Biophysical Investigations of Structural Features and Interactions of *Leishmania donovani* Peroxin 5

by

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Abstract

Parasites of the genus *Leishmania* cause a broad array of diseases, collectively termed leishmaniasis. These diseases range in morbidity; the cutaneous form is typically self healing, while the mucocutaneous and visceral manifestations require chemotherapeutic intervention to avoid lethality. At present there is no vaccine, and current methods of chemotherapeutic intervention have severe drawbacks, together creating a dire need for new options to combat these destructive diseases. An organelle within the parasite, the glycosome, has been identified as an attractive drug target. The glycosome compartmentalizes several enzymes from important biosynthetic and metabolic pathways, which has been shown to be necessary for the viability of the parasite. Although the organelle is structurally and evolutionarily related to peroxisomes of higher eukaryotes, the import machinery of the organelles differs significantly. The majority of proteins entering the glycosome contain a C-terminal PTS1 tri-peptide sequence, which is readily recognized and bound by the soluble cytosolic receptor LPEX5. The receptor binds PTS1 cargo in the cytosol, shuttling it to the glycosomal membrane where the protein interacts with LPEX14, a peripherally membrane bound protein. The interaction with LPEX14 at the glycosomal membrane, facilitated by several other biogenesis proteins, initiates the formation of a transient import pore. In this thesis research project the role of *Leishmania donovani* PEX5 (LdPEX5) in formation of this crucial import pore was analyzed. Using biophysical techniques, it was found that interaction of the receptor with a PTS1 did not cause major changes in secondary structure, although did provoke a conformational change in the protein, preceding and possibly facilitating its interactions with LdPEX14 at a glycosomal membrane. Using large unilamellar vesicles mimicking the glycosomal lipid composition, the domain of LdPEX5 necessary to interact with LdPEX14 was then narrowed to 268-302. Furthermore, using serial carbonate-urea extractions, the domain identified to be necessary for interaction with LdPEX14 at a glycosomal mimetic was also found to insert into the liposomal membrane, implying that the insertion of LdPEX14 into the glycosomal membrane could be drawing LdPEX5 into the membrane as part of

pore formation. In conclusion, this study has implicated LdPEX5 in having a central role in formation of the transient glycosomal import pore.

Abrégé

Les parasites du genre *Leishmania* provoquent un large éventail de maladies, appelées collectivement leishmanioses. Ces maladies varient en termes de morbidité ; la forme cutanée se conclut généralement par une auto-guérison, alors que les manifestations cutanéo-muqueuses et viscérales nécessitent une intervention chimiothérapeutique pour éviter le décès. À l'heure actuelle, il n'existe aucun vaccin, et les méthodes actuelles d'intervention chimiothérapeutique présentent de graves conséquences. Il existe aujourd'hui un besoin urgent de trouver de nouvelles options pour lutter contre ces maladies destructrices. Un organite dans le parasite, le glycosome, a été identifié comme une cible thérapeutique intéressante. Le glycosome compartimente plusieurs enzymes de biosynthèses et voies métaboliques importantes; il a été prouvé que cet organite est nécessaire pour assurer la viabilité du parasite. Bien que l'organite soit structurellement et évolutivement lié aux peroxysomes des eucaryotes supérieurs, le mécanisme d'importation des organites diffère sensiblement. La majorité des protéines entrant dans le glycosome contient une séquence tri-peptide PTS1 C-terminal, qui est facilement reconnue et liée par le récepteur cytosolique soluble LPEX5. Le récepteur se lie au cargo PTS1 dans le cytosol, le conduisant vers la membrane glycosomale où la protéine interagit avec LPEX14, une protéine liée à la membrane périphérique. L'interaction avec LPEX14 au niveau de la membrane glycosomale, facilitée par plusieurs autres protéines de biogenèse, initie la formation d'un pore d'importation transitoire. Dans ce projet de thèse, le rôle de PEX5 dans la formation de ce pore d'importations essentiel a été analysé chez *Leishmania donovani*. En utilisant des techniques biophysiques, il a été constaté que l'interaction du récepteur avec un PTS1 n'a pas causé de changements majeurs dans la structure secondaire, bien qu'elle ait provoqué un changement de conformation de la protéine, précédant et éventuellement facilitant ses interactions avec LdPEX14 à une membrane glycosomale. Grâce à l'utilisation de grandes vésicules unilamellaires mimant la composition lipidique glycosomale, le domaine de LdPEX5 nécessaire pour interagir avec LdPEX14 fut ramené à 268-302. En outre, en utilisant des extractions carbonate-urée en série, il

a été prouvé que le domaine identifié comme étant nécessaire pour l'interaction avec LdPEX14 au mimétique glycosomal, s'insère dans la membrane liposomale. De ce fait, l'insertion de LdPEX14 dans la membrane glycosomale pourrait tirer LdPEX5 dans la membrane dans le contexte de la formation de pores. En conclusion, cette étude a démontré que LdPEX5 possède un rôle central dans la formation du pore d'importation glycosomale transitoire.

Contribution of authors

Experiments presented in this document were carried out by Amanda Davidsen under the supervision of Armando Jardim. Armando Jardim was responsible for cloning LdPEX5 and LdPEX14, as well as the mutants ldpex5(Δ1-111), ldpex5(Δ1-205), ldpex5(203-391), ldpex5(Δ181-314), ldpex5(1-391) ldpex5(303-625), ldpex5(268-625), and ldpex14(1-120), used in circular dichroism, fluorescence spectroscopy, and sucrose density flotation experiments. Normand Cyr designed the protocol for the sucrose density flotation experiments and assisted with the circular dichroism experiments. Virginie Barrere kindly translated the abstract into French. This document was prepared and initially edited by Amanda Davidsen. Consultation and further editing of this document was provided by Rona Strasser and Armando Jardim.

Claims of Originality

1. This is the first study that has reported the tendency of *Leishmania* PEX5 to insert into large unilamellar vesicles (glycosomal mimetics).
2. In this study, the segment of LdPEX5 responsible for insertion was identified.
3. Using biophysical techniques, a conformational change with no structural rearrangement was observed in LdPEX5 at the point of interaction with a PTS1.

Abbreviations used

AKL - peptide with the sequence CNDRYRDLRHILILRDGDATRYPAKL

ANS – 1-anilino-8-naphthalene sulfonate

ATP – adenosine triphosphate

CD – circular dichroism

CL – cutaneous leishmaniasis

DALYs – disability adjusted life years

DCL – diffuse cutaneous leishmaniasis

DMSO – dimethyl sulfoxide

DOPC – dioleoylphosphatidylcholine

DOPE – dioleoylphosphatidylethanolamine

DOPG – dioleoylphosphatidylglycerol

DTT – dithiothreitol

EDTA – ethylenediaminetetraacetic acid

ELISA – enzyme-linked immunosorbent assay

FRET – fluorescence resonance energy transfer

Gp63 – glycoprotein 63

gRNAs – guide ribonucleic acids

HAART – highly active anti-retroviral therapy

HIV – human immunodeficiency virus

HRP – horseradish peroxidise

IEM – immunoelectron microscopy

I-TASSER – iterative-threading/assembly/renement modelling server

kDNA – kinetoplast deoxyribonucleic acid

LPG – lipophosphoglycan

LUVs – large unilamellar vesicles

MCL – mucocutaneous leishmaniasis

mRNA – messenger ribonucleic acid

NADH – nicotinamide adenosine dinucleotide

NMR – nuclear magnetic resonance

NTA – nitrilotriacetate

NTD – neglected tropical disease

PBS – phosphate buffered saline

PDB – protein database

PEX – peroxin

PI – phosphatidylinositol

PPT – pentapeptide WXXXY/F

PTSs – peroxisomal targeting signals

RNAi – RNA interference

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SL – splice leader

TBS – tris buffered saline

TCA – tricarboxylic acid

TPR- tetratricopeptide

Tris – tris(hydroxymethyl)aminomethane

UV – ultraviolet

VL – visceral leishmaniasis

WD – tryptophan-aspartic acid

WHO – world health organization

XPRT – xanthine phosphoribosyltransferase

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Chapter 1

General Introduction

1.1 Parasitism and *Leishmania*

Leishmania parasites are the causative agents of a broad spectrum of diseases, which have been collectively termed leishmaniasis. These diseases have differing tissue tropisms and differing severities. They range from cutaneous lesions which are self-healing and usually confer protective immunity, to the devastating visceral form, in which parasites disseminate to the vital organs, resulting in fatality without treatment. The burden of these diseases are predominantly in areas inhabited by the world's poorest populations, and have a great detrimental impact on the world's 'bottom billion' [1]. Furthermore, due to socioeconomic limitations in these endemic areas and a lack of appropriate control tools, these diseases are difficult to manage and the currently reported burden levels are thought to be great underestimates. To this end, leishmaniasis, and several other parasitic diseases (Chagas disease, African sleeping sickness, lymphatic filariasis, onchoceriasis, schistosomiasis, ascariasis, and trachoma) have been deemed neglected tropical diseases (NTD) by the World Health Organization (WHO). Compounding the dire situation in these endemic countries is the lack of a vaccine and a current arsenal of chemotherapeutics with serious drawbacks, such as high toxicity, high cost, drug resistance, and the need for intravenous administration. Global elimination of these devastating diseases will require large scale financial support, integrated health care in endemic countries, and continual research and development [2].

Current leishmanial research primarily examines the differences between the parasite and the mammalian host, which could potentially be exploited for rational drug design. Some key variations under investigation are cell surface glycoconjugates, metabolic pathways, and a unique organelle called the glycosome [3]. The glycosome is of particular interest as it is structurally and evolutionarily related to peroxisomes of higher eukaryotes, while its biogenesis machinery shares very low homology with these similar organelles [4-6]. Furthermore, dissimilar to higher eukaryotes, *Leishmania* and other kinetoplastids compartmentalize the first seven enzymes of glycolysis. This

compartmentalization has been shown to be necessary for the viability of the parasite [7], making the import machinery of this organelle an attractive drug target.

In *Leishmania*, proteins destined to enter the glycosome usually contain one of two topogenic signal sequences, a peroxisomal targeting signal (PTS)-1 or PTS2. The majority of proteins entering the glycosome contain a PTS1 sequence, which is a C-terminal degenerate tri-peptide with an archetypical signal sequence serine-lysine-leucine [8, 9]. The PTS2 is an N-terminal nonapeptide with the degenerate consensus motif [RK]-[ILV]-X_5-[HQ]-[LA] [10, 11]. The PTS1 and PTS2 sequences are recognized and bound by the soluble cytosolic receptors, peroxin 5 (PEX5) and peroxin 7 (PEX7), respectively. Cargo bound receptors have been observed to form large heteromeric complexes in the cytosol [6], before docking at the peripherally bound glycosomal protein, peroxin 14 (PEX14). Studies in our lab have shown that each of LdPEX5, LdPEX7, and LdPEX14 are necessary for the viability of the parasite, as viable knock-outs of any of the proteins cannot be produced. Furthermore, in the closely related *Trypanosoma brucei*, it has been demonstrated that knocking down any of these proteins results in a lethal phenotype [12, 13]. The convergence and docking of the cargo bound receptors to LdPEX14 is the pivotal first step of glycosomal protein import. Once convened at the glycosomal membrane the cargo proteins are imported into the lumen of the organelle by a yet undefined mechanism. The theory currently in favour describes the formation of a transient import pore facilitated by the peroxin proteins [14].

This study focused on proteins of the import machinery of *Leishmania* glycosomes, specifically the role of *Leishmania donovani* peroxin 5 (LdPEX5) in the shuttling of proteins to the surface and into the lumen of the organelle. Using biophysical techniques interactions preceding and facilitating glycosomal protein import were investigated. Furthermore, important protein-protein and protein-membrane interactions were analyzed using large unilamellar liposomes mimicking the glycosomal membrane. The results of this research adds to our

understanding of several important protein-protein interactions that precede transient pore formation, and adds insight into the important role of LdPEX5 in the formation of the transient glycosomal import pore.

1.2 Research Objectives and Project Goals

The aim of this thesis research project was to further elucidate the role of LdPEX5 in the formation of a transient import pore at the glycosomal membrane. To this end, the following research objectives had been determined:

- To investigate secondary structural changes that may accompany quaternary structural changes in LdPEX5 upon interacting with a PTS1.
- To describe conformational changes in LdPEX5 that occur as a result of association with a PTS1.
- To narrow in on the domain of LdPEX5 that interacts with LdPEX14 at a glycosomal mimetic.
- To investigate the ability of LdPEX5 to insert into the membrane of a glycosomal mimetic, and if so, determine the domain necessary and sufficient to do so.

Chapter 2

Literature Review

2.1 Leishmaniasis

Leishmania species are the causative agents of a spectrum of diseases, collectively termed leishmaniasis. Discovery of the protozoan parasite was by Dr. Charles Donovan in Madras, India, investigating a supposed malarial outbreak, while Sir William Boog Leishman was researching the causative agent of the supposed outbreak in Great Britain [15]. Their work showed that the disease was not malaria but rather a new parasite of the order Kinetoplastida which was subsequently named *Leishmania*. Leishmaniasis, in all forms, currently affects 12 million individuals, and threatens 350 million people in 88 tropical and sub-tropical countries around the world [16]. The spread of the disease is not subsiding, as an estimated 1.5-2 million new cases are reported each year in endemic countries, a number which is thought to be an underestimate [17, 18]. In 2001, the global burden of the disease was estimated at 2.4 million disability adjusted life years (DALYs), and estimated to cause over 70,000 deaths per year [16]. The disease presents three distinct clinical manifestations, which are: cutaneous, mucocutaneous, and visceral leishmaniasis, each having a different range of causative species and unique clinical symptoms in humans.

2.1.1 Disease manifestations

The predominant manifestation of the disease is the cutaneous form, with an estimated 1.5 million new cases per year. The cutaneous form is endemic in over 70 countries, with 90% of the burden focused in Afghanistan, Algeria, Brazil, Pakistan, Peru, Saudi Arabia, and Syria [19]. The most common etiologic agents of this infection are *L. major* and *L. tropica* in the Old World (Africa, Asia, and Europe), and *L. mexicana* in the New World (North and South America). Transmission of the parasite to its human host is via the bite of a sandfly vector [19]. In the Old World the sandfly is of the genus *Phlebotomus*, while in the New World is of the genus *Lutzomyia*. Typical infections manifest as localized cutaneous leishmaniasis (LCL). LCL initiates as an irritation that further develops into an ulcerative wound at the site of the sandfly bite. This wound usually self-

resolves and leaves behind a disfiguring scar and protective immunity [19]. The less common manifestation of cutaneous leishmaniasis is the diffuse form (DCL). DCL is characterized by non-ulcerative nodules that disseminate from the initial site of infection and has the potential and tendency to cover large areas of the body. DCL does not self-resolve, and requires chemotherapeutic intervention to prevent death [19]. The severity and length of infection greatly depends on the parasite species, as well as immune state of the host.

Mucocutaneous leishmaniasis (MCL) is a chronic form of infection in which the parasite attacks the mucosal membranes of the head. It is predominantly found in Latin America, usually resulting from *L. braziliensis* infection [20]. Initially, the infection manifests like CL, with a self resolving lesion. An estimated 5% of these cutaneous infections reactivate months to years later and metastasize to mucosal tissues by dissemination through the lymphatic or haematogenous system [19]. MCL causes a degeneration of the tissue of the nasal and pharyngeal mucosa, leading to respiratory disturbance, difficulty eating, and extreme disfigurement of the mouth and nose. MCL associated deaths usually are due to secondary bacterial infections of the destroyed facial tissues [20].

The most severe form of the disease, visceral leishmaniasis (VL), has a reported 500,000 new infections per year, and causes the majority of deaths associated with leishmaniasis. It is estimated that the visceral form causes 50,000 deaths per year, a number that is thought to be a gross underestimate due to inaccurate reporting [21]. VL is predominantly found in Bangladesh, India, Nepal, Sudan, Ethiopia, and Brazil [22]. For the Indian sub-continent and East Africa, *L. donovani* is the causative agent, while in the Mediterranean region and the New World, *L. infantum* and *L. chagasi* are the primary causative agents [23]. The parasite incubates for 2-6 months in the host, after which it induces a persistent systemic infection, causing symptoms of fever, fatigue, weakness, loss of appetite and weight loss, as well as distinctive swelling of the liver and spleen (hepatomegaly and splenomegaly) [22].

2.1.2 Life cycle of the parasite

Leishmania species have been implicated in both zoonotic and anthroponotic transmission, with dogs being a primary reservoir in urban settings, and a variety of sylvatic mammals in the wild [24]. Of the 30 *Leishmania* species that infect a wide range of mammalian species, 21 are able to infect humans. Over 70 species of sand flies are known vectors of *Leishmania* [25], with the parasite displaying vector specificity. Many *Leishmania* species can only be transmitted by a single sandfly species [25, 26]. Throughout its lifecycle the parasite alternates between two stages, a promastigote in the insect vector, and an amastigote in the mammalian host (Fig. 2.1).

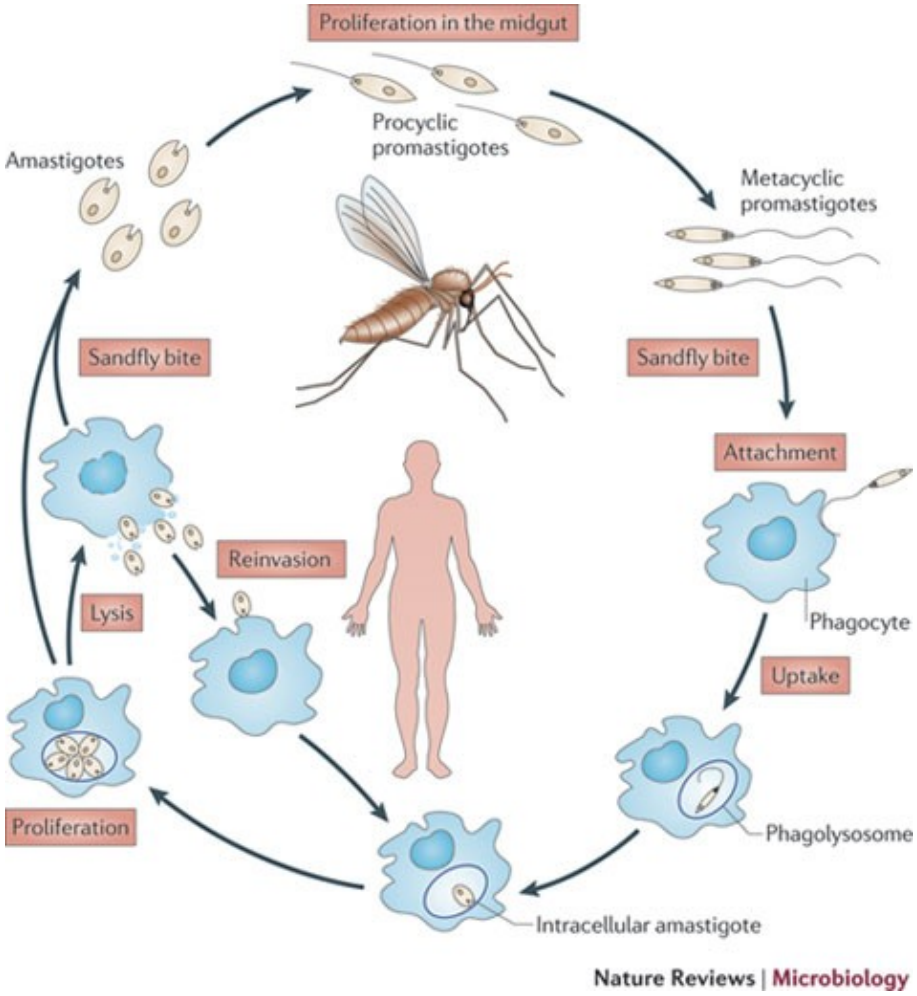


Figure 2.1 The lifecycle of *Leishmania* parasites. [27]

The life cycle begins when a female sandfly ingests macrophages infected with *Leishmania* amastigotes in a blood meal taken from an infected host. In the midgut of the sandfly the blood meal is digested and the intracellular amastigotes are triggered to transform into the promastigote stage [26, 28]. During the first five days post blood meal, the procyclic promastigotes remain in a peritrophic sac within the midgut, and undergo several developmental changes until final transformation into metacyclic promastigotes. [29]. The promastigote is able to degrade the peritrophic sac, and migrate within the sandfly to the pharyngeal valve [29, 30]. Finally, transmission back to the mammalian host occurs when the sandfly takes another blood meal and transmits the infectious promastigote parasites to a new mammalian host in regurgitated saliva.

