Man:Gal at this time was considered to be adequate, and no further attempt was made to improve the protocol.

An aliquot taken from the same SAcP sample was subjected to amino acid analysis, which had been done reproducibly on numerous occasions in the past. Five separate analyses were performed and the averaged data are presented in Table 5.5. Estimates of the molar quantity of SAcP in the sample were generated separately for most amino acids by division of the experimental value by its theoretical abundance within the protein. Serine was eliminated from these calculations as it is the site of PG modification and did not provide a reproducible estimate. Cysteine and Tryptophan were likewise eliminated, because they were destroyed during acid hydrolysis. These estimates were then averaged to give an accurate determination of the quantity of protein for use in the PG length calculation. The two sets of values compare well as shown by the small standard deviation, which is 15.2% of the mean value. An unrelated protein of similar size gave a much larger standard deviation when used to perform the same series of calculations, as demonstrated with BSA Table 5.5. Three separate experiments with three different batches of SAcP gave similar results, with accuracies in the range of 14-16%. This was a demonstration of good precision between measurements. Considering the reproducibility observed, it may be possible to improve the estimate by hydrolyzing a
Table 5.2 Quantitation of the SAcP by amino acid analysis.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>SAcP theoretical composition</th>
<th>quantity by amino acid analysis (pmol)</th>
<th>quantity of SAcP (pmol)</th>
<th>BSA composition</th>
<th>protein estimate using BSA (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>58</td>
<td>546</td>
<td>9.42</td>
<td>55</td>
<td>9.93</td>
</tr>
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<td>427</td>
<td>9.09</td>
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<td>5.41</td>
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<tr>
<td>Gly</td>
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<td>418</td>
<td>9.71</td>
<td>16</td>
<td>26.13</td>
</tr>
<tr>
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<td>14</td>
<td>95</td>
<td>6.81</td>
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<td>5.59</td>
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<td>219</td>
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<td>23</td>
<td>9.52</td>
</tr>
<tr>
<td>Thr</td>
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<td>535</td>
<td>7.04</td>
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<td>16.21</td>
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<td>656</td>
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<td>248</td>
<td>9.53</td>
<td>28</td>
<td>8.88</td>
</tr>
<tr>
<td>Tyr</td>
<td>25</td>
<td>150</td>
<td>6.01</td>
<td>20</td>
<td>7.50</td>
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<tr>
<td>Val</td>
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<td>328</td>
<td>6.82</td>
<td>36</td>
<td>9.11</td>
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<tr>
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<td>118</td>
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</tr>
<tr>
<td>Ile</td>
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<td>170</td>
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<td>12.14</td>
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<tr>
<td>Leu</td>
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<td>307</td>
<td>8.29</td>
<td>61</td>
<td>5.03</td>
</tr>
<tr>
<td>Phe</td>
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<td>156</td>
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<td>5.78</td>
</tr>
<tr>
<td>Lys</td>
<td>12</td>
<td>112</td>
<td>9.37</td>
<td>59</td>
<td>1.90</td>
</tr>
</tbody>
</table>

Average: 8.06  
Standard Deviation: 1.23

These protein quantitations were used to determine the molar quantity of PG modification sites present within each sample. The two SAcP genes differ slightly in the number of peptide repeats present in the serine rich regions. SAcP-1 had 22 repeats and SAcP-2 had 25 repeats, with each repeat containing one PG modification site. Since the relative expression level of the two genes was not known, the average value of 23.5 modification sites per SAcP molecule was used in the calculation. The results for trials one and two are shown in Table 5.6. In the final calculation, the ratio of both Gal and Man were compared to the number of PG sites contained within the same sample. This

Table 5.3 Calculation of the number of PG modifications contained within SAcP samples.

<table>
<thead>
<tr>
<th>pmol of SAcP/µl</th>
<th>Sample size (µl)</th>
<th>total SAcP (pmol)</th>
<th>number of PG sites per SAcP molecule</th>
<th>nmol of PG sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial 1</td>
<td>8.06</td>
<td>50</td>
<td>403</td>
<td>23.5</td>
</tr>
<tr>
<td>Trial 2</td>
<td>7.36</td>
<td>50</td>
<td>368</td>
<td>23.5</td>
</tr>
</tbody>
</table>
Table 5.4 Estimation of PG length by comparison of carbohydrate and protein quantities from SAcP samples.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Hexose quantity (nmol)</th>
<th>PG sites (nmol)</th>
<th>PG length estimation as a ratio of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Man</td>
<td>Gal</td>
<td>PG sites</td>
</tr>
<tr>
<td>Trial 1</td>
<td>316.8</td>
<td>175.0</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>296.9</td>
<td>153.3</td>
<td>9.5</td>
</tr>
<tr>
<td>Trial 2</td>
<td>210.3</td>
<td>171.4</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td>232.3</td>
<td>177.8</td>
<td>8.7</td>
</tr>
</tbody>
</table>

resulted in estimates of PG length that range from 20 to 33 phosphodisaccharide subunits (Table 5.7). The Gal estimates in trial one were low due to poor dephosphorylation and were discounted.

C. Discussion

Phosphoglycosylation is a rarely occurring form of post-translational modification. In the eukaryotic parasite Leishmania, modifications of this type are based on a repeating phosphodisaccharide composed of galactose and mannose that is specific to this organism. The SAcP from all species of Leishmania have been shown to be phosphoglycosylated (Lovelace and Gottlieb 1986), including L. major, in which the SAcP has only recently been described (Shakarian and Dwyer 2000). In this study, we have shown that the PG modifications of the SAcP in L. donovani resemble those of the LPG produced by this species. The structures consist of unbranched chains of Galβ-(1,4)-Manα-1-PO₄ subunits connected through phosphodiester bonds to the hydroxyl on carbon six of the Gal, which are terminally capped with neutral oligosaccharides of 2-4 residues containing only Man and Gal. The length of these chains is in the order of 20-30 subunits on average, which shows good correlation with the 14-30 subunits of the chains on LPG (McConville, Turco et al. 1992). The variation in PG length on the SAcP may be related to the age of the culture from which it is derived. The length of the saccharide units on LPG doubles as the cells undergo metacyclogenesis during the switch from logarithmic growth to stationary phase. It is likely that PG of the SAcP follow a similar pattern. Structural similarity suggests that aside from the protein attachment point, the same biosynthetic machinery is likely used in the construction of both protein and lipid-
bound PG in *L. donovani*. As such, changes in the structure of LPG should be reflected in
the structure of the SAcP.

The present study has demonstrated the presence of a consensus protein sequence
that directs the attachment of PG chains to the SAcP. This comes in the form of an eight
amino acid tandem repeat with the sequence (EG/DV)TT(A/T)SSS. The first serine in the
triplet contained within this sequence was invariably the site of phosphoglycosylation.
Multiple occurrences of this sequence within the SAcP results in abundant modification.
When combined with glycan microheterogeneity at each site, this resulted in substantial
structural variation, which was readily observed by SDS-PAGE. This required consensus
sequence also explained the absence of similar phosphoglycosylations on other proteins
secreted by *Leishmania*. The attachment of PG chains was shown to occur through a
phosphodiester bond to the indicated serine. Although commonly observed in
*Leishmania*, this type of glycosidic linkage is rarely observed in other organisms. It was
first demonstrated on a cysteine proteinase from *Dictyostelium discoideum* (Gustafson
and Milner 1980), which is decorated with single N-acetylglucosamine residues
phosphodiester linked to serine. More recently, a Gaβ-1-PO₄-Ser linkage has been
observed in a proteophosphoglycan of *Entamoeba histolytica* (Moody-Haupt, Patterson et
al. 2000).

*L. mexicana* differs from other *Leishmania* species (including *donovani*) by
producing a polymeric SAcP. It can be visualized by electron microscopy as a
filamentous structure similar in shape to a bottle-brush (Stierhof, Ilg et al. 1994). It is,
therefore, not surprising that the results presented here contrast with those observed in
*L. mexicana* (Ilg, Overath et al. 1994). The SAcP of *L. mexicana* is modified by shorter
PG chains consisting of 1-3 phosphodisaccharides, which are attached at multiple
consecutive serine residues. Although less frequent, the modifications of *L. donovani* are
much larger and lead to a higher glycosylation level overall. It is tempting to relate these
differences to the visible structural differences of the SAcP produced by different species.
Recently the SAcP from *L. mexicana* was cloned and expressed in a strain of *L. major*
(Wiese, Gorcke et al. 1999), a species which produces almost no SAcP of its own. Under
these circumstances the SAcP was monomeric and appeared to be more heavily
glycosylated than it was when produced by *L. mexicana*. This was observed as an
increase in the apparent molecular weight of the glycoprotein by SDS-PAGE. It would therefore seem that glycosylation patterns of the SAcP differ between New World and Old World species of *Leishmania*. Unfortunately, the significance of this difference remains unclear.