Within the vertebrate host the metacyclic promastigotes evade the immune responses by promoting receptor mediated phagocytosis by macrophages using the surface glycoconjugate lipophosphoglycan (LPG), and the Gp63 protease as ligands for interaction with the CR1 and CR3 complement receptors on the host macrophages [31]. The interaction promotes subvertive phagocytosis of the parasite into a phagocytic vesicle within the macrophage. After internalization the parasitophorous vacuole merges with lysosomes, effectively decreasing the pH of its local environment [28]. The decrease in pH, combined with the increased temperature of the mammalian host (37ºC compared to 26ºC in the vector) triggers the differentiation of the parasite into the round, aflagellated amastigote form [32]. *In vitro* studies have found that culturing promastigotes at 37ºC in an acidic pH (5-5.5) media is enough to trigger the morphological change of *L. donovani* and *L. mexicana* promastigotes into axenic amastigotes, and vice versa to reverse the process [33].

2.1.3 Treatment options

Due to the lack of a vaccine the conventional method of treating leishmaniasis is by chemotherapeutic strategy. For the past 70 years the most effective chemotherapeutic treatments has been the use of single drug

administration of pentavalent antimonials. However, the effectiveness of these drugs has been questioned in recent years due to high host toxicity [34, 35], and the widespread emergence of drug resistance, most notably in the Indian state of Bihar [36]. Additionally, antimonials require intravenous or intramuscular injection for 20-28 days, which is costly, requires healthcare professionals, and is not an optimal option for mass drug administration in the majority of countries where leishmaniasis is endemic [37]. A summary of the current drug arsenal in use is listed in (Table 2.1) and drugs in clinical trials are listed in (Table 2.2).

In areas of high antimonial resistance, the second line drug used for treatment of leishmaniasis is amphotericin B. Originally produced as an antifungal, the macrolide polyene antibiotic currently has the highest cure rate for leishmaniasis [38]. Amphotericin B acts by targeting sterols in cell membranes where it inserts and compromises the permeability barrier of the plasma membrane. This drug shows a higher affinity for ergosterol, the major sterol in the *Leishmania* plasma membrane and consequently it has a higher specificity for these parasites over the mammalian cell plasma membranes which predominantly contain cholesterol. However, due to the similarity of these sterols, and an affinity for mammalian lipoproteins, the antibiotic has proven to have a high renal toxicity [38]. Liposomal preparations of amphotericin B have been produced with significantly reduced toxicity and shorter treatment times, but this expensive mode of treatment is not an option in the impoverished areas endemic for leishmaniasis.

The newest compound with proven efficacy is the alkylphospocholine miltefosine (hexadecylphosphocholine), which was originally produced as a treatment for cancer. Miltefosine, is currently licensed in India, Germany, and Colombia, although it is still in clinical trials elsewhere [34]. It has shown efficacy in treating *L. donovani* in both tissue culture and rodent models [38], as well as in clinical trials. In a phase IV trial in India miltefosine demonstrated an 95% cure rate for visceral leishmaniasis [39]. Being that miltefosine is an oral treatment makes it an attractive option, however, it is excessively expensive, there

has been proven reproductive toxicity with its use, and resistant parasite strains have already been developed *in vitro* [40]. Clearly, additional new classes of anti-leishmanials with increased parasite specificity and reduced side effects are desperately needed.

Table 2.1 Current treatment options for leishmaniasis, specific manifestation of disease indicated. Adapted from [22, 23, 35, 38, 41]

Treatment	Advantageous Factors	Detrimental Factors	Disease Treated
Pentavalent Antimonials (*sodium stibogluconate, meglumine antimoniate*)	Affordable; effective	Toxicity; occasional cardiac arrhythmia; occasional acute pancreatitis; slow drug action; intravenous or intramuscular injection	Cutaneous; Mucocutaneous; Visceral
Amphotericin B	High efficacy	Intravenous or intramuscular injection; high cost, toxicity; anemia; fever; nausea; vomiting; hypokalemia; nephrotoxicity	Cutaneous; Mucocutaneous; Visceral
Liposomal amphotericin B	Reduced toxicity (*compared with non liposomal form);* High efficacy	Extremely expensive, inactive against CL and MCL; intravenous or intramuscular injection	Visceral
Miltefosine *(Registered in India, Colombia, and Germany; Phase III in Brazil and Guatemala)*	Oral dosage	Very expensive; teratogen; nausea; vomiting; diarrhea; hyperglycaemia; cardiotoxicity; raised creatinine; raised LFTs	Cutaneous; Visceral
Pentamidine	Affordable, effective	Toxicity to liver, kidneys, adrenal glands, and spleen; intravenous or intramuscular injection	Cutaneous; Visceral

Table 2.2 Treatment options for leishmaniasis currently in clinical trial, specific manifestation of disease indicated. Adapted from [22, 35, 38, 42]

Treatment	Advantageous Factors	Detrimental Factors	Disease Treated
Paramomycin *(Phase III with and without gentamicin)*	Affordable; effective	Nausea; abdominal pain; toxicity; vomiting; diarrhea; diabetes mellitus	Cutaneous
Sitamaquine *(Phase II)*	Oral dosage; efficacy	Renal toxicity	Visceral
Amphotericin B *(other preparations)*	Unknown	Unknown	Visceral

2.1.4 An important emerging disease (HIV co-infections)

The onset of the AIDS pandemic over the last 25 years has altered the spread and clinical aspects of leishmaniasis in mutually endemic regions [43, 44]. Co-infections of visceral leishmaniasis with HIV increases the parasite load in the blood and bone marrow, affecting the severity of the disease, and can reduce the accuracy of diagnostic serological tests [22]. Additionally, co-infection is detrimental to both HIV and leishmaniasis treatment regimes, increasing treatment failure in both and the potential for leishmaniasis relapse [45]. Co-infection has been reported in 35 countries, in people of low socio-economic standing [45]. Transmission of the co-infection has been strongly linked to intravenous drug use, as the parasite has been discovered in dried blood within used syringes [46], although transmission is also believed to occur via blood transfusions, congenital transmission and laboratory infections [45]. The use of highly active anti-retroviral therapy (HAART) has been shown to improve the response to treatment, but this expensive treatment is not an option in impoverished countries where the predominance of co-infection is seen [47].

2.2 Overview of kinetoplastid biology

Leishmania belongs to the order Kinetoplastida, organisms which are thought to have diverged early from the eukaryotic lineage. This order can be divided into two groups, Bodonida and Trypanosomatida. Organisms of the former are bi-flagellated and distinguished by the presence of a large kinetoplast; while the latter organisms have a single flagella and a smaller kinetoplast [48]. Bodonida are classified as free living or parasites of fish or snails. Trypanosomatida are strictly parasitic, being found in invertebrates, vertebrates, and plants. The single family of Trypanosomatidae contains the human parasites *Trypanosoma brucei*, causative agent of African sleeping sickness; *Trypanosoma cruzi*, causative agent of Chagas disease, and the *Leishmania* species [49]. The kinetoplastids exhibit several unique biological features that differentiate them from other eukaryotes. These organisms contain: a rare mitochondrion for which

they are named, the kinetoplast, which requires unique mRNA editing; transcripts of polycistronic mitochondrial RNA which require trans-splicing to generate monocistronic mRNA; and a novel organelle that compartmentalizes energy and carbohydrate metabolism, the glycosome.

Leishmania species contain a single mitochondrion with a dense matrix that branches into long slender tubules. The mitochondrial DNA (kDNA) forms a dense bar-shaped structure called the kinetoplast, enclosed within the mitochondrion near the base of the flagella. This single kDNA network accounts for ~30% of the total DNA content of these parasites and is composed of interlocking relaxed DNA in covalently closed plasmid like elements. These DNA structures consist of minicircles ~0.5-2.5 kb, of which there are several thousand, and the less abundant maxicircles, at a few dozen per cell, ranging from 20-40 kb. The maxicircles are identical in sequence, and are analogous to the higher eukaryotic mitochondrial DNA. However, these transcripts are non-functional without extensive post-translational editing to create complete and biologically active open reading frames [50, 51]. This is accomplished through the minicircles, which derive from hundreds of sequence classes, and encode guide RNAs (gRNAs), according to which this editing is done. The maxicircle editing begins with the insertion and deletion of uridylates (U's) by an enzyme cascade mechanism [52, 53]. Briefly, the gRNAs bind to the pre-edited maxicircle transcript, cleaving at the first mismatch site with an endonuclease, producing a 3' and 5' cleavage product. A uridine is then added to or removed from the 3' end of the 5' cleavage product, which is then reattached using a ligase powered by ATP hydrolysis [54]. The final step in mRNA processing is trans-splicing of the modified mRNA to convert polycistronic pre-mRNAs into monocistronic mRNAS. This requires two cleavages within intergenic regions, the first cleavage of a capped 39 nucleotide spliced leader (SL) at the 5' end, and the second 1kb upstream of the trans-splice acceptor site. The former creates the 5' cap 4 of the mature mRNA, necessary for initiation of translation, while the latter plays a role in polyadenylation, important for protection from degradation. The two processes act as a coupled event due to the polycistronic nature of the transcripts. Together

the cleavages form monocistronic mRNA containing a cap 4 structure at the 5' end and a poly(A) tail at the 3' end [55-57].

2.3 Glycosomes in trypanosomes

The glycosome is a microbody organelle unique to the kinetoplastids, and is evolutionarily related to peroxisomes in higher eukaryotes, glyoxysomes in plants, and Woronin-bodies of filamentous fungi [58-60]. In 1977, after the discovery of the peroxisome in mouse kidney cells [61], Opperdoes and Borst first described the glycosome in the bloodstream form of *Trypanosoma brucei* [62]. It was thus named due to the distinctive compartmentalization of several enzymes of the glycolytic pathway. The pathways used for the import of proteins into the glycosome share similarities with import pathways described for peroxisomes [62, 63], however, the glycosome of the medically important kinetoplastids lack the hallmark eukaryotic peroxisomal enzyme catalase. This enzyme has, however, been detected in the glycosome of the insect trypanosomes *Crithidia* spp. and *Leptomonas samueli* [64, 65]. Glycosomes are present in all members of the Trypanosomatidae family, and are known to have an electron dense matrix bound by a single phospholipid bilayer. These microbodies have a round or oval morphology and can be between 0.2 to 0.8 μm in diameter [66]. Glycosomes compartmentalize enzymes of the glycolytic pathway, as well as enzymes of glycerol metabolism [67], gluconeogenesis [68], β-oxidation of fatty acids pathway [69, 70], fatty acid elongation, biosynthesis of ether lipids [67], pentose phosphate pathway [71, 72], and are involved in isoprenoid synthesis [73] and oxidant stress protection [74].

2.3.1 Evolution of the Glycosome

Since the first description of glycosomes and their ability to compartmentalize many metabolically important enzymes there has been a great deal of debate regarding their origin [62, 75]. An initial hypothesis described the glycosome as deriving from a bacterial endosymbiont, similar to chloroplasts or

mitochondria, whose DNA was transferred to the nucleus where the targeting signals would have been later added [48]. This theory supported the compartmentalization of enzymatic pathways in the absence of glycosomal DNA, and replication of the glycosome, since a bacterial endosymbiont would have the necessary machinery to do so already. However, failure to find genes of prokaryotic lineage in the nucleus, together with biogenesis studies suggesting a monophyletic relationship between glycosomes, peroxisomes, glyoxysomes, and microbodies have decreased the appeal of the bacterial endosymbiont theory. Currently, the accepted theory is that glycosomes originated within a primitive phototrophic eukaryote, which then became endosymbiotic, giving rise to differing forms of the organelle [48]. Evidence for this hypothesis has increased rapidly due in part to the sequencing of several Trypanosomatid genomes and the discovery of many genes of a phototrophic eukaryote lineage [48, 76]. Although some of the resultant proteins have been found to associate with other organelles, several of these genes encode proteins of the pentose phosphate and glycolytic pathways, and posses a peroxisomal targeting signal [48]. Likely this endosymbiont was acquired by an ancestral Euglenozoan organism, before Kinetoplastida evolutionarily split away from the lineage (Fig. 2.2). Further adaptation of the glycosome likely was a result of horizontal gene transfer with bacteria and viruses within the insect intermediate [76].

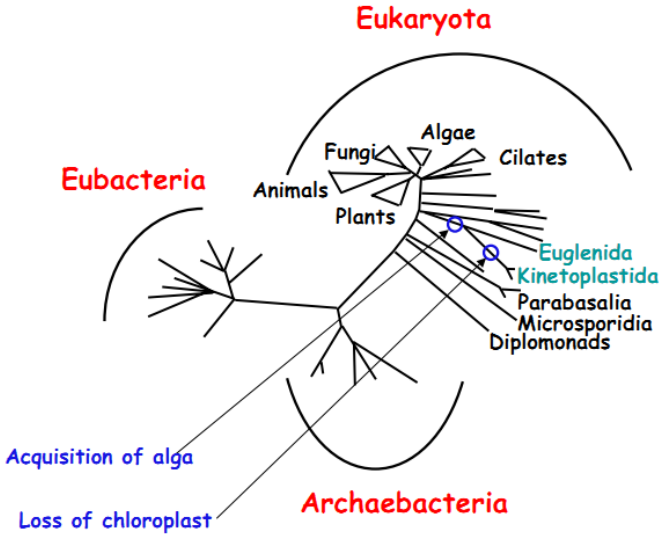


Figure 2.2 Modified tree of life, showing the theoretical point of acquisition of an algae endosymbiont before diverging from Euglenida, and subsequent loss of chloroplast after divergence. Adapted from [48].

2.3.2 Glycolysis and compartmentalization of enzymatic pathways in Kinetoplastids

Since the discovery of the compartmentalization of glycolysis in Trypanosomatidae there has been research into its role, and its implication in parasite viability. In the bloodstream form of *T. brucei*, the parasite is in glucose rich environment, which provides a primary carbon source for making ATP/NADH. Typical mitochondrion cellular processes for ATP production such as the citric acid cycle and the electron transport chain are shut down during this life stage and parasites rely on glycolysis for ~90% of the total energy production [77, 78]. Although glycolysis plays a paramount role in *T. brucei*, its role in *Leishmania* does not seem to be as crucial, since these organisms are proposed to use proline as an alternative carbon source [79]. Within the gut of the sandfly *Leishmania* promastigotes are engulfed in a blood meal rich in glucose. Once in the mammalian host the amastigote form resides in host macrophages within phagolysosomes, an environment where glucose is much lower in concentration, and therefore may explain why amastigotes primarily rely on β-oxidation of fatty acids for their energy needs [80-82]. Trypanosomatidae class members all compartmentalize the first seven enzymes of glycolysis, with the final three remaining enzymes found in the cytosol (phosphoglycerate mutase, enolase, and pyruvate kinase).

The role of compartmentalization of the various metabolically significant pathways is believed to be necessary for cell viability. In most eukaryotic organisms there is regulation of the two ATP consuming steps of glycolysis, those catalysed by hexokinase and phosphofructokinase, by feedback inhibition [78]. The trypanosome enzymes, however, lack this feedback inhibition and rely on glycosomal compartmentalization of glycolytic enzymes as an alternative to allosteric regulation [7, 83]. This prevents accumulation of toxic glycolytic intermediates in the cytosol, and unnecessary depletion of substrate due to futile pathway cycling. This lack of feedback inhibition is called a ‘turbo design’, where consumption of ATP is independent of downstream activities [78]. This lack of

regulation would be toxic to the parasite in the cytosol, hence the need to compartmentalize and control levels of lethal metabolic intermediates. Computational modeling found that without compartmentalization sugar phosphate levels in the parasite would become uncontrollable and the parasite would be unable to recover from glucose deprivation [84]. Thus, compartmentalization is thought to be important for regulating the ratios of ATP/ADP and NAD+/NADH within the glycosome.

2.4 Targeting and import of proteins to the glycosome

Glycosomes lack DNA or protein translation machinery; thus, proteins destined for the glycosome require signaling and import machinery to get proteins into this microbody organelle [12]. Targeting proteins to the glycosomes, and indeed to peroxisomes of higher eukaryotes, is accomplished primarily by two peroxisomal targeting signals (PTSs), the PTS1 and PTS2 [63, 85]. Proteins are translated in the cytosol on free ribosomes from nuclear encoded mRNA and imported post-translationally into the glycosome [12, 86].

The PTS1 signal peptide is located at the carboxy terminus of glycosomal proteins. *In silico* analysis of the PTS1 peptide showed a conserved degenerate tripeptide sequence containing serine, lysine, valine, alanine, cysteine, glycine, proline, asparagine, or threonine at the first position; lysine, serine, histidine, glutamate, aspartate, asparagine, or arginine at the second; and leucine, lysine, methionine, valine, isoleucine, or alanine at the third [8, 74]. Additionally, the sequence adjacent to the tripeptide has been demonstrated to occasionally play a role in proper recognition [74]. An *in silico* prediction model in *L. major* identified a potential of 191 PTS1 containing proteins, many which had homologues in *T. brucei* and *T. cruzi* [74].

Less frequently, proteins are targeted to the glycosomal matrix by the PTS2 topogenic signal sequence. This N-terminal topogenic sequence was identified in rat liver 3-ketoacyl CoA thiolase [10], and consists of an N-terminal conserved nonapeptide R-(L/V/I/Q)-xx-(L/V/I/H)-(L/S/G/A)-x-(H/Q)-(L/A) (Fig.

2.3) [87]. *In silico* analysis predicted 68 PTS2 containing proteins in the *L. major* genome, many of which had homologues in *T. brucei* and *T. cruzi* [74].

Although most proteins destined for the glycosomal matrix have a PTS signal, some lack a canonical targeting signal and are imported via other means. In higher eukaryotes some proteins contain non-conserved internal peroxisome targeting signals, while others "piggyback" into the peroxisome by interacting with PTS containing proteins [12, 63], since the peroxisomal membrane allows for the passage of folded and even oligomeric proteins. Additionally, some proteins are able to enter the peroxisomal matrix by interacting with cytosolic receptors via internal signals, or 'hot spots', such as the alcohol oxidase and castor bean isocitrate lyase of *H. polymorpha*, and acyl-CoA oxidase (which have two known internal signaling regions) of *S. cervisiae,* and *Y. lipolytica* [88, 89]. In trypanosomatids, phosphoglycerate kinase and triosephosphate isomerase of *T. brucei* both contain poorly defined internal PTS signals [90].