In spite of extensive structural analysis, the functional significance of the SAcP is unknown. It is an abundant enzyme with many glycosylations, and its construction represents a large energetic investment for the parasite. This suggests an important role for the enzyme in parasite survival. Although a nutritional role has been suggested, the enzyme is not upregulated by phosphate starvation. It also seems counterintuitive to use an enzyme containing 400 moles of phosphate per mole of protein as a means of countering low phosphate availability. While the enzyme may ultimately contribute to the available phosphate pool, it is unlikely to be involved as an emergency response. With the high degree of glycosylation, one might expect the SAcP to display an affinity for lectins produced by its host organisms. This does not appear to happen during the mammalian portion of the life cycle. SAcP knockout mutants retain their ability to invade and proliferate within macrophages, and remain virulent to mice (Wiese 1998). No information is available concerning their passage through the insect host, but since it is the promastigote that produces most of the SAcP, it is likely that this represents the environment in which the enzyme finds its greatest utility. Ultimately, the best functional hypothesis will be one which explains the coexistence of phosphatase activity and phosphoglycosylation within the same molecule. One possibility is that the phosphatase activity acts in the disruption of host signalling pathways, and that the PG directs this activity to an appropriate location at the surface of arthropod host cells. Until potential targets are identified, this possibility cannot be confirmed. Since the SAcP is released into the gut of the sandfly, the surface of the cells making up the gut lining would be the most likely site of such a target. The availability of large quantities of pure SAcP could assist in future attempts to probe this location.
6. Partial Characterization of the mannosyl phosphate transferase of *L. donovani*

**A. Introduction**

The abundance of phosphoglycosylations and the variety of different structures to which they are bound suggests that a complex biosynthetic pathway is at work. Early research into the biosynthesis of LPG, the first phosphoglycosylated molecule identified, showed that the polymer grows in a stepwise fashion through the alternating addition of mannose α-1-phosphate and β-galactose (Carver and Turco 1991). This proceeds by way of a galactosyltransferase and a mannosyl phosphate transferase (MPT). The galactosyltransferase activity has been detected, but the enzyme responsible has not been studied in detail. The MPT has received more attention. Work in several laboratories has shown that there are several enzymes which display MPT activity. There seem to be separate molecule for the addition of the first Man-1-PO₄ to the hexasaccharide core of LPG and for the addition of all subsequent Man-1-PO₄ to the growing polymer. Respectively, these are named the initiating MPT and the elongating MPT (Descoteaux, Mengeling et al. 1998). The fact that this modification is present on protein as well as glycolipid suggests that there may be a third enzyme, which adds the initial Man-1-PO₄ to protein acceptor molecules. The alternative would be to have a single enzyme with an active site capable of accepting a structurally diverse range of substrates. There are advantages to both possibilities. One would allow separate control mechanisms for different types of phosphoglycosylation, the other offers energetic rewards by utilizing a single biosynthetic pathway to accomplish multiple functions. Studies of these processes have been successfully performed using natural (Carver and Turco 1991; Carver and Turco 1992), and synthetic (Brown, Millar et al. 1996; Descoteaux, Mengeling et al. 1998) carbohydrate and glycolipid acceptor molecules. These experiments showed that the biosynthetic underpinnings of phosphoglycosylation can be effectively investigated in *vitro*. The following chapter describes the development of an assay for the detection of protein directed MPT activity, and partial characterization of the responsible enzyme.
B. Results

Designing an enzymatic assay for the mannosyl phosphate transferase

Designing a suitable substrate for the MPT of *L. donovani* was the first step in defining a workable assay for the enzyme. The identification of a repetitive consensus sequence for the attachment of PG chains allowed the production of synthetic peptides for this purpose. A peptide was produced with the sequence acetyl-WSSEGTTASSS-amide (sacp-1). The tryptophan was included in the sequence to improve interaction with reverse phase columns, and to make it possible to detect the peptide by UV absorption at 280 nm. The conditions of the MPT assay were based on work initially done with LPG (Carver and Turco 1991). These researchers have shown that PG biosynthesis does not make use of lipid linked sugar donors such as dolichol-P-Man. Instead GDP-Man and UDP-Gal are required during this process. Subcellular fractionation by both differential centrifugation and zonal sucrose density gradient centrifugation was used to isolate the Golgi from *Leishmania* cells, which were incubated with the sacp-1 peptide and GDP-Man. The reaction products were subsequently analyzed by negative mode ESI-MS. The doubly charged peptide was observed at 572.1 amu, but no modifications of this compound were observed. In addition to the main component, small amounts of truncated peptides, which arise due to errors in synthesis, were also present in this sample. Close examination revealed a very small peak with a m/z of 596.1 that corresponds to a peptide with the sequence Ac-SSEGTTASSS modified with a single phosphomannose. The association of mannosyl phosphate addition with tryptophan deletion suggested that the bulky, hydrophobic nature of this amino acid interfered with the interaction of sacp-1 and the MPT active site. In spite of this, the feasibility of using a defined peptide substrate to test for MPT activity was demonstrated by the *in vitro* modification of the deletion peptide.

Data collected using sacp-1 led to the synthesis of a second substrate peptide with the sequence SSSEGTTASSSEGTTASSS-amide (sacp-2). The interfering Trp was omitted. Unlike sacp-1, this peptide contains three possible attachment sites and all possible orientations of the Ser triplet within the consensus sequence. If there are specific requirements for the orientation of residues adjacent to the serines they should be
provided by this peptide. Analysis of this peptide following modification proved to be problematic. The removal of Trp from the sequence caused the peptide to display poor affinity for reverse phase columns and as a result, was unsuitable for chromatographic purification and analysis. It was subsequently found that fluorescamine labeling prior to chromatographic separation could overcome this difficulty. Fluorescamine covalently modifies primary amines (Udenfriend, Stein et al. 1972) and in the case of sacp-2, only the N-terminus was available, ensuring that the modification would only occur once. The new product was hydrophobic, readily binding to a RP column and absorbed UV light at 280nm, making it an ideal replacement for Trp. The labeled peptide was commonly observed to elute as a triplet from a C8 column (Figure 6.1). It was not clear why this was the case, but each of these peaks appeared identical and correct by MALDI-TOF mass spectrometry (Figure 6.2).

Figure 6.2 MALDI-TOF MS of fluorescamine modified sacp-2. This sample was prepared by reverse phase HPLC as shown in Figure 6.1.

Figure 6.1 Reverse phase separation of fluorescamine labeled sacp-2. Separation was performed on a C8 column. The labeled peptide is consistently observed eluting as a cluster of three peaks as indicated by the asterisk.
Figure 6.3 RP-HPLC analysis of phosphomannosylated sacp-2. Following incubation of sacp-2 with Leishmania Golgi membranes and GDP-[\(^{14}\text{C}\)]-Man, the peptide was modified with fluorescamine and chromatographed on a C18 reverse phase column. In panel A the elution of the peptide is detected by UV absorbance, while the corresponding elution of radioactivity is shown in panel B. SDS-PAGE analysis of this material is shown in panel C. Lane 1 contains GDP-[\(^{14}\text{C}\)]Man. Lanes 2 and 3 represent the void fraction and the peak eluting at 22 min respectively. A separate reaction, in which no sacp-2 was added, was chromatographed under identical conditions. Lanes 4 and 5 represent the void and 22 min fractions from the control.
Sacp-2 was subjected to a similar glycosylation reaction as sacp-1, except the radiolabeled sugar donor GDP-[\(^{14}\)C] Man was used. Following labeling and fluorescamine treatment, the peptide was chromatographed on a C\(_8\) column. Individual peaks were collected and a portion of each was subjected to scintillation counting. The results shown in Figure 6.3 (panels A and B) demonstrate the elution of radiolabeled peptide from the column. These fractions were then subjected to SDS-PAGE using the tricine buffer system designed for separating small molecular weight peptides (Schagger and von Jagow 1987) and a radiolabeled band was observed eluting at a position slightly before the main peptide peak (Figure 6.3, panel C). This band was not observed when the synthetic peptide was omitted from the reaction. The apparent molecular mass of approximately 5.0 kDa is significantly higher than anticipated (theoretical mass = 2.3 kDa). This was likely due to decreased binding of SDS once the negatively charged mannosylphosphate was attached. We have observed other phosphopeptides to demonstrate higher molecular weights than expected in this system. As a final test of the glycosylated peptide, a sample of mannosylphosphate labeled sacp-2 (not fluorescamine labeled) was subjected to negative mode ESI-MS. A doubly charged species was observed at 979.4 m/z Figure 6.4. This computes to a mass of 1960.8 Da, and is consistent with sacp-2 modified by a single mannosylphosphate (1961.7 Da).

![Figure 6.4](image_url) Negative ion ESI-MS of phosphomannosylated sacp-2. The doubly charged ion representing unmodified sacp-2 is observed at 858.3 (theoretical, 858.31) while its phosphomannosylated counterpart is observed as a doubly charged ion at 979.4 (theoretical, 978.97).
Partial characterization of the subcellular compartment in which the MPT was found

It was noted during initial experiments that the MPT was sedimented in a 100,000 x g microsomal pellet. This supported the belief that the enzyme was located in the Golgi. To investigate this further, a sucrose step gradient was prepared by layering eight 1.0 ml layers of increasing sucrose concentration (0.25 – 2.0 M) on a cushion of 2.2 M sucrose. A 1500 x g cleared cell lysate was layered on top of this and the sedimentation was performed by centrifugation at 70,000 x g. A sample at each layer was assayed for MPT activity, which was observed to sediment primarily at a density below 1.0 M sucrose (Figure 6.5). The Golgi is a heterogeneous mixture of vesicles, which vary in density (Hayes, Freeze et al. 1993). The cis- and trans-Golgi can be partially separated by sucrose density gradients, as there is a decrease in density across the stack (Farquhar and Palade 1998). The sedimentation of the MPT in this region of the sucrose density gradient is consistant with its presence primarily in the cis-Golgi. This relies on the assumption of similarity in vesicle density between Trypanosomatid and mammalian cells. Based on these findings, subcellular components containing MPT were purified by pooling all of the material sedimenting at a density less than 1.0 M sucrose. This fraction was tested for the presence of the markers α-mannosidase (lysosomes) and acid phosphatase (plasmalemma). Neither activity was recovered in any significant quantity in the MPT enriched fraction (Figure 6.6). These membrane fractions, which are both sources of degradative enzymes, were not observed.
to copurify with the putative Golgi fraction. Strangely, the MPT could not be detected in cell lysates prior to Golgi enrichment. This may have been due to enzyme dilution, or the activity of an α-mannosidase removing the radiolabeled mannose from sacp-2.