Upon exiting the cytosolic ribosomes the PTS1 or PTS2 containing proteins interact with their respective receptors Peroxin 5 or Peroxin 7 (PEX5 or PEX7). These two pathways are believed to converge at the PEX14 protein, a glycosomal peripheral membrane protein [91].

2.4.1 PTS1 targeting in trypanosomes via peroxin 5 (PEX5)

After synthesis on cytosolic ribosomes PTS1 proteins are rapidly bound by PEX5, for transport to the glycosomal membrane. Homologues of the *Leishmania* PEX5 protein exist in *Trypanosoma*, as well as mammals, plants, and yeast [63, 92]. The first homolog to be discovered was PAS8 in the yeast *Pichia pastoris*, identified by mislocalization of the PTS1 proteins catalase, methanol oxidase, and dihydroxyacetone synthase to the cytosol using a non-functional mutation in the *PAS8* gene [93]. Similarly, studies using RNA interference to deplete PEX5 in *T. brucei* displayed mistargeting of PTS1 proteins to the cytosol and death of the parasite upon loss of PEX5, revealing the importance of this receptor in trypanosomatid systems [12].

PEX5 has several interaction domains. The PTS2 receptor, PEX7, interacts with PEX5 between residues 111-148 [6]. Likewise, the region necessary for association with PEX14 has been mapped to residues 290-323 [94]. The highly conserved C-terminal of PEX5 contains seven tetratricopeptide repeats (TPR), localized between residues 324 and 588 in *L. donovani*, which form a pocket that interacts with the PTS1 peptide sequence. Each TPR domain is composed of 34 amino acids arranged in an α-helix-turn-α-helix configuration [95]. The first three TPR domains are arranged together in a fold. Similarly, TPR 5-7 are arranged in a three domain fold, while TPR 4 acts as a hinge between the two folds, adopting a continuous α-helix [95]. Recognition of the PTS1 signal is facilitated by a binding pockets at the interaction site of the receptor, and is mediated by a set of Asn residues located in the first helix of TPR6 (TPR6α1), TPR7α1, and a loop of TPR3 [95]. Binding of this degenerate PTS1 signal by cargo proteins is further facilitated by binding association with water molecules [96]. Crystal structures of the seven TPR motifs of TbPEX5 interacting with several PTS1 peptides were elucidated, confirming the localization of the PTS1 binding region on PEX5 and significant residues for binding [97]. The N-terminal region of PEX5 does not share the same conservation across species, aside from several pentapeptide (PPT) WXXXY/F domains, which differ in abundance and location within the proteins. In *L. donovani* PEX5 there are 3 PPT domains, while mammalian PEX5 have seven PPT domains. In mammalian cells and the closely related *T. brucei*, these aromatic domains are necessary for PEX5 interaction with the docking proteins PEX14 and PEX13 [98, 99]. In *Leishmania*, however, WXXXY/F domains do not seem to be significant for LdPEX5-LdPEX14 interactions, as site-directed mutagenesis of these aromatic motifs did not ablate LdPEX5-LdPEX14 binding [94]. Taking into consideration the differences in PPT functionality it is not surprising that bioinformatic analysis has revealed that *L. donovani* shares low sequence homology with PEX5 in mammals and yeast, although still retaining major common structural elements.

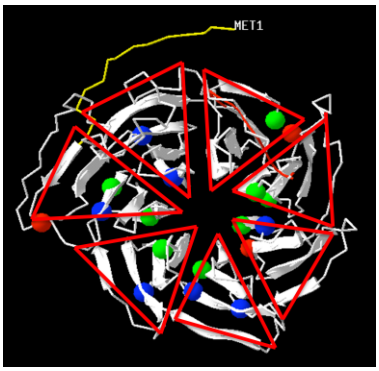
Unique to mammalian cells, PEX5 exists in two isoforms due to alternative splicing, a long form (PEX5L) containing a 37 amino acid insertion,

and a short form (PEX5S) [100]. These isoforms are believed to exist as homotetramers in solution, similar to oligomerization of PEX5 in kinetoplasts [98]. The long isoform, PEX5L, is required for PTS2 protein import and mediates the interaction with PEX7 in the process [100]. Furthermore, in rescue studies it has been shown that PEX5L and PEX5S are able to recover PTS1 import, while only PEX5L is able to recover PTS2 import [101]. In kinetoplasts, however, no PEX5L has been discovered, and it has been demonstrated that PEX7 is able to interact with PEX5 and PEX14 directly [4, 6, 102]. In the mammalian system it has been shown that PEX5 competes with PEX19 for binding to the N-terminal of PEX14, exposed at the peroxisomal membrane [103]. If a similar competition for binding exists in *Leishmania* remains to be determined.

In the absence of PTS1 proteins, the soluble 625 amino acid, 69.7kDa LdPEX5 forms a homotetramer, composed of a dimer of dimers. On binding a PTS1 with cargo there is a conformational change and dissociation of LdPEX5 resulting in a single dimer of LdPEX5 [4]. In this conformation LdPEX5 is believed to shuttle PTS1 proteins to the glycosomal membrane, where an LdPEX5-LdPEX14 interaction occurs prior to translocation of the cargo proteins across the membrane. It has been demonstrated that when LdPEX5-PTS1 interacts with LdPEX14 there is a marked decrease in LdPEX5 affinity for the PTS1 cargo, implying the release of PTS cargo proteins promotes transport into the lumen of the glycosome [104]. Confocal microscopy studies and subcellular fractionation experiments have revealed that indeed LdPEX5 is predominantly found in the cytosol [4]. This is not the case in all systems, however, as in yeast PEX5 was shown to dissociate from its PTS1 within the peroxisome after association with PEX8 [105]. Although the exact mode of translocation is disputed in both mammals and *Leishmania* it is known that an importomer complex is formed at the glycosomal surface. This complex involves PEX5, PEX14, PEX7, PTS1 proteins, and the RING finger proteins (PEX2, PEX10, PEX12), as well as PEX13 [98, 99]. The PEX13 in trypanosomatids shares low sequence identity with others, although it has been shown to be essential for viability of the parasite [106].

2.4.2 PTS2 targeting in trypanosomes via peroxin 7 (LPEX7)

Like PTS1 proteins, PTS2 proteins are translated on cytosolic ribosomes, where they interact with the soluble PTS2 receptor PEX7 for transport to the glycosomal membrane. PEX7 belongs to the family of WD40-repeat proteins, defined as having a common β-propeller structure formed by 30-40 amino acid sequence repeats that terminate with a tryptophan-aspartic acid dipeptide [107]. In *Leishmania*, it has been found that LPEX7 possesses five WD40 repeats, with conserved consensus sequences 40 amino acids long [6]. Although no crystal or NMR structure has been elucidated, an *in silico* model of PEX7 was generated using the I-TASSER server, showing a seven β-propeller blade arrangement typical of WD40 proteins (Fig. 2.3). The torroid structure creates a central channel possessing hydrophobic residues towards its interior and hydrophilic residues towards its exterior [108]. In vitro, however, *Leishmania* PEX7 has been observed to have numerous hydrophobic amino acids on its surface, causing purification of the recombinant protein to be problematic. Initial attempts to purify the protein generated PEX7 which was co-purified with the bacterial chaperone protein GroEL [6]. Only recently has a purification method been produced that overcomes this issue [108]. Proteins analogous to *Leishmania* PEX7 exist in other organisms containing peroxisomes or glyoxysomes, although sequence homology between the WD-40 proteins is very low [6].

(A) 

(B) 

Figure 2.3 *In silico* model of *L. major* peroxin 7 protein produced using the I-TASSER server of predicted secondary structure, further manipulated using the Swiss PdB software. The seven β-propeller structure, made of seven-strands of WD40 repeats, are outlined in red (A). A side view of predicted structure can be seen in (B) (Adapted from [108]).

After transporting the PTS2 containing protein to the glycosomal membrane PEX7 has been demonstrated to interact with both PEX14 and PEX5 via its N-terminus [108]. Based on localization studies, it's thought that, through a mechanism yet to be elucidated, the receptor cycles into the lumen of the glycosome, delivering its cargo, before recycling back to the cytosol (Fig. 2.4) [12, 109]. *Leishmania* PEX7 interaction assays have demonstrated that LPEX7 can associate directly with LPEX14, both in the presence and absence of LPEX5 or a PTS2 protein [6]. However, in higher eukaryotes the PEX7-PEX14 complex is known to require the PTS2 protein as well as several associated peroxin proteins, such as PEX18 and PEX21 in *S. cerevisiae* [110, 111].

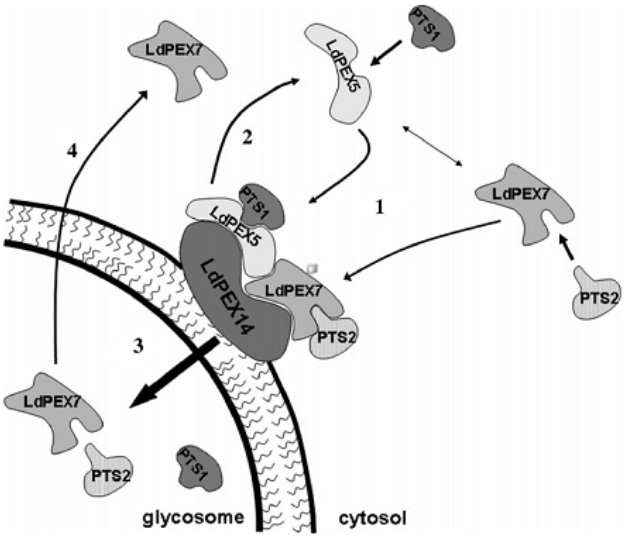


Figure 2.4 Proposed model for PTS1 and PTS2 import in *L. donovani*. PTS1 and PTS2 proteins are recruited by LdPEX5 and LdPEX7, respectively, in the cytosol, and are shuttled to the glycosomal surface. At the glycosomal surface LdPEX5-PTS1 and LdPEX7-PTS2 interact with each other and LdPEX14, which is sitting peripheral to the membrane. Upon interaction, LdPEX14 experiences a conformational change, resulting in a decrease in affinity of LdPEX5 for PTS1, and LdPEX7-PTS2 for LdPEX14. The PTS1 protein and LdPEX7-PTS2 are translocated into the glycosome [6].

2.4.3 The role of peroxin 14 (PEX14) in glycosomal protein targeting in trypanosomes

The convergence point for the PTS1 and PTS2 targeted protein trafficking pathways is peroxin 14 (PEX14). PEX14 plays an important role in both protein docking at the glycosomal membrane and trafficking into the glycosomal lumen [91]. Mapping studies have revealed that LdPEX14 contains a LdPEX5 binding domain in the conserved N-terminal region between residues 23-63 [94], and a LPEX7 binding domain in a proline rich region between residues 120-148 [6]. Downstream of the LPEX7 binding domain between residues 148-179 is a hydrophobic domain, shown to play a role in membrane interaction [112], and a leucine zipper motif near the C-terminus between residues 270-321, implicated in homo-oligomerization [113]. In mammalian cells it has been demonstrated that PEX14 acts as an integral membrane protein. Treating this membrane associated

PEX14 with increasing strength of ionic buffers was not sufficient to extract PEX14 from the membrane. When using protease treatments of PEX14 with peroxisomes it was found that although the cytosolically located C-terminus suffered degradation, the N-terminus survived proteolysis, likely due to association with the membrane [114, 115]. In contrast, *Leishmania* PEX14 behaves as a peripheral membrane protein when in the absence of other importomer complex members [5]. Membrane associated LdPEX14 has been shown to be resistant to alkaline carbonate extraction, as is expected for integral membrane proteins, although after urea and Triton-X114 treatments LdPEX14 was found in the supernatant, indicative of a peripheral membrane protein [116]. Protein sequence alignments of *Leishmania* PEX14 with human, *P. pastoris*, and *S. cereviseae* revealed that this protein shared only 16-20% sequence identity and 33-43% sequence similarity [5]. In mammalian cells PEX14 forms homo-oligomeric structures via the hydrophobic domain and the coiled-coil motif [117], while in *Leishmania* LdPEX14 has been shown to form similar structures via the leucine zipper [113].

It has been theorized that PEX14 is involved in the formation of a putative pore that allows folded PTS1 and PTS2 proteins to be transported into the glycosome. *In vitro*, the interaction of LdPEX5 and LdPEX14 causes conformational changes in LdPEX14 which reveals hydrophobic residues, adding support to the possibility of transient pore formation [113, 118]. Furthermore, interaction of the LdPEX5-PTS1 and LPEX7-PTS2 complexes with each other and with LdPEX14 results in alteration of binding affinities. In *T. brucei*, RNA interference (RNAi) knock down of TbPEX5 and TbPEX7 receptors resulted in death of the parasite [12], revealing the necessity of proper translocation for parasite survival. Similar studies of TbPEX14 resulted in death of the parasite in glucose containing media, as well as mislocalization of the glycolytic enzymes hexokinase and phosphofructokinase, implying the significance of PEX14 in glycosomal targeting [13]. Similar studies cannot be undertaken in *T. cruzi, L. major,* or *L. donovani* as these species lack RNAi activities [119, 120]. Finally, viable parasites with knocked out LdPEX7, LdPEX5, and LdPEX14 are unable to

be produced, implying the necessity of each protein for survival of the parasite *(Jardim Lab, unpublished)*.

2.4.4 The transient pore model for glycosomal protein import

Proteins destined for the peroxisomal or glycosomal matrix are unique in their ability to traverse the membrane as large proteins, or in a folded or oligomeric conformation. There are theories of how this feat is accomplished, such as invagination of the membrane followed by vesicle release within the matrix, although current models of peroxisomal protein import include formation of an importomer complex and a transient pore [14]. The model of transient pore formation describes that a cargo-loaded import receptor will be able to associate with and insert into the membrane, becoming integral as part of the translocation machinery. This shift in translocation machinery will trigger a cascade of protein-protein interactions at the membrane that leads to ubiquitinylation and ATP dependant dissociation of the receptor from the peroxisomal membrane to the cytosol [118]. For this model the translocation pore must be composed of an integral membrane protein, as least temporarily, which will be able to associate with cargo proteins, and regulatory proteins, for the rapid disassembly of the pore. Additionally, these proteins should form large complexes to produce channels large enough for folded or oligomeric proteins, while not being so large as to permit the exchange of solutes [118]. Receptor recycling is necessary to facilitate another round of import, and to maintain the permeability barrier of the membrane, which would be compromised by a permanent pore. Elaborate mechanoenzymes are thought to be required to disassemble the pore and dislocate the receptors. In humans it is thought that Pex5-PTS1 and Pex7-PTS2 converge in the cytosol before shuttling cargo to the membrane, interacting with Pex14 and Pex13, thus forming a translocating channel composed of Pex5-Pex14. This channel is believed to be conjugated to export machinery consisting of the apparatus for receptor ubiquitinylation (Pex2, Pex10, and Pex12) and the AAA-ATPase complex (Pex1 and Pex6), which is attached to the membrane by Pex26 and is thought to be required for the release of the receptor from the membrane

[118, 121]. The transiently integral membrane protein is thought to be Pex5p; as suggested by biochemical treatment and protease protection assays which showed that a portion of the protein behaves as an intrinsic membrane protein. In addition, it has been demonstrated that Pex5p in association with Pex14p forms a dynamic gated ion-conducting channel that has been shown to fluctuate between 0.6 – 9 nm in diameter [122]. However, while some of these peroxin proteins have homologues in *Leishmania*, there are several that have not yet been identified. Therefore, it is reasonable to assume that there are differences in the exact import mechanism between *Leishmania* and higher eukaryotes. For *Leishmania*, current *in vitro* experiments using recombinant LdPEX5 and LdPEX14 in association with liposomes are in agreement with human proteins, depicting the ability of LdPEX5 to reversibly act as an integral membrane protein (*Jardim Lab, unpublished*).

2.5 Interactions of proteins with lipid bilayers

To traverse the membrane of an organelle a protein must form an interaction with the membrane. The membrane of glycosomes, and related organelles, is in the form of a lipid bilayer. This form is favoured in aqueous media due to the amphipathic nature of phospholipids and the physical constraint of two fatty acid tails [123]. The formation of such bilayers is driven by hydrophobic interactions, stabilized at the hydrocarbon tails by van der Waal forces, and further stabilized at the polar head groups by electrostatic and hydrogen bonding. Proteins interacting with lipid membranes can be categorized as either peripheral or integral, depending on the nature of their interaction with the membrane and the ease of their dissociation [124, 125]. In *Leishmania*, LdPEX14 has been shown to make interactions with phospholipids of the glycosomal membrane that are not solely electrostatic. The LdPEX14 protein showed increased binding with higher temperatures, and has been shown to act as peripheral membrane protein [5]. This is in contrast with the mammalian form of the protein, which has been shown to act as an integral membrane protein [114, 115]. Regions of proteins that traverse the membrane are rich in non-polar amino

acids and favour interaction with the hydrophobic core of the lipid bilayer, while soluble regions of proteins rich in hydrophilic amino acids interact with the aqueous environment. Regions of proteins that span the membrane have distinct sequences that may be transmembrane helices or beta-barrels, and can usually be predicted from the primary amino acid sequence by analyzing a hydropathy plot of free energy change, looking for peaks indicative of such a domain [126].

It has been shown that LdPEX5 is able to interact with membranes when in the presence of LdPEX14 [112]. When analyzing the primary sequence of LdPEX5, however, it can be seen that there are no regions obviously acting as a transmembrane domain(s) (Fig. 2.5). Therefore, part of this study was further analyzing the protein-membrane relationship of LdPEX5 with liposomes to better understand what type of interaction was being made and how this interaction may play into the bigger picture of glycosomal protein import. Although not evident in the hydropathy plot, the possibility that this protein interacts with membranes via an amphipathic domain cannot be discounted.

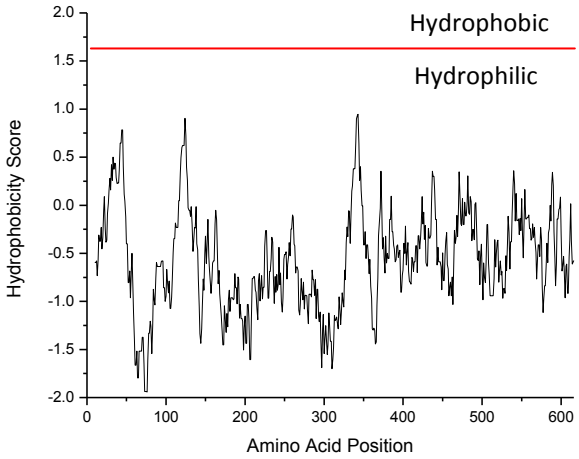


Figure 2.5 Hydropathy plot of the primary sequence of *L. donovani* peroxin 5 [127]. The window size was 19, and the threshold is set at 1.6 (displayed as a red line separating hydrophobic from hydrophilic sequences). The LdPEX5 protein does not have any obvious transmembrane domain(s).

2.6 Spectroscopic techniques used to investigate protein-protein interactions

2.6.1 Circular Dichroism

Circular dichroism (CD) is spectroscopic tool that assists in the determination of protein secondary structure, as well as conformational changes to secondary structure [128]. CD is a method of analyzing the differential absorption of left and right circularly polarized light. The ability for selective absorption is a product of necessary chirality in both the light wave and the protein sample. Plane polarized light has an electric field in one dimension depending on a magnetic field oscillating in a single plane. Circularly polarized light, however, has an electric field that turns about the direction of propagation, with a magnetic field moving at a right angle to the changing electric field, creating a circular light wave. If viewed from the front, right, and left, circularly polarized light would trace out circles, one rotating clockwise, and the other counterclockwise, respectively. When colliding with a sample this light plane will be distorted and become elliptically polarized light, striking a detector which measures the angle change in millidegrees, thus recording this change in ellipticity as spectra [128].