**Solubilization of the mannosyl phosphate transferase**

The MPT could only be detected once the vesicles with which it was associated had been disrupted by either sonication or detergent solubilization (Figure 6.7). This indicated that the enzyme was not only membrane bound, but luminally oriented within these vesicles. A comparison was made of a variety of different nonionic and zwitterionic detergents at varying concentrations to determine which were most compatible with MPT activity. N-dodecyl β-D-maltoside (β-DDM) is a detergent that has been shown to be most effective in solubilizing the LPG specific MPT from *Leishmania major* (Brown, Millar et al. 1996). This detergent, CHAPS, Triton X-100 and octylthioglucoside were tested at concentrations of 0.1% and 1.0%. Figure 6.8 shows that the MPT retains a portion of its activity in all of these detergents. β-DDM appeared to be the most gentle
Figure 6.7 The detection of MPT activity requires vesicle disruption. The transfer of radiolabeled mannose to sacp-2 was only observed when either sonication or detergent (0.1% n-dodecyl β-D-maltoside) was used to disrupt the Golgi membrane. These data are representative of more than three separate experiments. Each bar represents a single determination.

detergent because it supported the highest levels of MPT activity as measured by $[^{14}\text{C}]$-Man incorporation. Since purification of this enzyme was of interest, we further tested these detergents for their ability to solubilize the MPT. Following detergent disruption, the samples were subjected to sedimentation at 100,000 x g as a test for solubility. The results in Figure 6.9 showed that, although n-dodecyl β-D-maltoside was the most compatible with MPT activity, it was unable to generate freely soluble enzyme. Triton X-100 was the only detergent of those tested that was able to completely solubilize the enzyme and 1.0% Triton X-100 was used in all subsequent experiments.
Figure 6.8 The effect of various detergents on the activity of the MPT. Purified Golgi were solubilized prior to MPT assay by the addition of two different concentrations (w/v or v/v) of each detergent. The final sample in the series was a control containing no detergent. [\(^{14}\)C]-Man labeled peptide was isolated by RP-HPLC and quantified by scintillation counting.

Figure 6.9 Solubilization of the MPT as assayed by high speed centrifugation. Each sample was centrifuged at 100,000 \(x\) g for 60 min. Supernatants and pellets were assayed separately for MPT activity. The negative control received 0.1% \(\beta\)-DDM, but no \(\text{sacp-2}\).
Affinity purification of the MPT

The ability to solubilize the MPT while still maintaining enzyme activity suggested that enzyme purification was feasible. Since the enzyme seemed to specifically recognize sacp-2, the peptide was used to construct an affinity column. Sacp-2 was covalently linked to CNBr activated sepharose via the primary amine at its N-terminus. The peptide (13.5 mg) was reacted with 1 g of the CNBr sepharose resin, and peptide binding was assessed by UV absorption before and after the reaction. Only 38.8% of the peptide was recovered, indicating a peptide loading of 8.26 mg per gram of resin. The rehydrated resin had a final volume of 2.6 ml. Thus the final concentration of peptide on the resin was 1.9 μmol/ml. As a control, a second column was prepared by covalently attaching glycine to the same resin.

![Flowchart](image)

**Figure 6.10** Flowchart detailing the procedures used in the affinity purification of the MPT from *L. donovani*. 
Solubilized Golgi were centrifuged at 100,000 x g to remove insoluble material and the resulting supernatant was applied to the affinity column. The binding conditions were identical to those used in the standard MPT assay, with the exception that 0.5 M NaCl was added to prevent non-specific charged interactions with the column. Several elution protocols were attempted in an effort to recover active MPT from the column. A flowchart is presented (Figure 6.10), which is a composite of these protocols showing all of the conditions used. MPT binding was performed in high and low salt conditions. Elution was attempted using extremes of pH, high salt (following low salt binding), and excess sacp-2. Finally, 8 M urea was used to ensure complete removal of any proteinaceous material from the column. MPT assays were performed prior to and following incubation of the solubilized fraction with the affinity resin. Also, assays were performed on eluted samples following desalting and neutralization. Figure 6.11 shows the results of the MPT assays performed on samples collected from the sacp-2 Sepharose column (panel A). Not all of the enzyme activity was adsorbed to the column, but a reduction in activity recovered in the void was repeatedly observed, which ranged from 65-78% recovery in three separate experiments. This behavior was not observed when the glycine control column was used (Figure 6.11, panel B). However, no enzyme activity could be eluted from the column, and it is likely that the elution conditions used resulted in denaturation of the enzyme. SDS-PAGE was used to ascertain the presence or absence of protein components in these samples. Most of the fractions did not contain protein (Figure 6.12) but the 8 M urea fraction that was collected from the sacp-2 Sepharose column contained a pair of protein bands that were not observed in any other fraction. This result was reproduced in two separate experiments. These bands had calculated molecular masses by SDS-PAGE of 58.3 kDa and 65.9 kDa. Unfortunately, Edman sequencing of each of these proteins failed, indicating that they were N-terminally blocked. Comparison by amino acid analysis shows that these two proteins are similar in composition (Table 6.1), and are likely related. It may be that two isoforms exist for this enzyme, or more likely that it is susceptible to limited chemical or proteolytic degradation. Asp-Pro bonds are known to be acid labile, and the acidic conditions that were employed during affinity column development could result in cleavage at one of
Figure 6.11 Recovery of MPT activity during affinity chromatography. Panel A shows the pattern of MPT elution from the sacp-2-sepharose affinity column. The soluble fraction refers to the material recovered in the supernatant following centrifugation at 100,000 x g. The control received no sacp-2. The other samples were collected from the affinity column using the indicated elution conditions. Panel B shows the result when glycine-sepharose is used to perform the same experiment. These data are representative of two separate experiments.
Figure 6.12 SDS-PAGE analysis of the material eluted from the sacp-2-sepharose affinity column. The samples shown here are the affinity void (lane A), and elution using low pH (lane B), high pH (lane C), sacp-2 (lane D), and 8 M urea (lane E). The two bands which presumably represent the MPT are indicated by arrows.

These sites if any are present. These compositions were compared against the Swiss-Prot database using the AACompIdent program found on the ExPASy Molecular Biology Server website (http://ca.expasy.org/tools/aacomp/). Constellation 2 was used, as it is most appropriate for the data. It uses a single value for the amino acid pairs Asn/Asp and Gln/Glu, and excludes Cys and Trp. No significant matches were obtained by this analysis of affinity purified MPT by in situ digestion and Q-TOF sequencing.

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procedure, and although not as refined as an amino acid sequence similarity search, these data were in support of these proteins being novel molecules.

The protein bands that were obtained by SDS-PAGE following affinity purification were used to prepare samples for mass spectrometric analysis on an orthogonal quadrupole TOF MS (API QSTAR™ Pulsar Hybrid LC/MS/MS System, Applied Biosystems, Foster City, CA). The protein bands were excised from the acrylamide gel and subjected to in situ digestion with bovine trypsin. The two bands were processed together, reasoning that if the two bands were derived from the same protein, this would double the sample quantity and increase the likelihood of obtaining useful sequence. From this sample, three unique sequences were obtained. The MS/MS spectra of the individual peptides are presented in Figure 6.14, along with the determined sequences. Each of these peptides has been analyzed using the database matching software available at the Protein Prospector (http://prospector.ucsf.edu/), Matrix Science (http://www.matrixscience.com/cgi/index.pl?page=/search_form_select.html), and PROWL (http://prowl.rockefeller.edu/) websites. However, no matches were identified, indicating once again that these proteins in all probability do not presently exist in the database.
Figure 6.13 Q-TOF MS sequencing of tryptic peptides derived from the affinity purified MPT. The MPT was digested in situ and subjected to CID analysis. The identified sequence ions are indicated in each trace as are the sequence tags deduced from them.
C. Discussion