Intrinsic protein CD, which is measured by internal chromophores, can be specifically targeted by wavelengths in the far UV range [129]. In the far UV (below 250nm) the electronic transitions are dominated by the amide chromophore, making this wavelength range appropriate for protein backbone analysis. Transition moments originating from different amide chromophores interact to produce CDs indicative of the geometric relationships between them. Depending on the composition of secondary structure and special arrangements of amides the CD spectrum will vary [129]. Some of the expected peaks at corresponding wavelengths are depicted figuratively below (Fig. 2.6), and tabulated below (Table 2.3).

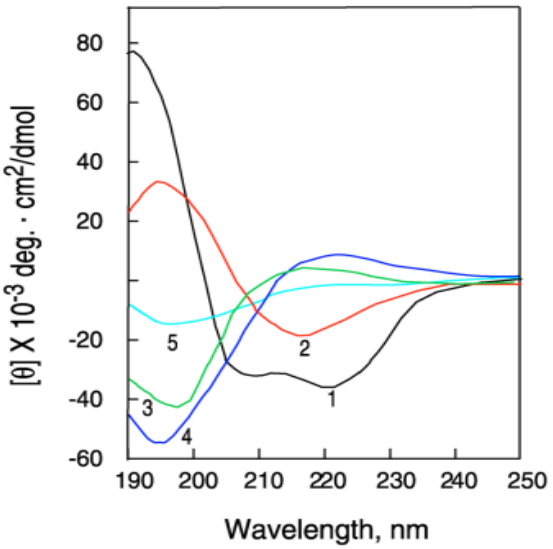


Figure 2.6 A CD spectra of polypeptides and proteins that have representative secondary structures; a CD spectra of a typical α-helical conformation (1), β-sheet conformation (2), disordered conformation (3), native triple helical (4), and denatured forms (5) [128].

Table 2.3 Structural elements of characteristic CD spectra [128]

Secondary Structure	Peak Orientation	Wavelength (nm)
α-helix	Positive	190-195
	Negative	208
	Negative	222
β-sheet	Positive	195-200
	Negative	215-220
Random	Negative	200
	Positive	220

2.6.2 Fluorescence Spectroscopy

Fluorescence spectroscopy is a type of electromagnetic spectroscopy which facilitates analysis of intrinsic or extrinsic sample fluorescence. In fluorescence, excitation of electrons by a beam of light causes the emission of light at a longer wavelength. This emission of light is triggered by an initial excitation of the electron causing it to move from its ground electronic state to a vibrational level of an excited electronic state. Here the electron falls down through several vibrational states, until it reaches the lowest vibrational level of the excited state, and then drops to a vibrational level in the ground state, emiting a photon (Fig. 2.7) [130].

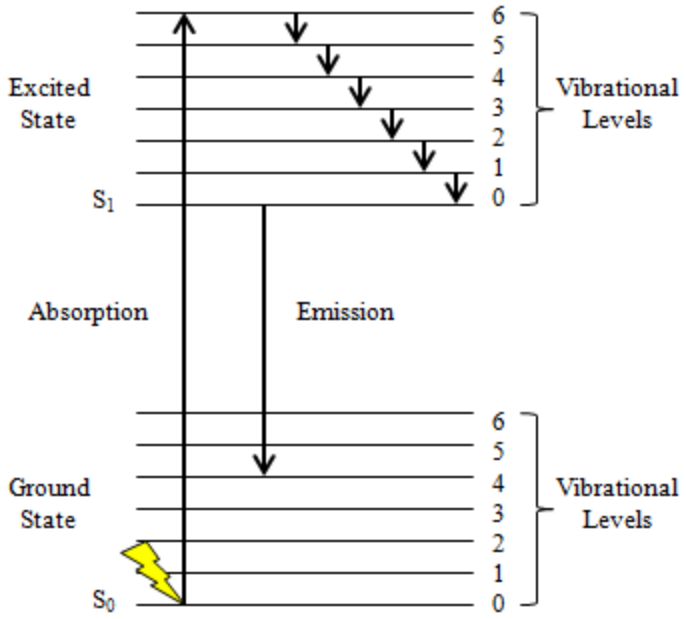


Figure 2.7 Energy-level diagram illustrating the excitation of an electron from its ground state to an excited state, and the fluorescence emission observed when this electron drops through several vibrational states to finally reside in a vibrational level of the ground state.

Intrinsic protein fluorescence measures the fluorescence emission of amino acids within the protein of interest. These three naturally occurring fluorescent amino acids are phenylalanine (F), tyrosine (Y), and tryptophan (W), due to the resonance of their aromatic functional group. The amino acids can be specifically analyzed due to differing excitation and emission wavelengths. The quantum yield of phenylalanine is very low, causing the amino acid to be rarely used in fluorescence analysis, and although all aromatic, the emission from tryptophan and tyrosine is far stronger than that of phenylalanine [130]. In this study tryptophan fluorescence will primarily be used for analysis. The fluorescence of the aromatic amino acids is acutely affected by their immediate environment. Changes in the local environment, such as a decrease in polarity, cause both wavelength and intensity shifts to the spectrum. Protein-protein interactions can cause revelation of buried tryptophans from the hydrophobic protein interior to a more polar environment of the solvent, which can alter the

environment immediately surrounding the aromatic residue. The strong polar bonds of the solvent are destabilized by interactions with the indole group of tryptophan, altering polarity of the reaction, as well as intensity and position of the spectra.

Extrinsic protein fluorescence analyzes sample fluorescence by means of extrinsic fluorophores. The anionic extrinsic probe 8-anilino-1-napthalene-sulfonate (ANS) is commonly used, due to its ability to bind tightly to hydrophobic regions when within 10Å [131, 132]. ANS can be used to analyze potential conformational changes due to protein-protein interactions. In the absence of sample the probe has an excitation in polar solvents at 350 nm and a minimal emission peak at 510 nm, however, when in non-polar environments the probe will produce a significant fluorescence peak and shift of the spectra [133]. When in the presence of tryptophans which have been excited at their respective wavelength the fluorophore will act as an acceptor of florescence resonance energy transfer (FRET) from the excited tryptophans if within 10 nm, and will then fluoresce in its usual range. The intensity of the energy transfer directly correlates with the distance between donor and acceptor. Revelations of buried aromatic regions will interact with the probe when in its nearby vicinity, altering the polarity of its immediate environment and causing a shift in the spectrum to shorter wavelengths and an increase in signal intensity.

2.7 Connecting statement

In addition to serving as an attractive drug target, the biogenesis machinery of *L. donovani* is part of an interesting biological system that is poorly understood. Previous analyses in *L. donovani* have suggested that soluble import shuttling proteins interact with a protein peripheral to the glycosomal membrane and may play a role in formation of an import complex that creates a transient import pore.

In the next chapter I will further investigate the role of the soluble cytosolic receptor LdPEX5 in the process of glycosomal protein import. Using biochemical techniques I will analyze the relationship between LdPEX5 and LdPEX14 at a glycosomal mimetic, to further our understanding of how these proteins form a pre-pore complex. Moreover, using biophysical techniques we will further probe structural and conformational changes that precede the formation of the pre-pore complex, that occur at the interaction of LdPEX5 with a PTS1.

Together these analyses will further demonstrate the important role that LdPEX5 plays in the formation of an import pore, necessary for shuttling of proteins into the glycosomal lumen.

Chapter 3

Insertion of the *Leishmania donovani* peroxin 5 into glycosomal membranes.

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3.1 Abstract

Leishmania donovani parasites contain a peroxisomes-like organelle called a glycosome that compartmentalizes multiple metabolic and biosynthetic pathways. Correct targeting and compartmentalization of this enzymatic machinery is essential for viability of the parasite. The import receptor LdPEX5 rapidly binds proteins tagged with a PTS1 signal destined for glycosomal import and is required for trafficking of the cargo and docking with LdPEX14 in the formation of an import pore. We demonstrated that recombinant LdPEX5 and LdPEX14 bind to larger unilamellar liposomes that mimic the glycosome lipid bilayer composition. Moreover, these protein-membrane interactions cause LdPEX5 to undergo a conformational change from a soluble to an integral membrane protein that is resistant to alkaline carbonate extraction. Using biophysical and molecular techniques with both full length and truncated LdPEX5 in liposome membranes containing LdPEX14 we have mapped the domain of LdPEX5 necessary for both interacting with LdPEX14 and inserting into the glycosomal membrane as part of pore formation to residues 268-302.

3.2 Introduction

Leishmania are protozoan parasites that are the causative agents of a spectrum of devastating diseases, collectively termed leishmaniasis. These diseases range from self-healing ulcerative skin lesions caused by the cutaneous form, to devastating systemic shock, organ failure, and eventual death without treatment caused by the visceral form [134]. *Leishmania spp.*, along with the other medically important kinetoplastid parasites *Trypanosoma brucei and Trypanosoma cruzi*, contain an organelle called a glycosome, which is structurally and functionally related to peroxisomes of higher eukaryotes [78, 135]. The glycosome compartmentalizes enzymes required for many important metabolic and biosynthetic pathways such as the pentose phosphate pathway, purine salvage, gluconeogenesis, β-oxidation of fatty acids, ether lipid biosynthesis, and glycolysis, for which the organelle was named [62, 66, 67]. Proper compartmentalization of these pathways is necessary for the viability of the parasite [12, 81, 136], making the glycosomal import machinery an attractive drug target [49].

Glycosomal matrix proteins are encoded on the nuclear genome, and synthesized on free cytosolic polyribosomes. For proteins to enter the glycosome they usually require one of two topogenic signals, a PTS1 (peroxisomal targeting signal 1) or a PTS2 (peroxisomal targeting signal 2). The PTS1 is a degenerate C-terminal tripeptide with an archetypical signal sequence serine-lysine-leucine [8, 9], while the PTS2 is an N-terminal nonapeptide with the degenerate consensus motif [RK]-[ILV]-X_5-[HQ]-[LA] [10, 11], found within the first 20 to 30 residues of the protein. The PTS1 and PTS2 sequences are tightly bound by the *Leishmania* trafficking receptors peroxin-5 (LPEX5) and peroxin-7 (LPEX7), respectively. These receptors have been shown to also form heteromeric complexes, a process which is stabilized by the binding of their respective PTS proteins [6, 104]. These receptors have been shown to be necessary for parasite viability and attempts to generate *ldpex5* or *ldpex7* null mutant cell lines have not been successful. However, in the closely related parasite *T. brucei* it has been

shown that knocking down either of these receptors by RNAi results in a conditional lethal phenotype [12]. This contrasts with studies done in mammalian and yeast cell lines, where knocking down PEX5 or PEX7 levels resulted in mistargeting of the PTS1 and PTS2 proteins to the cytosol without causing a lethal phenotype [137-139].

PEX5 receptors have been characterized in: mammals, yeast, fungi, plants, and kinetoplastid parasites [8, 85, 102, 140-142]. Mammalian cells contain two isoforms of PEX5 generated by an alternate splicing event, a short isoform (PEX5S) which is involved in PTS1 targeting, and a long isoform (PEX5L) which is required for import of PTS1 and PTS2 proteins into peroxisomes. PEX5L contains a 37 amino acid insert that corresponds to the PEX7 binding site [137, 138, 143, 144]. Plants and kinetoplasts contain one isoform of PEX5, which is essential for the import of PTS1 and PTS2 proteins into peroxisomes/glyoxysomes and glycosomes, respectively [4, 6, 12, 142]. LdPEX5, like other PEX5 homologues, is a soluble receptor with multiple interaction domains. The C-terminal half of PEX5 makes up the PTS1 binding pocket which is composed of six to seven tetratricopeptide repeats (TPRs) that form helical hairpin repeats [4, 92, 95, 98]. The N-terminal region of PEX5 has been shown to be important for intramolecular and intermolecular interactions that facilitate PTS1 import. Mapping studies of the LdPEX5 N-terminus show that this region contains critical sequences for LdPEX7 and LdPEX14 interactions [6, 94]. The LdPEX14 interaction domain differs from the system seen in mammals, *Saccharomyces cerevisiae*, plants, and *T. brucei* where the PEX5-PEX14 interaction is mediated by three WXXX/F motifs [99, 145-147]. In LdPEX5, however, abolition of these diaromatic motifs did not disrupt the PEX5-PEX14 binding [94]. In the absence of a PTS1 ligand, LdPEX5 has been shown to exist as a homotetramer in solution [4]. This tetramer separates into a corresponding dimer of dimers upon interacting with a PTS1 ligand, and then further into monomers when the concentration of PTS1 increases [148].

Glycosomal import is initiated by docking of the cargo loaded receptors to peroxin 14 (LdPEX14), a peripheral membrane protein anchored to the cytosolic face of the glycosomal membrane. The convergence and docking of the cargo-laden receptors at PEX14 (and PEX13 in peroxisomes) is the pivotal first step in protein import [117, 149-151]. Furthermore, functional PEX14 has been shown to be necessary for the viability of the parasite. Mutations altering cellular levels of PEX14 lead to accumulation of PTS1 and PTS2 proteins in the cytosol [151, 152]. Additionally, PEX14 has been shown to be vital for glycosome biogenesis, as creating functional mutants in *T. brucei* by RNAi resulted in a conditional lethal phenotype. Once converged at the glycosomal membrane the cargo proteins are translocated into the glycosomal lumen by a yet undefined mechanism. There are theories of how this feat is accomplished, but the currently accepted model of peroxisomal protein import includes formation of an importomer complex and a transient pore [14].

The current model of transient pore formation suggests that LdPEX5-PTS1 and LdPEX7-PTS2 interact with LdPEX14 at the glycosomal membrane, causing a conformational change in LdPEX14 and facilitating the insertion of LdPEX14 and LdPEX5 into the membrane to create a tightly regulated pore. This pore allows passage of LdPEX5, LdPEX7 and the cargo proteins into the luminal space [153]. In *Leishmania*, however, the localization of PEX5 during import is disputed, as only trace amounts of LdPEX5 have been detected to associate with the glycosomes, while LPEX7 was found to have a dual distribution in the cytosol and glycosomal lumen [6]. Pore formation is believed to involve recruitment of the RING subcomplex, composed of the subunits PEX2, PEX10, and PEX12 [154-156]. These proteins have been implicated in, translocation of cargo proteins across the peroxisomal membrane, ubiquitination of PEX5 which tags this receptor for recycling back to the cytosol or degradation by proteasome, and finally in the biogenesis of peroxisomes. Translocation through the pore appears to be energy independent for peroxisomes, while recycling of the receptors back into the cytosolic compartment requires ubiquitinylation and ATP, as well as the proteins PEX1 and PEX6 [157]. After transport of their PTS ligands into the

matrix, PEX5 and PEX7 are recycled back to the cytosol [6], by a mechanism yet to be elucidated. A contributing factor to this transient behavior is likely the high fluidity of the glycosomal membrane caused by the high percentage of unsaturated fatty acids [112].

An important regulator in the glycosomal import pathway is the actual membrane of the organelle. This poorly permeable membrane is necessary for the viability of the parasite in its compartmentalization of many important and potentially toxic enzymes from biosynthetic and metabolic pathways within the parasite. Proteins of the import pathway make important interactions with the membrane as part of the import process. LdPEX14, an integral membrane protein in higher eukaryotes, behaves as a peripheral membrane protein in *Leishmania* and has been shown to make interactions with phospholipids of the glycosomal membrane that are not solely electrostatic [114, 115]. In higher eukaryotes PEX5 spontaneously inserts into peroxisomal membranes as part of import, while in *Leishmania* LdPEX5 is unable to independently interact with glycosomal mimetics and has been observed to only minimally co-purify with glycosomes. However, in this study we have observed the ability of LdPEX5 to behave as an integral membrane protein on liposomes in the presence of LdPEX14 and have queried what role this might play in transient pore formation.

Here we demonstrate that a segment at 268-302 of LdPEX5 plays an important role in the process of glycosomal protein import. Using an *in vitro* model system we have shown that LdPEX5 requires the docking protein LdPEX14 to interact with large unilamellar vesicles (LUVs) and the domain at 268-302 of LdPEX5 for insertion of the protein into large unilamellar vesicles (LUV) in an integral manner that is resistant to serial extraction techniques. Furthermore, we have identified changes to the conformational structure of LdPEX5 in the process of PTS1 import, which could be important for revealing this insertion domain. Finally, we have performed binding studies at a biologically relevant liposomal membrane, thereby providing insight into the role

of LdPEX5 in the formation of a reversible import pore at the glycosomal membrane.

3.3 Materials and Methods

3.3.1 Materials

Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibodies were purchased from GE Healthcare. HRP-conjugated goat anti-mouse and donkey anti-guinea pig secondary antibodies, and cholesterol were purchased from Sigma-Aldrich. Synthetic phospholipids and natural lipids were obtained from Avanti Polar Lipids. All other reagents were of the highest quality commercially available.

3.3.2 Protein expression

L. donovani PEX5 and *L. donovani* PEX14 were previously cloned into the *Escherichia coli* cloning and expression vectors pTYB12 and pET30b(+), respectively. The ldpex5 mutants ldpex5(Δ1-111), ldpex5(Δ1-205), ldpex5(203-391), ldpex5(Δ181-314), and ldpex5(1-391) were previously cloned into pTYB12; and ldpex5(303-625), ldpex5(268-625), and ldpex14(1-120) were previously cloned into pET30b(+) [4, 5, 94].

Recombinant LdPEX5/ldpex5 and LdPEX14/ldpex14 proteins were expressed in *E. coli* ER2566 as fusion constructs containing an N-terminal chitin-binding domain fused to LdPEX5/ldpex5 proteins, and a C-terminal hexahistidine tag for LdPEX14.

For purification of the recombinant proteins bacterial cell pellets were resuspended in 25 ml of 20mM Tris-HCl pH 8.0 and lysed by three passes through a French Press. NaCl was added to the lysate to a final concentration of 500 mM before clarification by centrifugation at 24,000 × g for 20 minutes. The supernatant of LdPEX5/ldpex5 and LdPEX14/ldpex14 were then applied to 2.0 ml chitin columns or 2.0 ml Ni^{2+} -NTA resin (Qiagen) columns, respectively, and equilibrated with 40 mM Tris-HCl- 500 mM NaCl pH 8.0 (TBS500). LdPEX5/ldpex5 on chitin columns were thoroughly washed with TBS500,

washed with 50 mM DTT-Tris buffer, and left to cleave in 10 ml 50 mM DTT-TBS500 for 40 hours. Purified protein was removed in the cleavage buffer and with a subsequent 15 ml wash with TBS500. LdPEX14/ldpex14 on Ni^{2+} -NTA resin columns were sequentially washed with 100 ml TBS500, 100 ml TBS500-containing 10 mM imidazole, 100 ml of TBS500 containing 20 mM imidazole, and 50 ml of TBS500 containing 40 mM imidazole. The purified protein was eluted with 15 ml of TBS 500 containing 160 mM imidazole, and then TBS500 containing 500 mM imidazole. [4, 5, 94]. Purification of ldpex14(120-200) was performed as described previously [112].