_Leishmania_ utilize several enzymes in the construction of the wide range of phosphoglycans that they routinely display. We have partially characterized a GDP-Man: protein mannosylphosphate transferase (MPT) from _L. donovani_ that is required for initiating the synthesis of PG chains bound to the SAcP of this organism. This included the derivation of an assay for the detection of the MPT based on its biochemical function. This assay employed the transfer of mannosyl phosphate from the nucleotide sugar donor GDP-Man to a synthetic peptide comprised of a portion of the SAcP sequence. The product was detected as a radiolabeled molecule and its identity was confirmed by mass spectrometric analysis. It is interesting to note that an assay for the N-acetylglucosamine-1-phosphate transferase from _Dictyostelium discoideum_ (Freeze and Ichikawa 1995), also employed recognition of a protein substrate. PG are constructed by adding Man-1-phosphate in a single step, rather than as a consecutive addition of phosphate and mannose. The reason for this and for the development of the MPT may be related to the means by which _Leishmania_ store energy. These parasites produce mannose polymers or mannan in the same way that mammalian cells produce glycogen. The mannose is released from these mannan chains in the form of Man-1-phosphate. Thus a ready supply of Man-1-phosphate is always available for the coupling to GMP to regenerate the GDP-Man used by the MPT. As mentioned, mannosyl phosphate transfer in _Leishmania_ is actually mediated by several enzymes, including the initiating MPT (iMPT) and elongating MPT (eMPT) involved in LPG synthesis. The conditions used to assay the GDP-Man:protein MPT were similar to those used for the eMPT and iMPT from _Leishmania_ (Carver and Turco 1991; Descoteaux, Mengeling et al. 1998). It is almost certain that these enzymes are related and that their mechanism of action is very similar. They differ primarily in the substrates with which they interact, but even here there are similarities. A study of the eMPT involved in LPG synthesis was recently performed using synthetic glycolipid acceptors (Routier, Higson et al. 2000). This study showed that a nearby phosphate group was absolutely required for eMPT recognition of the synthetic substrates that were tested. The SAcP sequence to which PG chains are added contains regularly spaced acidic residues. It is reasonable to hypothesize that the
negative charges provided by these residues are involved in MPT recognition of its target sequence. Similarly, the high concentration of hydroxylated amino acids in this region could interact with the MPT in a fashion similar to the carbohydrate acceptors that are recognized by the eMPT and iMPT. These questions could easily be addressed through the use of a panel of synthetic peptide substrates based on sacp-2 and the SAcP.

The MPT described here was a membrane bound protein. It was compatible with a variety of detergents, which were required for the detection of activity, but only Triton X-100 was capable of freely solubilizing the enzyme as tested by high speed centrifugation. This detergent is known for its gentle solubilization, often maintaining subunit interactions as well as native conformations (Vance and Vance 1991). The ease with which the MPT is solubilized suggests that it may be a simple membrane protein, with only a single transmembrane domain. The enzyme is contained in the microsomal fraction following cell disruption and appears to be Golgi derived. Although no direct Golgi markers have been tested for copurification, the enzyme does sediment at a density normally associated with the cis-Golgi (Findlay and Evans 1987). However, the Golgi is not a rigorously defined compartment within the cell. Being involved in protein modification and sorting, it is constantly changing as components pass through en route to other final destinations (Farquhar and Palade 1998). There are no distinct boundaries and caution should, therefore, be taken when trying to pinpoint the location of an enzyme within this space. It should also be noted that the use of GDP-Man as a sugar donor, rather than dolichol-Man is further indication of Golgi localization, as dolichol donors are located in the endoplasmic reticulum, while Golgi glycosyltransferases traditionally use nucleotide activated sugar donors (Stryer 1995).

Purification of the MPT was successfully accomplished using an affinity column constructed from the synthetic peptide substrate used in the MPT enzyme assay. The use of synthetic peptides for affinity chromatography is rare and we have been unable to locate other examples in the literature. Nevertheless, the enzyme was purified to apparent homogeneity by this procedure and a pair of protein bands were produced which ran at 58.3 kDa and 65.9 kDa by SDS-PAGE. This molecular weight is similar to other glycosyltransferases that use nucleotide sugar donors as substrates (Joziasse, Shaper et al. 1990; Basu, Weng et al. 1996). The appearance of two bands is likely due to limited
degradation. This has been previously observed for other glycosyltransferases such as the UDP-glucose:glycoprotein glucosyltransferase from Schizosaccharomyces pombe (Fernandez, 1994) and the murine galactosyltransferase GalT-4 (Basu, Weng et al. 1996).

While successful, the affinity protocol was not ideal. MPT interaction with the column was poor and much of the activity could not be extracted. Because the affinity ligand is protein based, it is possible that specific secondary structural elements are required for MPT recognition. This is supported by the fact that mannosylphosphate transfer to sacp-2 in vitro is less efficient than the observed transfer in vivo, which appears to be 100%.

Longer peptide sequences could compensate for this, but the difficulties associated with the synthesis of large peptides make this an unattractive option. Nucleotide sugar analogs could be used as an alternative ligand for affinity column construction. This procedure has received widespread use in the purification of other glycosyltransferases. GDP-hexanolamine is an analog of GDP-Man (Mudgapalli, Roy et al. 1994) that would be ideal for this application. These ligands have the benefit of being simpler than peptides at a structural level, with no possibility of higher-order folding requirements. UDP-hexanolamine sepharose has been successfully applied to the purification of numerous enzymes, such as an α-1,3-galactosyltransferase (Joziasse, Shaper et al. 1990) and a UDP-Gal:glucosylceramide β-1,4-galactosyltransferase (Nomura, Takizawa et al. 1998).

Much work remains to be completed with respect to the MPT of L. donovani. The identification of peptide sequence from the MPT should facilitate cloning of the enzyme, and preliminary work has been initiated using a PCR based cloning strategy that may eventually lead to the isolation of the MPT gene. This type of strategy relies on the presence of the mini exon, a short nucleotide sequence that is introduced upstream of every mRNA produced by trypanosomatid parasites. This supplies a ready forward primer for use with a reverse primer based on the experimentally derived sequence. The 5’ end of the MPT gene can be isolated from a cDNA library in this fashion, and a similar approach can be used to isolate the 3’ end of the gene, by manually inserting a known nucleotide sequence at the end of every cDNA for use in a second PCR experiment. The gene sequence might also be accessible by means of a phage display approach. The peptide that has been used as an affinity ligand could be used to screen a phage display library produced from this species. It would also be of value to use the peptide substrate
in crosslinking studies, which represent a viable approach for labeling the MPT and could assist in the identification of sequences at or near the active site of the enzyme. Alternatively, commercially available photoreactive analogs of GDP-Man could be used for this type of experiment, a process that has been previously employed in the analysis of other glycosyltransferases (Mudgapalli, Roy et al. 1994).
Conclusions

The characterisation of the SAcP has been a complex problem requiring a variety of technical approaches to achieve its completion. I have developed an optimized procedure for the purification of this enzyme, which has contributed to the success of this study. This procedure provides microgram quantities of SAcP for structural analysis and results in greater than 40-fold purification of this secreted glycoprotein. Structural analysis has demonstrated the presence of both N-linked and O-linked glycosylations on this molecule. Hence, the variety of post-translational modification leads to substantial structural heterogeneity as demonstrated by smearing during SDS-PAGE analysis. The N-linked carbohydrates are primarily high-mannose type as indicated by their monosaccharide composition and size. These modifications are present at seven of the eight occurrences of the consensus sequence for N-linked glycosylation (Asn-X-Ser/Thr). The eighth site was undetected by the procedures used in this work. The O-linked glycosylations are a unique structure not previously observed bound to a protein molecule. They are structurally and antigenically related to the lipophosphoglycan produced by all species of Leishmania. In L. donovani these modifications are arranged as unbranched, linear chains of the phosphodisaccharide 4-O-(beta-D-galactopyranosyl)-alpha-D-mannopyranosyl-1-phosphate connected to one another by phosphodiester bonds. They are capped at the nonreducing end by small neutral oligosaccharides composed entirely of mannose and galactose ranging from one to four residues in size. These phosphoglycan chains are attached to the protein by a rare phosphodiester linkage between mannose and specific serine residues within the protein. These serine residues appear to occur solely within the peptide sequence Asp/Glu-Val/Gly-Thr-Thr-Ala-Ser*-Ser-Ser at the residue marked with an asterisk. This sequence appears to be a consensus sequence that directs the addition of these carbohydrates to the protein molecule and restricts the addition of phosphoglycan to the SAcP.

Additional studies have been directed at the analysis of phosphoglycan biosynthesis with respect to the SAcP. I have performed a preliminary investigation of the enzyme responsible for the initial step in protein bound phosphoglycan synthesis.
This enzyme is known as the mannosyl phosphate transferase (MPT). The MPT catalyses the transfer of mannose-alpha-l-phosphate from GDP-Man to select serine residues within the SAcP sequence. This enzyme is associated with the Golgi apparatus as shown by sucrose density sedimentation experiments. It is a membrane bound enzyme that can be solubilised using the detergent Triton X-100. Detection of the enzyme can be accomplished by monitoring the transfer of radiolabeled mannose from GDP-[14C]Man to a peptide substrate based on the sequence of the SAcP. The enzyme can be purified via an affinity chromatography approach using a solid support constructed by the attachment of the synthetic peptide used in the MPT assay to a Sepharose resin. The proteins purified in this manner migrate to an apparent mass of 58 and 66 kDa by SDS-PAGE. Amino acid compositions and MS derived sequences were obtained from these protein bands, but did not match any entry in current public databases. It is likely that these proteins are novel molecules that have not been previously defined.

These studies could be further complimented by analysis of the MPT involved in PG addition to the SAcP of *L. donovani*. The affinity isolation of the MPT could be repeated to attain additional sequence information for comparison to current databases. The data already obtained could also be further utilised in the analysis of the MPT. Specifically, the internal sequence obtained by MS analysis could be used in the construction of primers for PCR amplification of the gene sequence of the MPT from a cDNA library. Another approach that could lead to the isolation of the gene sequence would be to use the affinity protocol designed during this study to probe a phage display library derived from *L. donovani*. This approach has been attempted, but no significant results have yet been obtained. Finally, a number of biochemical studies of the MPT could be performed using the procedures outlined here to determine functional characteristics of the enzyme.
Bibliography


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