The model PTS1 peptide, AKL, was a peptide corresponding to the C-terminus of the xanthine phosphoribosyl transferase (XPRT), with the sequence CNDRYRDLRHILILRDGDATRYPAKL, and was synthesized by stepwise elongation (Biomatik).

3.3.3 Circular dichroism (CD) analysis

Purified proteins were extensively dialyzed at 4ºC against 10 mM phosphate buffer pH 7.6. CD measurements were performed on a Jasco 810 spectropolarimeter at 20ºC at a scan rate of 50 nm/min, using a cuvette with a pathlength of 0.1 cm. Five spectra were collected and averaged for each condition in units of millidegrees, which were then deconvoluted into units of delta epsilons using the CDSSTR analysis algorithm [158, 159] using the web based server DICHROWEB [160]. Reference protein set 7 was used for accurate normalization, as it is composed of proteins with known structures similar to the proteins of interest in the far UV spectrum [161, 162]. For all trials, data was collected at wavelengths between 260 nm and 190 nm. LdPEX5/ldpex5(Δ1-111) were diluted in dialysis buffer to a concentration of 2 μM, and AKL was added to a concentration of 1μM, and then 2μM to obtain an initial molar ratio of 2:1, and a final ratio of 1:1 of LdPEX5/ldpex5(Δ1-111):AKL.

3.3.4 Fluorescence spectroscopy

Fluorescence, spectra were acquired on a Varian Cary Eclipse spectrofluorometer (Palo Alto, CA) at 25ºC. Spectra of intrinsic LdPEX5/ldpex5 fluorescence were recorded at a scan rate of 120 nm/min with excitation and emission slit widths of 5 nm. These spectra had an excitation wavelength of 290 nm and were recorded from 300-550 nm. Solutions of 1.25 μM LdPEX5/ldpex5 in 40 mM Tris-HCl-150 mM NaCl pH 8.0 were titrated with increasing concentrations of AKL peptide to a final concentration of 50 μM. Dilution effects were corrected for using a cell with buffer additions in parallel. Spectra of extrinsic LdPEX5/ldpex5 fluorescence were recorded at a scan rate of 120 nm/min with excitation and emission slit widths of 5 nm. These spectra were excited at 290 nm and 350 nm and emission spectra recorded from 400-550nm. Spectra of 1.25 μM LdPEX5/ldpex5 in 40 mM Tris150 containing 50 μM of anionic probe 8-anilino-1-napthalene-sulfonate (ANS) [132] were similarly collected at increasing intervals of AKL.

3.3.5 Liposome preparation

Mixtures of DOPE:DOPC:DOPG:PI:cholesterol (53:24:15:4:4) were dissolved in chloroform and evaporated to form a thin film using a delicate nitrogen gas stream. The lipid ratio resembled the composition of the glycosomal membrane of *L. donovani*, determined previously [112]. Multilamellar vesicles were formed by vigorous re-suspension of the lipid film in PBS, to a phospholipid concentration of 5 mg/ml. To produce large unilamellar vesicles (LUV) the suspension was extruded through a 0.2 μm polycarbonate membrane (Whatman) 11 times. This produced LUV with a diameter of 200 nm, which is comparable to the diameter of glycosomes.

3.3.6 Sucrose density flotation centrifugation

LUVs composed of 53% dioleoylphosphatidylethanolamine (DOPE), 24% dioleoylphosphatidylcholine (DOPC), 15% dioleoylphosphatidylglycerol (DOPG),

4% phosphatidylinositol (bovine liver PI), and 4% cholesterol were incubated with recombinant proteins (molar ratio of 500:1 phospholipid:protein for LdPEX14/ldpex14 and 700:1 phospholipid:protein for LdPEX5/ldpex5) in 300 µl of PBS for 40 minutes at 23ºC. Proteins were added to the reaction in an order that is predicted to mimic glycosomal protein interaction in *Leishmania*. After incubation 1.2 ml of 66% sucrose in PBS (w/v), and added to the bottom of a 5.2 ml ultracentrifuge tube which was then overlaid with 3.0 ml of 40% sucrose in PBS, and 1.0 ml of PBS. Flotations were centrifuged at 28,000 rpm for 16 h at 4ºC in a Beckman-Coulter SW55 rotor. After centrifugation the gradient was fractionated (0.65 ml fractions) and the proteins precipitated by the addition of sodium deoxycholate (0.2%), and trichloroacetic acid (15%). Protein pellets were washed with 1 ml of acetone prior to resuspension in SDS-PAGE sample buffer. Proteins were visualized by Western blotting using anti-LdPEX5 rabbit antisera or anti-LdPEX14 rabbit antisera (1:5000) and goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:10000; Sigma-Aldrich).

3.3.7 Alkaline carbonate extraction

LUVs loaded with recombinant LdPEX5/ldpex5/LdPEX14/ldpex14 after sucrose density flotations were isolated and treated sequentially with 500 mM NaCl, 100 mM sodium carbonate pH 11.5, and 100 mM sodium carbonate pH 11.5 containing 4.0 M urea at 0ºC for 30 minutes. After each treatment samples were separated into supernatant and pellet fractions by centrifugation at 65,000 rpm for 30 minutes at 4ºC in a TLA 100.3 rotor on a Beckman-Coulter table top ultracentrifuge. After each round of extraction the liposomes remained in the pellet fraction along with any protein that was unable to be extracted from the membrane fraction. If the treatment was sufficient to remove the protein from the membrane fraction it was separated into the supernatant fraction. Aliquots of each sample were isolated, proteins were precipitated via 15% TCA, and visualized using Western blotting.

3.3.8 Limited proteolysis

LdPEX5-LdPEX14 and LdPEX14 were pre-floated with liposomes (as previously described); the top two fractions were collected and pooled. Liposome-protein complexes were pelleted using a TLA-100 rotor in the Beckman-Coulter Optima MAX ultracentrifuge; fractions were then washed using PBS. Control samples were solubilized using a 7.5:1 molar ratio of Triton X-100 to protein [163]. Liposome samples treated with and without Triton X-100 were treated with sequencing grade porcine trypsin (Promega, Madison, WI) at a ratio of 200:1 (w/w) at 20ºC. Aliquots (50 µl) were sequentially removed at specific time points and added to 25 µl of an EDTA-free protease inhibitor cocktail (1 tablet/ml PBS, Roche) to terminate proteolysis. Digests were analyzed by Western blot analysis.

3.3.9 Labelling of proteins with fluorescent probes

LdPEX5 and LdPEX14 were labelled with Oregon Green®488 (Invitrogen) and Texas Red® (Invitrogen), respectively. The peptide AKL was labelled with N-(1-pyrene) maleimide (Invitrogen). The proteins were dialyzed against 0.1M sodium bicarbonate-150 mM NaCl pH 8.3 buffer. For labelling of LdPEX5, 2.2 mg of protein was added to 0.9 mg of Oregon Green®488 resuspended in 10 µl of DMSO. For labelling of LdPEX14, 2.0 mg of protein was added to 1.0 mg of Texas Red® resuspended in 10 µl of DMSO. Labelling of AKL was performed by mixing 2.0 mg of peptide with 1.0 mg of pyrene maleimide resuspended in 10 µl of DMSO. The reaction mixtures were stirred overnight at 4ºC. Reaction mixtures were centrifuged at 10,000 g for 10 minutes to remove precipitate and the supernatants were applied to a 15 mL Sephadex G50 gel filtration columns. All fractions collected and ran on an 8% SDS-PAGE; fractions containing labelled protein were isolated using a UV transilluminator. The labelling efficiency (moles of dye/moles of proteins) of LdPEX5 and LdPEX14 were 0.46 and 0.66, respectively. Fractions containing protein were pooled and quantified using a spectrophotometer, correcting for emission from the probes, before use in fluorescence experiments.

Spectra of LdPEX5-Oregon Green, LdPEX14-Texas Red, and AKL-pyrene were recorded at 72 nm/min at 700 V with excitation and emission slit widths of 5.0 nm and 2.5 nm, respectively. Spectra containing LdPEX5-Oregon Green were excited at 496 nm and recorded from 510-550 nm. Spectra containing LdPEX14-Texas Red were excited at 595 nm and recorded from 605-650 nm. Spectra containing AKL-pyrene were excited at 345 nm and recorded from 350-460 nm. All spectra containing liposomes were corrected for scattering effect.

3.4 Results

3.4.1 Association of LdPEX5 with glycosome mimetic membranes is LdPEX14 dependent

Glycosomal mimetics were used in flotation assays to model the association of LdPEX5 with the glycosomal membrane. For these studies large unilamellar liposomes of DOPE:DOPC:DOPG:PI:cholesterol (53:24:15:4:4) [112], were used for sucrose density centrifugation analyses. After fractionation of each flotation the fractions of proteins with liposomes were then visualized using Western Blotting with polyclonal antibodies specific to each LdPEX5 and LdPEX14. The antibody for LdPEX5 is specific to the entire protein, while the antibody for LdPEX14 is specific to residues 23-63 [94]. It has been previously shown that LdPEX14 interacts with the liposomal membrane and is found in the top fraction after flotation [112]. Further, the region of LdPEX14 at residues 149-179 has been implicated in this membrane association [112], and in previous trials it has been shown that while ldpex14(1-200) is able to interact with liposomes ldpex14(1-120) is not. In this study it is found that recombinant LdPEX5 is found in the bottom fractions after centrifugation alone, with liposomes, or with the AKL peptide and liposomes (Fig. 3.1), indicating the protein is not able to interact directly with the liposomal membranes when alone or in conjugation with a PTS protein. To examine the necessity of LdPEX14 for the binding of LdPEX5-AKL to glycosomal mimetic membranes, liposomes were first loaded with LdPEX14 prior to incubation with LdPEX5-AKL. In flotations of LdPEX5-LdPEX14 and LdPEX5-AKL-LdPEX14 the LdPEX5 protein was found in the top fractions after centrifugation (Fig. 3.1), indicating that either the protein-protein interaction is facilitating the floating of LdPEX5 or LdPEX5 is able to interact with the liposomal membrane when in the presence of LdPEX14. Conversely, in flotations of LdPEX5-ldpex14(1-120) and LdPEX5-AKL-ldpex14(1-120) LdPEX5 was found in the bottom fractions after centrifugation (Fig. 3.1), implying that although LdPEX5 interacts with ldpex14(1-120) [94], this interaction is not sufficient for the proteins to interact with the lipid membrane. These observations

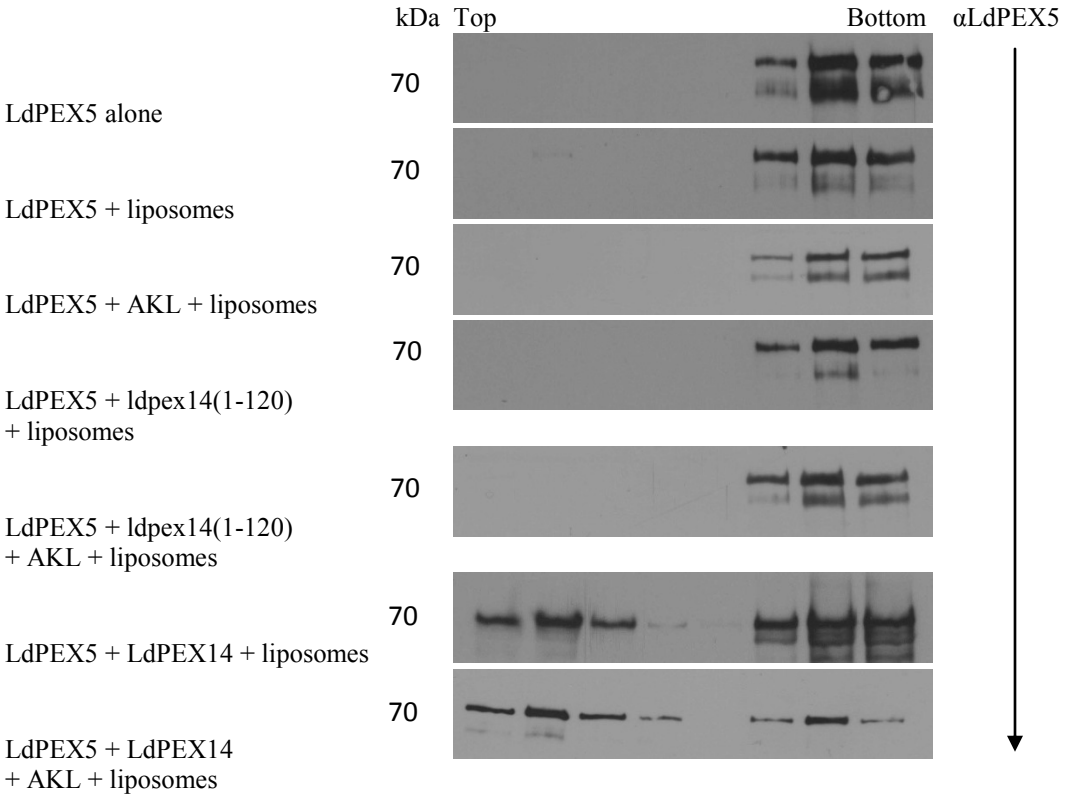


Figure 3.1 Analysis of the interaction of LdPEX5 with lipid bilayers. Flotation results for LdPEX5 incubated alone; LdPEX5 incubated with (large unilamellar vesicles) LUVs; LdPEX5 incubated with AKL and liposomes; LdPEX5 incubated with ldpex14(1-120) loaded liposomes; LdPEX5 incubated with AKL and ldpex14(1-120) loaded liposomes, LdPEX5 incubated with LdPEX14 loaded liposomes, and LdPEX5 incubated with AKL and LdPEX14 loaded liposomes. For all flotations, LUVs were incubated with samples (molar ratio of 500:1 phospholipid:protein for LdPEX14, 700:1 phospholipid:protein for LdPEX5 and 300:1 phospholipid:peptide for AKL) in 300 µl of PBS150 for 40 minutes at 23ºC. The mixtures were brought to a final concentration of 50% sucrose, layered under a sucrose gradient and were centrifuged at 28000 rpm for 16 h at 4ºC in a Beckman-Coulter SW55 rotor. The gradients were fractionated (0.65 ml fractions), proteins were precipitated with sodium deoxycholate (0.2%) and trichloroacetic acid (15%), and pellets were washed with acetone. Precipitated proteins were then visualized via Western blotting. Fractions from the top to the bottom of the flotation column are 1-8 from the left to the right in the figure.

were expected, as we have shown previously that recombinant LdPEX5 is recovered in the glycosomal fraction after sedimentation studies when LdPEX5 is added to glycosomes containing native LdPEX14 [104].

3.4.2 Mapping the LdPEX14 association domain on LdPEX5

Since LdPEX5 requires LdPEX14 for binding to liposomal membranes we then performed mutational analysis to identify the LdPEX5 motif necessary for interacting with membrane bound LdPEX14. In this study we used several truncated forms and one deletion model of LdPEX5 (Fig. 3.2) in flotation assays using liposomes pre-incubated with LdPEX14 to analyze this protein-protein association (Fig. 3.3). Considering that both LdPEX5 and LdPEX5-AKL were able to float in the presence of LdPEX14-liposomes (Fig 3.1), we assume that at a liposomal membrane LdPEX5 interacts with LdPEX14 similarly with and without a PTS1. Since we have used a simplified model of this protein-protein interaction these mapping experiments were done using only the LdPEX5/ldpex5 and LdPEX14 proteins. In addition to LdPEX5 the following proteins were detected in

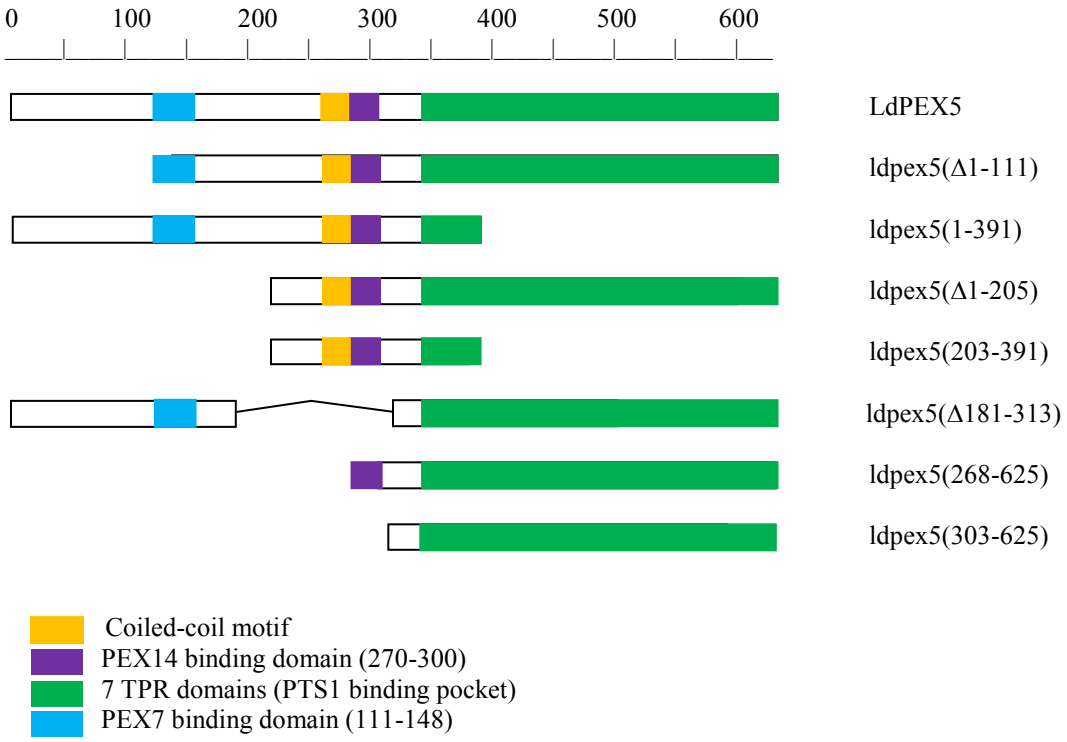


Figure 3.2 Schematics of LdPEX5/ldpex5 constructs used in analyses. Schematic representation of LdPEX5/ldpex5 constructs with domains indicated by colour coding: LPEX7 binding domain is in blue, the coiled-coil motif is in yellow, LdPEX14 binding domain is in purple, and the PTS1 binding pocket is in green.

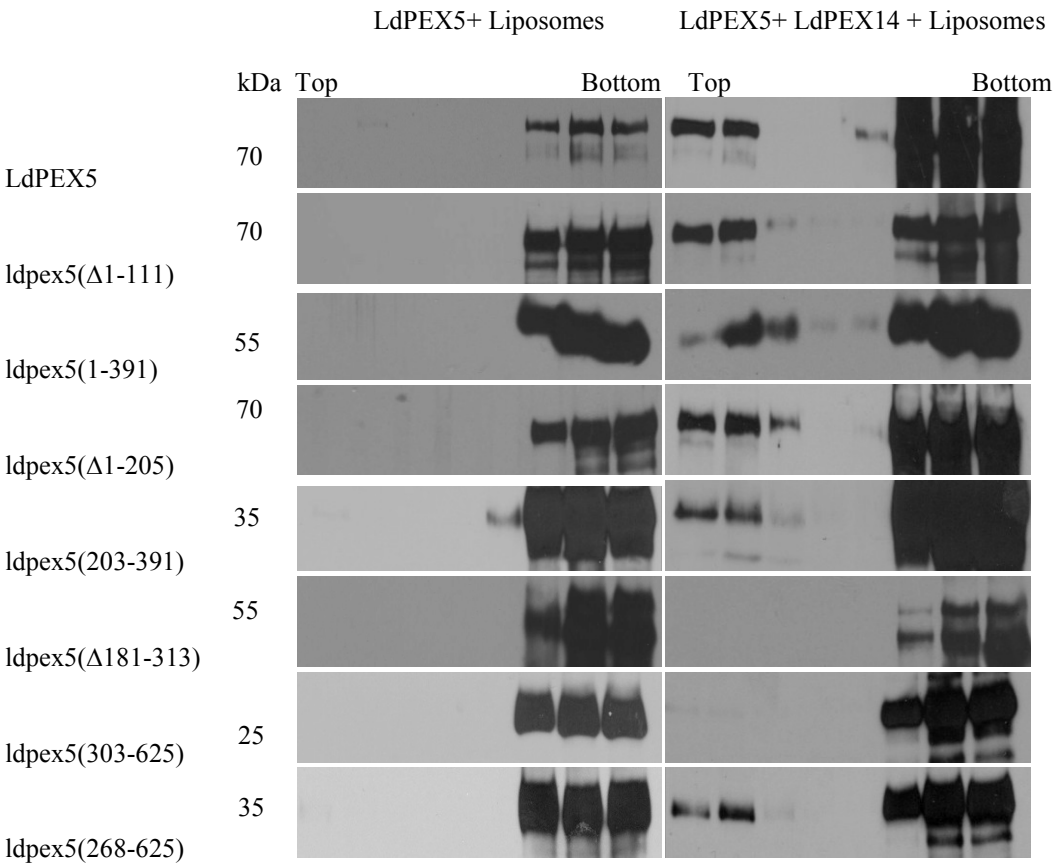


Figure 3.3 Flotations of LdPEX5/ldpex5-LdPEX14 with liposomes. Western blots of LdPEX5, ldpex5(Δ1-111), ldpex5(1-391), ldpex5(Δ1-205), ldpex5(203-391), ldpex5(Δ181-313), ldpex5(303-625), ldpex5(268-625), and ldpex5(268-625) floated alone with LUVs or with LdPEX14 loaded liposomes. The left column is LdPEX5/ldpex5 floated with LUVs alone, the right column is LdPEX5/ldpex5 floated with LdPEX14 loaded liposomes. For all flotations, LUVs were incubated with samples (molar ratio of 500:1 phospholipid:protein for LdPEX14 and 700:1 phospholipid:protein for LdPEX5/ldpex5) in 300 µl of PBS for 40 minutes at 23ºC. The mixtures were brought to a final concentration of 50% sucrose, layered under a sucrose gradient and were centrifuged at 28000 rpm for 16 h at 4ºC in a Beckman-Coulter SW55 rotor. The gradients were fractionated (0.65 ml fractions) and proteins were precipitated with the addition of sodium deoxycholate (0.2%) and trichloroacetic acid (15%). Protein pellets were washed with acetone and resuspended in SDS-PAGE sample buffer. Precipitated proteins were visualized via Western blotting. Fractions from the top to the bottom of the flotation column are 1-8 from the left to the right in the figure, respectively.

the liposomal fraction: LdPEX5, ldpex5(Δ1-111), ldpex5(1-391), ldpex5(Δ1-205), ldpex5(203-391), and ldpex5(268-625) (Fig. 3.3). Therefore, these proteins were all able to interact with LdPEX14 at a liposomal membrane. In contrast, the

proteins ldpex5(Δ181-313) and ldpex5(303-625) (Fig. 3.3) were found in the bottom fractions after flotation and hence were lacking the domain necessary for this protein-protein interaction. This pattern of "floating" implies that the domain necessary for interaction with LdPEX14 at a liposomal membrane is in the regions of 268-302. This corroborates previous studies using ELISAs and pull down assays that the segment necessary for interaction with LdPEX14 is at 270-300 [94], and implies that the region of LdPEX5 identified is necessary for the LdPEX5-LdPEX14 interaction whether at a surface or a more biologically relevant liposomal membrane.

3.4.3 Mapping the membrane binding segment of LdPEX5

To further examine the ability of LdPEX5 to associate with a liposomal membrane containing LdPEX14 we analyzed the nature of this association to determine if the interaction was solely protein-protein or an LdPEX14 mediated insertion event. To analyze this potential protein-membrane association we used a serial membrane extraction technique after isolation of liposomes loaded with LdPEX14 or LdPEX5/ldpex5-LdPEX14 complex by flotation (Fig. 3.4). Treatment of liposomes with 500 mM NaCl, which removed proteins associated with the lipid bilayer by electrostatic interactions, failed to release LdPEX5 from the liposomal membrane (Fig. 3.4). Similarly, treatment with 100 mM alkaline carbonate, which compromises membrane integrity releasing luminal proteins and reducing protein-protein interactions, was again insufficient to remove LdPEX5 from the membrane fraction. Finally, adding the strong denaturant 4 M urea disrupts loosely bound protein-membrane interactions, such as proteins bound peripherally [164]. LdPEX5 remained in the pellet fraction after the most stringent extraction (Fig. 3.4). In a separate trial the final pellet was solubilized with Triton X-100, resulting in removal of LdPEX14 and LdPEX5 into the supernatant fraction (Fig. 3.5). Together, these results indicate that LdPEX5 makes interactions with the hydrophobic core of the membrane, behaving as an integral membrane protein as part of the pore formation. This was unexpected, as when the hydrophobicity plot of LdPEX5 was analyzed there were no domains

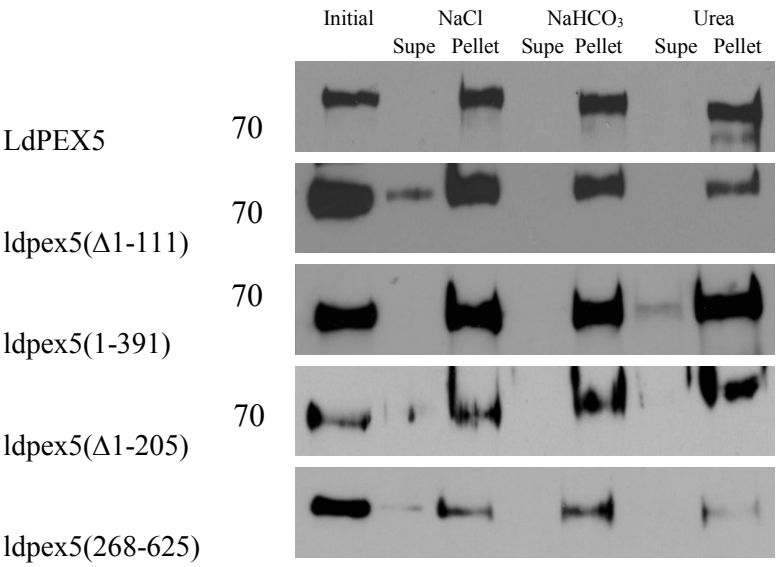


Figure 3.4 Alkaline-urea extractions of LdPEX5 from LUVs. Serial carbonate urea extractions of LdPEX5, ldpex5(Δ1-111), ldpex5(1-391), ldpex5(Δ1-205), and ldpex5(268-625) from LdPEX14-liposomes after flotation. For all extractions LUVs loaded with LdPEX14 and LdPEX5/ldpex5 after sucrose density flotations were isolated and treated sequentially with 500 mM NaCl, 100 mM sodium carbonate pH 11.5, and 100 mM sodium carbonate pH 11.5 containing 4.0 M urea at 0º C. Each treatment samples were separated into supernatant and pellet fractions, denoted by supe (supernatant) and pellet respectively, by centrifugation at 65000 rpm for 30 minutes at 4ºC in a TLA 100.3 rotor on a Beckman-Coulter Optima MAX ultracentrifuge. Aliquots of each sample treatment were removed; proteins were precipitated via 15% TCA, and visualized using Western blotting.

clearly indicative of an amphipathic domain, according to the Kyte-Doolittle algorithm [127].

To map the protein segment necessary for the insertion of LdPEX5 into liposomal membranes several truncations and a deletion mutant of LdPEX5, previously described, were analyzed using serial extractions. Deletion of the first 111, 205, or 267 amino acids from the N-terminus of LdPEX5 is not sufficient to remove the protein from the pellet fraction after stringent extraction (Fig. 3.4). Similarly, deletion of the final 234 amino acids from the C-terminus of the protein did not facilitate removal of the protein into the supernatant fraction. This implies that these truncations do not affect the ability of the protein to insert into the liposomal membrane or disrupt the interaction with the hydrophobic core of the

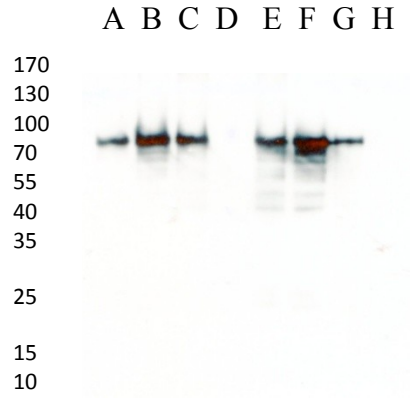


Figure 3.5 Triton X-100 solubilization of liposomes with LdPEX14-LdPEX5 after flotation. After serial carbonate-urea extraction of LdPEX14/LdPEX14-LdPEX5 from liposomes post-flotation controls were set aside and samples were solubilized using a 7.5:1 molar ratio of Triton X-100 to protein. Controls and samples were then pelleted using a TLA100.3 rotor in the Beckman-Coulter Optima MAX ultracentrifuge. Aliquots of each sample treatment were removed; proteins were precipitated via 15% TCA, and visualized using Western blotting.

A- Supernatant – LdPEX14 + LdPEX5 + liposomes (α-PEX14)
B- Pellet – LdPEX14 + LdPEX5 + liposomes (α-PEX14)
C- Supernatant – LdPEX14 + LdPEX5 + liposomes + Triton X-100 (α-PEX14)
D- Pellet – LdPEX14 + LdPEX5 + liposomes + Triton X-100 (α-PEX14)
E- Supernatant – LdPEX14 + LdPEX5 + liposomes (α-PEX5)
F- Pellet – LdPEX14 + LdPEX5 + liposomes (α-PEX5)
G- Supernatant – LdPEX14 + LdPEX5 + liposomes + Triton X-100 (α-PEX5)
H- Pellet – LdPEX14 + LdPEX5 + liposomes + Triton X-100 (α-PEX5)

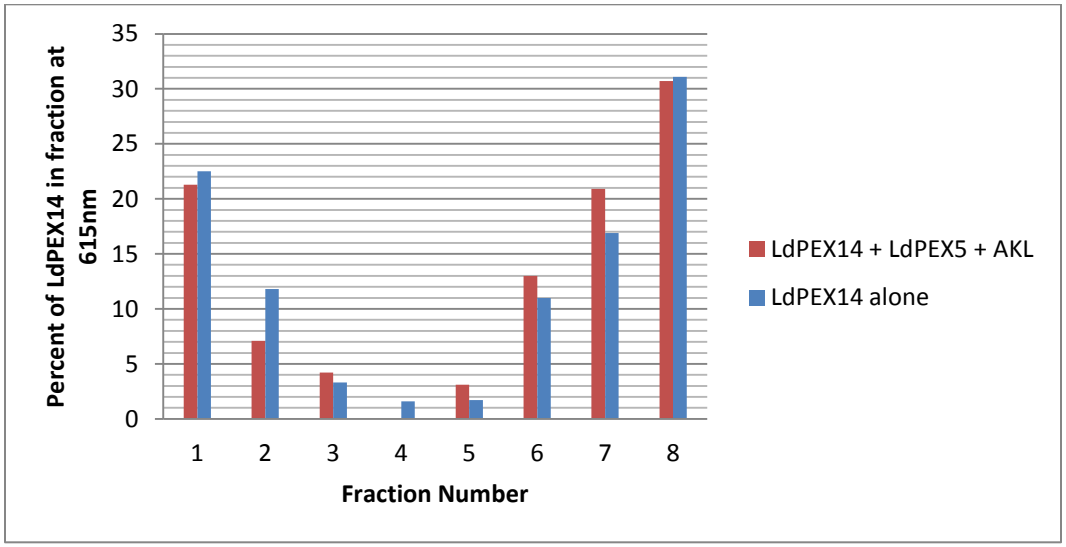
membrane, upon interaction with LdPEX14. These results demonstrate that the segment of LdPEX5 necessary for interacting with LdPEX14 is also the region required for insertion into the liposomal membrane, residues 268-302. This suggests that LdPEX5 binds to LdPEX14 at the glycosomal membrane, and upon conformational changes in LdPEX14 is pulled into the membrane by its interacting domain during the process of pore formation.

3.4.4 Biochemical characterization of the interaction of LdPEX5 with a lipid bilayer

To further characterize the interaction of LdPEX5, LdPEX14, and AKL with the liposomal membrane the proteins and peptide were labelled with

molecular probes (LdPEX5-Oregon Green, LdPEX14-Texas Red, AKL-pyrene), floated, and analyzed via the fluorescent spectrophotometer. When LdPEX14 was floated alone approximately 38% of the protein was found in the top fractions, able to float. This amount decreased to approximately 33% when LdPEX14 was first incubated with LdPEX5 and AKL before floating (Fig. 3.6A), implying that upon interacting with LdPEX5 some LdPEX14 was unable to interact with the liposomal membrane. When LdPEX5 was floated alone approximately 11% of the protein was found in the top fractions. The percent of protein able to float remained at 11% when LdPEX5 was floated after incubation with AKL, implying that interaction with a PTS1 is not sufficient to promote interaction with a liposomal membrane. Conversely, when incubated with LdPEX14 and AKL there was an increase of LdPEX5 floating to approximately 14% (Fig. 3.6B), suggesting that LdPEX14 is indeed necessary for interaction of the protein with the liposomal membrane.

A.



B.

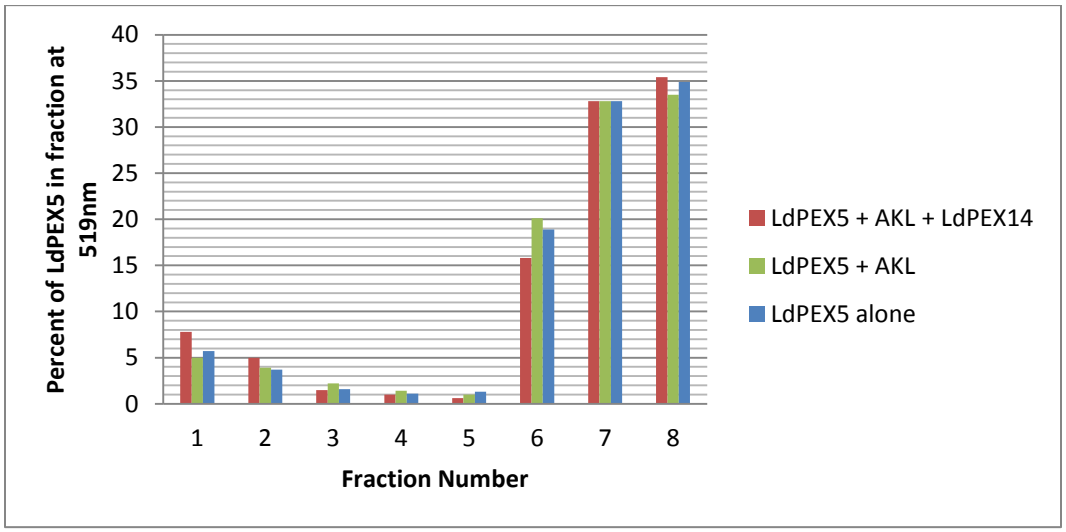


Figure 3.6 Flotations of fluorescently conjugated proteins with liposomes. A. Percent of total LdPEX14 in each of eight fractions at an emission wavelength of 615 nm after an overnight centrifugation using 20 µg (0.004 m) of LdPEX14-texas red, 29 µg (0.004 m) of LdPEX5-oregon green, 2.62 µg (0.008 m) of AKL-pyrene, and 400 µg of lipids in a sucrose gradient; analyzed using a Varian-Cary fluorescence spectrophotometer at 700 V, with constant excitation at 595 nm, taking a scan of emission at 72 nm/min, with an excitation slit width of 5.0 nm, and an emission slit width of 2.5 nm. B. Percent of total LdPEX5WT in each of eight fractions at emission of 519 nm after an overnight centrifugation using 20 µg (0.004 m) of LdPEX14WT-texas red, 29 µg (0.004 m) of LdPEX5-oregon green, 2.62 µg (0.008 m) of AKL-pyrene, and 400 µg of lipids in a sucrose gradient; analyzed using a Varian-Cary fluorescence spectrophotometer at 700 V, with constant excitation at 496 nm, taking a scan of emission at 72 nm/min, with an excitation slit width of 5.0 nm, and an emission slit width of 2.5nm.

Analysis of LdPEX5 has predicted an intrinsically disordered N-terminus, which has been shown to be highly sensitive to proteolytic degradation [4], and a C-terminus composed of seven tetratricopeptide repeats that have been postulated to fold in a manner which is highly resistant to proteolysis. Treatment of LdPEX5 with trypsin reduced the protein to a fragment of ~30 kDa corresponding to the organized C-terminus spanning residues 301-625 [4]. Taking into account that the region of LdPEX5 necessary for insertion is residues 268-302, we then performed limited proteolysis of LdPEX5 and LdPEX14 after flotation with liposomes to determine the degree of protection from degradation conferred from insertion into the liposomal membrane. For samples of LdPEX14-liposomes solubilized with Triton X-100 there was almost complete degradation after proteolysis, with no retention of the wild type protein (Fig. 3.7A). In contrast, samples of LdPEX14-liposomes without a solubilizing agent retained a small portion of wild type LdPEX14 after degradation with trypsin (Fig. 3.7B). This implies that the association of LdPEX14-liposomes is conferring some degree of protection to the wild type protein, although the majority of the protein is degraded, which is consistent with previous findings of LdPEX14 acting as a peripheral, exposed membrane protein [5]. Degradation at time zero was likely due to degradation of the proteins during the incubation at room temperature before flotation. Proteolysis of liposomes containing the LdPEX5-LdPEX14 complex solubilized with Triton X-100 resulted in a final degradation fragment size of ~30 kDa (Fig. 3.7C), corresponding to the highly ordered C-terminus portion of LdPEX5. The majority of LdPEX5 degradation observed was in the N-terminus, which is predicted to have a highly disordered structure [4]. However, treatment of intact liposomes with trypsin showed an altered degradation pattern that generated a fragment of ~50 kDa (Fig. 3.7D), which could be due to increased protection from proteolysis by membrane insertion or from an altered interaction with LdPEX14 bound to the liposomal membrane. These data suggest that the interaction of LdPEX5 with LdPEX14 at a liposomal membrane triggers LdPEX5 to become more resistant to proteolysis, possibly due to insertion into the membrane.

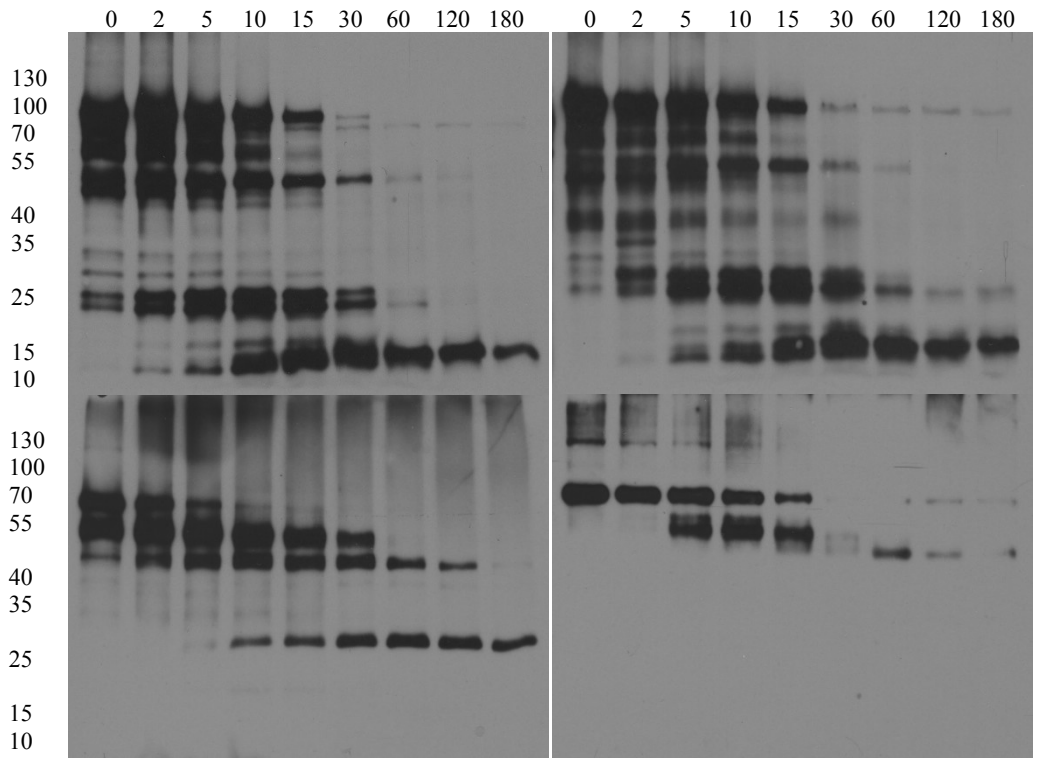


Figure 3.7 Limited proteolysis of LdPEX14 and LdPEX14-LdPEX5 after flotation. After LdPEX5-LdPEX14 and LdPEX14 were floated with LUVs the top two fractions were collected and pooled. Liposome-protein complexes were pelleted using a TLA-100 rotor in the Beckman-Coulter Optima MAX ultracentrifuge; fractions were washed using PBS-150. Control samples were solubilized using a 7.5:1 molar ratio of Triton X-100 to protein [163]. Controls and samples were treated with sequencing grade porcine trypsin at a ratio of 200:1 (w/w) at 20ºC. Aliquots of 50 µl were sequentially removed at specific time points, detailed at the top of the blots, and added to 25µl of an EDTA-free protease inhibitor cocktail (1 tablet/ml PBS) to terminate proteolysis. Digests were analyzed by Western blot.

A. (Top left) LdPEX14 control, probed for αPEX14
B. (Top right) LdPEX14 sample, probed for αPEX14
C. (Bottom left) LdPEX14-LdPEX5 control, probed for αPEX5
D. (Bottom right) LdPEX14-LdPEX5 sample, probed for αPEX5

3.4.5 Loss of N-terminus does not affect LdPEX5 secondary structure

Considering the putative insertion domain of LdPEX5 is at 268-303 we then worked to structurally characterize LdPEX5 and an N-terminus truncation of LdPEX5 using the Greenfield method [128]. Tryptophan is the dominant amino

acid that interplays in the far UV region of CD, and the difference in content between LdPEX5 and ldpex5 is the sole tryptophan at position 53 (Fig. 3.8). Taking CD readings in the far UV we found that at a 1:1 molar ratio the predicted alpha-helical percentages for LdPEX5 and ldpex5 were 60 and 57, and experimental percentages were 61 and 62, respectively (Table 3.1; Fig 9A). Therefore there was not a great discrepancy between predicted secondary structures, generated using the online GOR4 algorithm [165], and experimental percentages for either LdPEX5 or ldpex5. Moreover, direct comparison of LdPEX5 and ldpex5 reveals insignificant differences between the two proteins, implying that no major structural rearrangement has taken place.

It has been previously demonstrated that LdPEX5 forms a homotetramer in solution. Upon interaction with a PTS1 ligand this tetramer dissociates into dimers of LdPEX5:PTS1 with a 2:1 ratio. When the PTS1 concentration is increased these dimers are removed to monomers with a LdPEX5:PTS1 ratio of 1:1, which are stabilized by two domains; one at the coiled-coiled motif near the centre of the protein, and one in the N-terminus of the protein [148]. We then investigated possible structural rearrangements caused by the LdPEX5-PTS1 interaction, which could be facilitating a change in LdPEX5 necessary for membrane insertion. Using circular dichroism it was found that for LdPEX5/ldpex5:PTS1 at a 1:1 molar ratio the alpha-helical content for LdPEX5 and ldpex5 was 50% and 56%, respectively, and the disordered content was 29% and 24%, respectively (Table 3.1; Fig. 3.9B).

```
  1 mdcntgmqlg qqfskdatmm hggvpmsgam seqdalmvga qvaganpmma aqwaqnfqqq
 61 qamqamrqqh emeqafqnsq qqqaaaaqsr qmlgmagpqq qqfmaqqqqa smmnaammsq
121 gmmaanmglg mmmprtqyqp lpnlsalqpk qqqplanlap aaqdsawadq lsqqqwstdy
181 sqvqtfsapg medktveeri kdsefykfmd qvknkevlid eekgelvqgp gpevgvpeda
241 eylrhwaeme glhmpesvfq spppasamts pengdpdayv kemdmaandv edwaqeyaem
301 qerlqkvtns tdypfepnnp ymfhdfpfde gmemlqlgnl aeaalafeav chkdssneka
361 wqilgttqae nekdglaiia lnnarklnpr nlevhaalsv shtnernada amdslkawli
421 nhpeyeqlas vsippnaeld vqetfffadp srmreartly eaaiemnpsd sqlftnlgvl
481 hnvahefdea aecfrkaval hpddpkmwnk lgatlanggh pdqaleaynr aldinpgyvr
541 amynmavays nmsqynmaar qivkaiasqq ggtkpsgegs imatrnmwdl lrmtlnlmdr
601 ddlvqltyne qlepfvkefg leghv
```

Figure 3.8 Primary sequence of LdPEX5. Primary sequence of LdPEX5 with tryptophan residues highlighted in yellow, and the sole tryptophan disparity between LdPEX5 and ldpex5(Δ1-111) circled in red.

	Predicted LdPEX5 (GOR4) [165]	Predicted ldpex5(Δ1-111) (GOR4) [165]	2μM LdPEX5	2μM ldpex5(Δ1-111)	2μM LdPEX5 + 2μM AKL	2μM ldpex5(Δ1-111) + 2μM AKL
Helix	60	57	61	62	50	56
Strand	6	5	7	5	7	6
Turns			11	12	12	14
Disordered	34	38	22	20	29	24
Total	100	100	101	99	99	100

Table 3.1 Circular dichroism analyses. Comparison of percent composition of alpha-helices, β-strand, β-turn, and disordered structure for: LdPEX5 generated using the GOR4 algorithim; ldpex5(Δ1-111) generated using the GOR4 algorithim; 2 μM LdPEX5; 2 μM ldpex5(Δ1-111); 2 μM LdPEX5 with 2 μM AKL (1:1 molar ratio); and 2 μM ldpex5(Δ1-111) with 2 μM AKL (1:1 molar ratio). Samples were extensively dialyzed at 4ºC against 10 mM phosphate buffer pH 7.6. All sample spectra were generated on a Jasco 810 spectropolarimeter at 20ºC with a scan rate of 50 nm/min and a cuvette with a pathlength of 0.1 cm. Spectra were deconvoluted using CDSSTR algorithim in DICHROWEB with the reference protein set 7. Scans were collected from 260-190 nm.

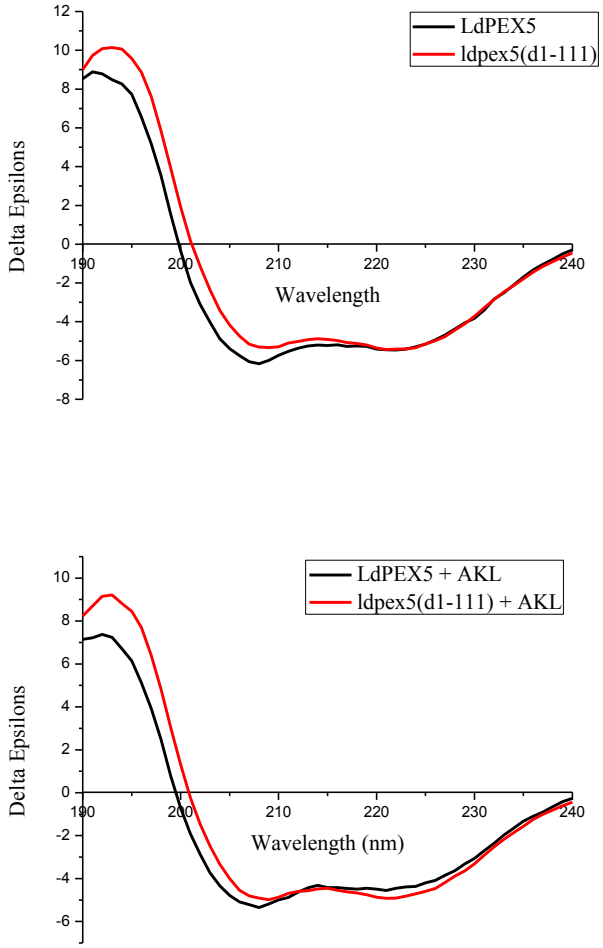


Figure 3.9 Analyses of LdPEX5/ldpex5 secondary structure. All samples were extensively dialyzed at 4ºC against 10 mM phosphate buffer pH 7.6. All sample spectra were generated on a Jasco 810 spectropolarimeter at 20ºC with a scan rate of 50 nm/min and a cuvette with a pathlength of 0.1 cm. Spectra were deconvoluted using CDSSTR algorithm in DICHROWEB with the reference protein set 7. Scans were collected from 260-190 nm.

A. (Top) 2 µM LdPEX5; 2 µM ldpex5(Δ1-111)
B. (Bottom) 2 µM LdPEX5 with 2 µM AKL (1:1 molar ratio); 2 µM ldpex5(Δ1-111) with 2 µM AKL (1:1 molar ratio).

3.4.6 Loss of the N-terminus affects conformational change in LdPEX5 on binding a PTS1 ligand

To further investigate the interaction of LdPEX5-PTS1 and ldpex5-PTS1, to attempt to correlate the increase in alpha-helical content with a conformational change, we analyzed intrinsic protein fluorescence. This biophysical technique takes advantage of the naturally occurring fluorescence of the aromatic amino acids. We specifically focused on the tryptophan fluorescence, as this environmental probe is sensitive to its immediate environment. The difference in tryptophan content is one amino acid at position 53, from 9 tryptophans in LdPEX5 to 8 tryptophans in ldpex5(Δ1-111), highlighted by the red circle in Fig. 3.8. This amino acid was used as a diagnostic probe to look for differences in conformational change between the two proteins. At increasing concentrations of AKL there is a shift in the spectra of LdPEX5 (Fig. 3.10A) to a more non-polar environment, while there no shift observed for ldpex5 (Fig. 3.10B). To clarify this disparity, λ_{max} was plotted at increasing concentrations of AKL (Fig. 3.10C), verifying the shift in LdPEX5 spectra and suggesting there is a conformational change occurring in LdPEX5 at increasing concentrations of AKL that is not observed for ldpex5(Δ1-111).

Since one probe is not sufficient to analyze conformational change, we used the anionic extrinsic probe ANS (1-anilino-8-napthalene sulphonate). ANS is sensitive to hydrophobic patches on a surface or in a crevasse. The excitation of tryptophans at 290 nm produces an emission at 350 nm that excites the ANS, producing an emission spectrum. In solution with LdPEX5, there is an increase in ANS intensity observed when the concentration of PTS1 is increased (Fig. 3.11A), as well as a shift in maximum intensity to a more non-polar environment that saturates (Fig. 3.11B). These results reveal a dramatic conformational change in LdPEX5, exposing a hydrophobic domain upon interaction with the PTS1 AKL. This was not surprising, as during dissociation of the LdPEX5 tetramer a conformational change was predicted. When ANS was in solution with ldpex5 there was also a marked increase in intensity when the concentration of PTS1 was

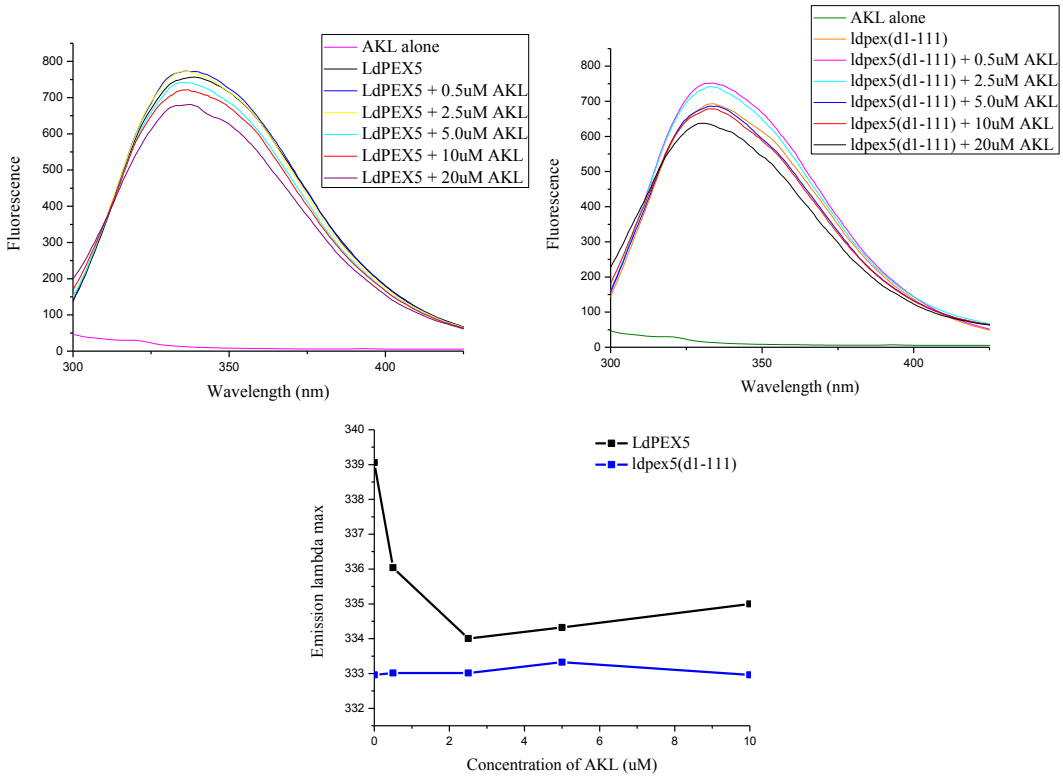


Figure 3.10 Intrinsic fluorescence analyses of LdPEX5 and ldpex5(Δ1-111). Intrinsic fluorescence spectra were produced on a Varian Cary Eclipse spectrofluorometer at 25ºC using an excitation wavelength of 290 nm, a scan rate of 120 nm/min with excitation and emission slit widths of 5nm. Spectra were recorded from 300-550 nm (displayed from 300-425 nm). Solutions of 1.25 µM LdPEX5/ldpex5 in 40 mM Tris-HCl-150 mM NaCl pH 8.0 were titrated with increasing concentrations of AKL peptide to a final concentration of 50 µM. Dilution effects were corrected for using a cell with buffer additions in parallel.

A. (Top left) Spectra of LdPEX5 at increasing concentrations of AKL
B. (Top right) Spectra of ldpex5(Δ1-111) at increasing concentrations of AKL
C. (Bottom) Plot of the shift in λ_{max} of fluorescence spectra observed at increasing concentrations of AKL peptide in LdPEX5 and ldpex5(Δ1-111) plotted against increasing concentrations of AKL.

increased (Fig. 3.11C), and a shift in maximum intensity to a more non-polar environment that saturated (Fig. 3.11D).

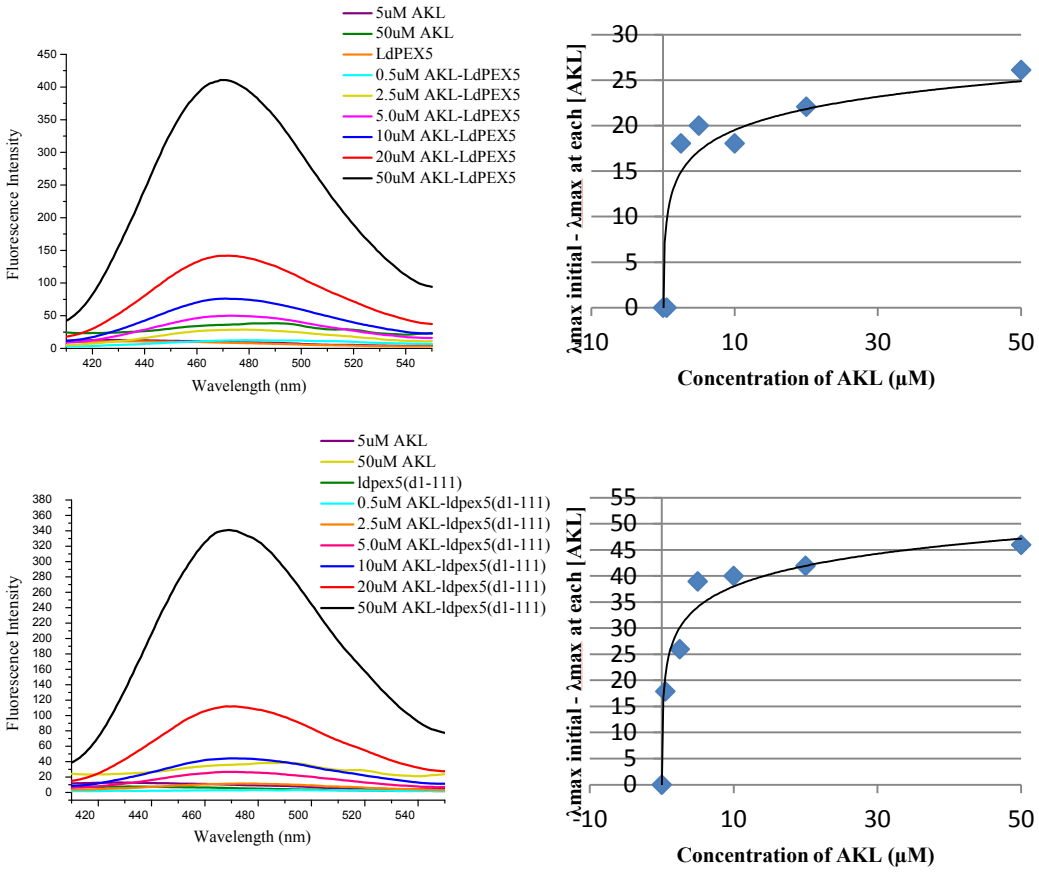


Figure 3.11 Extrinsic fluorescence analyses of LdPEX5 and ldpex5(Δ1-111) using ANS. Fluorescence spectra were acquired on a Varian Cary Eclipse spectrofluorometer at 25ºC using an excitation wavelength of 290 nm, a scan rate of 120 nm/min with excitation and emission slit widths of 5 nm. Spectra were recorded from 400-550 nm. Solutions of 1.25 µM LdPEX5/ldpex5 in 40 mM Tris- 150 mM NaCl contained 50 µM of the hydrophobic, anionic probe 8-anilino-1-napthalene-sulfonate (ANS) were collected at increasing intervals of AKL.

A. (Top left) Plot of LdPEX5 at increasing concentrations of AKL, displayed from 410-560 nm
B. (Top right) Shift in lambda max of LdPEX5 spectra (from A) plotted against increasing concentrations of AKL.
C. (Bottom left) Plot of ldpex5(Δ1-111) at increasing concentrations of AKL, displayed from 410-560 nm.
D. (Bottom right) Shift in λ_{max} of ldpex5(Δ1-111) spectra (from C) plotted against increasing concentrations of AKL.

3.5 Discussion

PEX5 is a vital component of the peroxisomal/glycosomal protein import machinery and has been demonstrated to play an important role in both shuttling proteins to the peroxisomal/glycosomal membrane and in facilitating their entry into the organelle [12, 143, 153, 155, 166]. Our findings suggest that in the process of glycosomal protein import LdPEX5 inserts into the glycosomal membrane in a reversible manner.

The import machinery of peroxisomes/glycosomes is novel from that of other organelles in its ability to translocate folded and even oligomeric proteins across the membrane without affecting the integrity of the organelle's permeability barrier [167, 168]. This tight regulation is particularly important in kinetoplasts, as leakage of intermediates from the glycosomal matrix may be toxic to the parasite [13, 81, 169]. Several mechanisms for protein translocation have been postulated [170, 171], although most models describe the formation of large transient import pores [172]. Indeed, in mammalian cells large conductance channels have been identified in peroxisomes using patch-clamp techniques [173, 174]. In the process of glycosomal protein import the docking of PEX5-PTS1 and PEX7-PTS2 to PEX14 and to a lesser degree PEX13 has been suggested to be an important first step in creation of these putative import pores [104, 175]. To examine the LdPEX5-LdPEX14 interaction at a membrane we used sucrose density flotation experiments using LUVs with a phospholipid composition mimicking the glycosomal membrane [112]. We found that when LdPEX5 was centrifuged alone, with liposomes, or with AKL and liposomes the protein was unable to float (Fig. 3.1), suggesting that the LdPEX5 alone and LdPEX5-PTS1 are unable to interact with liposomes. This was surprising, as in yeast and mammalian PEX5 has been shown to spontaneously insert into lipid membranes [155, 166]. However, this finding is supported by previous reports of very low amounts of LdPEX5 co-purifying with glycosomes [104], suggesting that in the pre-import complex LdPEX5 is only making transient interactions. In the following flotation LdPEX5 was added to LdPEX14-liposomes pre-centrifugation.

After centrifugation it was observed that both LdPEX5 and LdPEX14 were found in the top fractions, and therefore were able to float (Fig. 3.1), indicating that LdPEX14 is required on liposomal membranes in order for LdPEX5 to associate. Similarly, when the previous flotation was repeated with the addition of AKL it was found that both LdPEX5 and LdPEX14 were able to float, implying that the addition of a PTS does not affect the ability of LdPEX5 to interact with LdPEX14 at a liposomal membrane. Interestingly, in flotations of LdPEX5-ldpex14(1-120) or AKL-LdPEX5-ldpex14(1-120) LdPEX5 was found in the bottom fractions, confirming the necessity of LdPEX14 to interact with the membrane prior to LdPEX5 association. This result agrees with sedimentation analyses with glycosomes which showed that recombinant LdPEX5 is able to sediment with the glycosomal fraction containing native LdPEX14 [104], but not alone. The necessity of LdPEX14 in LdPEX5 floating was further verified using fluorescently labelled proteins in flotations. These analyses demonstrated that the overall abundance of LdPEX14 that floated was decreased when LdPEX14 was first incubated with LdPEX5, suggesting that the proteins are interacting. The decrease in LdPEX14 abundance could be due to LdPEX14-LdPEX5 complexes crashing out of solution before being able to interact with the liposomal membrane, exemplified by the high percentage of proteins observed in the bottom fractions (Fig. 3.6A). Furthermore, it was observed that the abundance of LdPEX5 in the top fractions after centrifugation increased when the LdPEX14-LdPEX5 complex was floated, compared with LdPEX5 alone (Fig. 3.6B), thus confirming the necessity of LdPEX14 for LdPEX5 to interact with a liposomal membrane. To map the region of LdPEX5 necessary for this interaction we used several truncation and deletion constructs of LdPEX5 (Fig. 3.2) in flotation assays with LdPEX14 at a liposomal membrane (Fig. 3.3). The observed pattern of floating, especially that ldpex5(268-625) floated and ldpex5(303-625) did not float, identified the region of 268-303 as crucial for the LdPEX5-LdPEX14 interaction at a liposomal membrane. This corroborates previous findings of the LdPEX14 domain spanning residues 270-300 of LdPEX5, determined using ELISAs and pull-down assays [94]. This implicates the region identified as being

necessary for the protein-protein interaction at the liposomal membrane, but cannot be implicated in the association of LdPEX5 directly with the liposomal membrane, without further analyses.

To further characterize the interaction of LdPEX5 with LdPEX14 on lipid bilayers serial carbonate-urea extractions were conducted to examine the interaction of LdPEX5 with membranes with a phospholipid composition similar to the glycosomal membrane. Following these extractions both LdPEX14 and LdPEX5 remained in the pellet fraction, meaning that these stringent conditions were not sufficient to dissociate the protein lipid interaction (Fig. 3.4). In a subsequent extraction it was demonstrated that solubilization of the liposomes using Triton X-100 was sufficient to remove both proteins from the pellet fraction, indicating that the proteins are interacting with the membranes in a reversible manner (Fig. 3.5). Together, these results are diagnostic of LdPEX14 and LdPEX5 association with the membrane being stabilized by hydrophobic interactions with the nonpolar lipid bilayer core, a feature that is typically observed with integral membrane proteins. These findings support previous reports with the yeast Pex5p showing changes in its topology when this receptor transitions from a soluble cytosolic receptor to an integral membrane bound form, an event that was central to assembly of a 9 nm ion-conducting channel in association with Pex14p [176]. In comparison, slightly larger 10-40 nm diameter bowl-like structures were observed using IEM in *L. donovani* promastigotes [112]. What is surprising, however, is that hydropathy plots of LdPEX5 failed to show the presence of amphipathic or transmembrane domains helical structure(s) that would be able to bind the hydrophobic core of the lipid bilayer. In mammalian cells PEX5 has been suggested to translocate into the peroxisomal lumen where it releases the PTS1 cargo, recycling back into the cytosol [153]. To map the motif required for the LdPEX5-membrane interaction carbonate urea extractions were performed on LdPEX5/ldpex5-LdPEX14 flotations. The N-terminal truncations ldpex5(Δ1-111), ldpex5(Δ1-205), and ldpex5(268-625), and a C-terminal truncation ldpex5(1-391) were all able to resist extraction and remain in the pellet fraction after stringent urea treatment (Fig. 3.4), suggesting that these truncations

of ldpex5 were making hydrophobic interactions with the lipid core of the membrane. Together these results suggest that the region of LdPEX5 identified at 268-302 is necessary for protein-protein interactions with LdPEX14 at a liposomal membrane and also for interacting directly with the core of the membrane. It's not clear if LdPEX5 is dipping or fully penetrating into the membrane, but it seems clear that at the point of interaction with LdPEX14 there is a change in LdPEX5 facilitating insertion of the protein into the glycosomal membrane.

This insertion event was further verified using a protease protection assay. As controls LdPEX14-liposomes were floated; an aliquot of LdPEX14-liposomes were solubilized with Triton X-100 and an aliquot of liposomes unsolubilized were then both treated with trypsin. The solubilized sample showed almost complete degradation, while the unsolubilized sample displayed some retention of wild type LdPEX14 (Fig. 3.7A/B), indicative of a peripheral membrane protein that is conferred some protection from proteolysis by loose association with the membrane [5, 104]. Following this, the LdPEX5-LdPEX14 complex was floated; a solubilized and an unsolubilized sample were treated with trypsin, with aliquots being removed at specific time points. At three hours in the solubilized sample there was a reduction of LdPEX5 to ~30kDa (Fig. 3.7C), representing the highly organized stable C-terminal, while in the unsolubilized sample there was reduction of LdPEX5 to ~50kDa (Fig. 3.7D). This increase in final digestion product is likely due to protection from the membrane upon insertion, although it could also be due to an altered interaction LdPEX14-LdPEX5 interaction. This suggests that the region of LdPEX5 interacting with the membrane is upstream of the C-terminal, agreeing with results of the serial extraction.

In the glycosomal import pathway LdPEX5 makes at least two important interactions, first with its PTS1 cargo, and then docking at LdPEX14 at the glycosomal membrane. At this point we looked at interactions preceding the insertion event, to look for potential structural rearrangements that could be indicative of a change facilitating insertion into the membrane. *In vivo*, LdPEX7

likely plays an important role in the shuttling and docking of cargo, but in these *in vitro* experiments was left out to focus on the role and potential conformational changes of LdPEX5. For these analyses LdPEX5 was compared with an N-terminal truncation ldpex5(Δ1-111) to assess the affect of truncating the protein downstream of the putative insertion region. CD readings taken in the far UV show that at a 1:1 molar ratio there is not a great discrepancy between predicted and experimental percentages of LdPEX5 and ldpex5 nor between LdPEX5 and ldpex5 (Fig. 3.9A; Table 3.1). This suggests that the truncation is not causing any major structural rearrangement(s). However, when comparing the CD readings for each of the proteins with PTS1 at a 1:1 molar ratio it was observed that ldpex5 displayed a decrease in disordered content and an increase in alpha-helical content (Fig. 3.9B; Table 3.1). This decrease was expected, as it has been shown that the N-terminus of LdPEX5 is highly disordered and sensitive to proteolytic degradation. Considering that the domain truncated is downstream from the predicted insertion domain the increase in alpha-helical content in ldpex5 compared with LdPEX5 is interesting and could be linked to a change in function. With increasing concentration of PTS1 a shift in spectra maxima was visualized using intrinsic protein fluorescence for LdPEX5 but not for ldpex5 (Fig. 3.10). Together with the results from CD this implies that there are major changes in the N-terminus region. LdPEX5 is binding AKL, sending a message altering the N-terminus of the protein, causing the tryptophan at position at 53 to move into a hydrophobic environment. Furthermore, when analyzing extrinsic protein fluorescence a shift in spectra maxima and robust increase in intensity was observed (Fig. 3.11A-D). The ANS probe demonstrated that the binding of a PTS1 is altering the structure of LdPEX5, causing the exposure of a hydrophobic, non-polar domain. When this was repeated with an N-terminal truncation a conformational change was also seen, indicating that this hydrophobic domain exposed is downstream of residue 111.

Here we have demonstrated that at the point of interaction with a PTS1 ligand LdPEX5 undergoes a marked conformational change and minor structural rearrangement, which is likely indicative of a change in function. Once shuttled to

the membrane LdPEX5 requires LdPEX14 for incorporation in the pre-import complex, although once part of the importomer complex we have shown that this protein inserts into the membrane in an integral manner facilitated by a domain at residues 268-302. This insertion event is coupled to the insertion of LdPEX14 into the glycosomal membrane [113], suggesting that LdPEX14 could be drawing LdPEX5 into the membrane via the interaction domain and that the insertion of the two plays an important role in pore formation. Previous work has demonstrated that insertion of the two proteins is not sufficient to import a PTS1 into the glycosome [112], suggesting that homologues of importomer machinery identified in other systems are likely needed for pore functionality and import, such as the RING subcomplex PEX2/PEX10/PEX12 [144, 155, 156, 177], and PEX13 [99, 106, 117, 166].

Protease protection assays further confirmed that a domain beyond the C-terminal was remaining protected by insertion into the membrane. Results of fluorescence spectroscopy suggest that an important conformational change occurs at the point of interaction between LdPEX5 and a PTS1 revealing a hydrophobic patch. This revealed domain could play an important role in membrane insertion. Using CD we were able to further characterize a minor structural change at the point of interaction that also could be important in insertion. Together these results indicate that a domain situated in near the centre of LdPEX5 is involved in mediating both the LdPEX14-LdPEX5 interaction and the LdPEX5-membrane interaction. Finally, this work provides insight into the role of LdPEX5 in PTS1 glycosomal import.

Chapter 4

Summary and Conclusions

4.1 Summary

Leishmaniasis has been deemed an NTD by the WHO due to the poor socioeconomic standing in endemic countries, compounded with a lack of vaccine or affordable efficacious treatment options. Although interest in combating the NTDs has been increasing, such as strategies outlined in the United Nations Millennium Goals [178] and incentives by the Bill and Melinda Gates Foundation [179], the funding for NTDs that are not easily treated has not increased proportionately, leaving leishmaniasis, African trypanosomiasis, and Chagas' disease behind. Therefore, leishmaniasis remains a significant NTD, with an estimated 59,000 deaths attributed each year. Sadly, this mortality rate is likely an underestimate, due to a lack of available diagnostic tools, and inaccurate reports of the diseases severity [180, 181]. Together, these limitations in the battle against leishmaniasis necessitate further research into the biology of the parasite, to examine fundamental differences that could potentially be exploited in rational drug design.

An organelle within the parasite, the glycosome, has been identified as having an import pathway that is not only an interesting biological system, but also an attractive drug target. This organelle shares structural and morphological features with peroxisomes of higher eukaryotes, although proteins of the import pathways share very low homology. These proteins have been shown to be necessary for the viability of the parasite, as no mutant lines lacking any member of the import pathway have been successfully generated, and knocking down any of the proteins in the closely related *T. brucei* is fatal. At the glycosomal membrane, members of the import pathway form a transient import pore, which allows for tightly regulated passage of proteins into the lumen of the organelle. Although parts of the import mechanism have been elucidated, for the large part this pore formation in *Leishmania* is poorly understood.

In this study we focused on key proteins of the import machinery of *Leishmania* glycosomes, specifically the role of *L. donovani* peroxin 5 (LdPEX5) in the shuttling of proteins to the surface and into the lumen of the organelle. The

relationship of the import receptor LdPEX5 with a model PTS1 was analyzed by use of biophysical techniques. Using circular dichroism, it was found that the interaction did not induce changes in the secondary structure of the protein, while fluorescence analyses of the relationship revealed a marked conformational change, which could be an important event preceding and promoting interactions made at the glycosomal membrane. Moreover, using biochemical investigations at a glycosomal mimetic, the domain of LdPEX5 necessary to interact with LdPEX14 at a glycosomal membrane was mapped to residues 268-302. Furthermore, using serial extractions this domain was also implicated in inserting into the glycosomal membrane suggesting that LdPEX14 could be drawing LdPEX5 into the membrane as part of transient pore formation. This research adds to our understanding of several important protein-protein interactions that precede transient pore formation, and add insight into the important role of LdPEX5 in the formation of this import pore.

4.2 Conclusions

The glycosomal import machinery of *L. donovani* has been identified as an attractive drug target, yet the mechanism of import is still poorly understood. Results from this study have added to our understanding of the import mechanism. Our revised model is as follows:

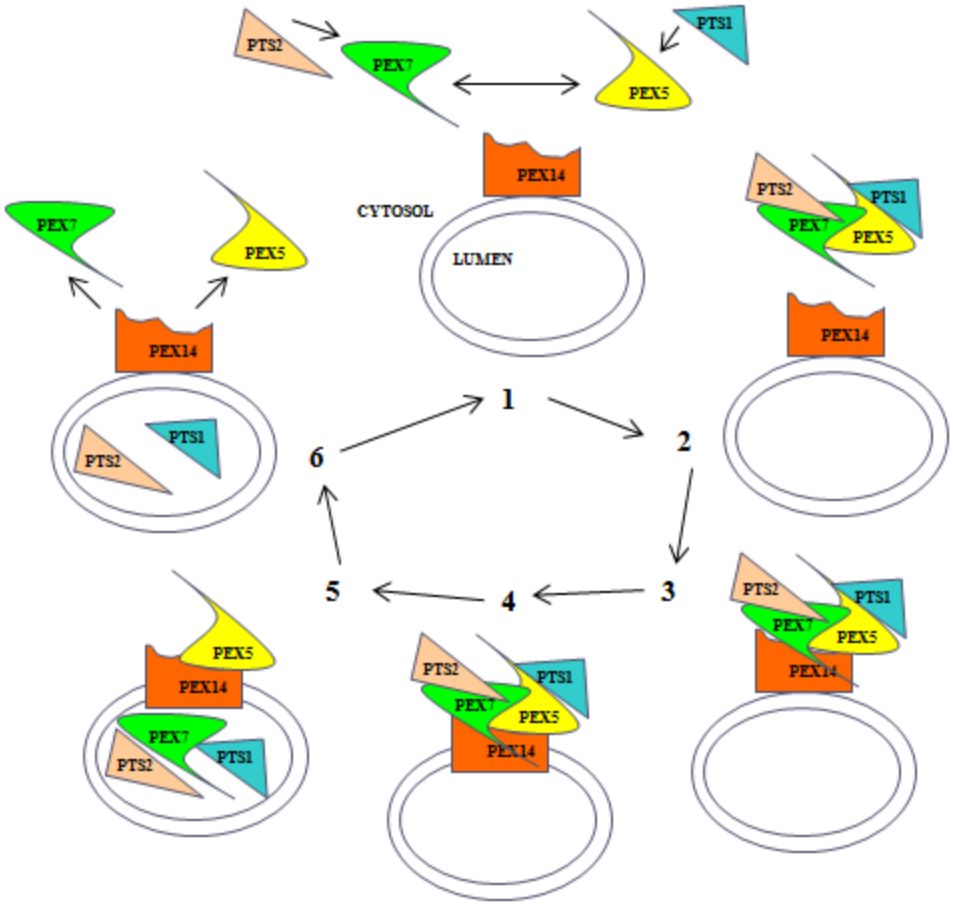


Figure 4.1. Revised glycosomal import model in *Leishmania*. An updated model of glycosomal import using previous data and results from this study. (1) In the cytosol, LdPEX7 recruits PTS2 proteins, LdPEX5 recruits PTS1 proteins. The interaction with PTS1 cargo does not cause structural changes in LdPEX5, although provokes a marked conformational change. (2) LdPEX5-PTS1 and LdPEX7-PTS2 form heteromeric complexes in the cytosol. (3) These complexes dock at LdPEX14, sitting peripheral to the glycosomal membrane. LdPEX5 interacts with LdPEX14 at a domain located at 268-302. (4) LdPEX14 undergoes a conformational change facilitating its insertion into the glycosomal membrane and a creation of a transient import pore. This insertion of LdPEX14 draws LdPEX5 into the membrane, as part of pore formation. (5) LdPEX7 and the PTS proteins are transported into the glycosomal lumen. (6) LdPEX14 resumes its position peripheral to the glycosomal membrane. LdPEX5 and LdPEX7 are recycled into the cytosol by a yet undefined mechanism, ready to resume the import cycle [4-6, 94, 104, 108, 112, 148] (Adapted from Rona Strasser's figures with kind permission).

This revised model will facilitate future studies looking to identify and understand the steps required for glycosomal import. The full complement of proteins necessary for glycosomal protein import remains unclear, although results from this study support LdPEX5 as playing a central role in this process.

5. References

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