McGILL UNIVERSITY

Role of *Leishmania donovani* peroxin 14 in glycosomal import machinery

by

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Declaration of Authorship

I, NORMAND CYR, declare that this thesis titled, 'ROLE OF *LEISHMANIA DONOVANI* PEROXIN 14 IN GLYCOSOMAL IMPORT MACHINERY' and the work presented in it are my own. I confirm that:

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"Trying to determine the structure of a protein by UV spectroscopy was like trying to determine the structure of a piano by listening to the sound it made while being dropped down a flight of stairs."

Francis Crick (1916-2004)

Abstract

Peroxisomes are organelles found in eukaryotic cells where several oxidative processes take place. Enzymes destined for the peroxisome generally contain either a C-terminal or a N-terminal peroxisomal targeting signal, named PTS1 and PTS2 respectively. These signals are cytosolically recognized by their correspondent receptors PEX5 and PEX7 prior to being recruited at the surface of the peroxisomal membrane where the complex docks onto PEX14. Then, enzymes are transported in a natively folded fashion inside the organelle where they are released. Such general mechanism also exist in the protozoan parasite *Leishmania* and in the evolutionarily related trypanosomatids (including the medically relevant parasites *Trypanosoma brucei* and *Trypanosoma cruzi*). However, in these micro-organisms, other metabolic pathways, including glycolysis, are also segregated in the organelle, which has been renamed the glycosome to better reflect its purpose. Giving the fact that these parasites cause debilitating diseases in human and in animals of veterinary importance, such unique feature becomes attractive as a potential drug target.

To gain a better understanding about this transport and translocation machinery that exist in *Leishmania donovani*, we have first looked at the quaternary structure of the docking protein LdPEX14, and the implication of several domains on the protein using various biochemical and biophysical techniques. Using gel permeation chromatography, analytical ultracentrifugation and chemical cross-linking, we observed that LdPEX14 was forming large oligomeric structures of varying sized both in solution and within the parasite. By domain mapping studies, we identified three domains on LdPEX14, the LdPEX5 binding site, an hydrophobic region and a predicted leucine zipper, to all being implicated in the formation of such oligomer. Moreover, we investigated the structural changes that take place on LdPEX14 upon binding LdPEX5.

Both isothermal titration calorimetry and fluorescence spectroscopy revealed that the hydrophobic region of LdPEX14 became more exposed to the solvent upon attachment to LdPEX5. Likewise, circular dichroism studies demonstrated that a portion of LdPEX14 was losing some secondary structure upon binding to LdPEX5, which was also linked to the formation of a more compact complex based on analytical ultracentrifugation experiments. We hypothesized that these structural features and conformational changes could be link to the formation of pore that will facilitate the translocation of proteins targeted to the glycosome.

Such oligmeric structure was also confirmed by immunoelectron microscopy using parasite sections. LdPEX14 clusters adopted round, rosette-like shapes of about 30-40 nm in diameter and were found mainly at the surface of the glycosomal membrane. A lipidomic investigation of the glycosomal membrane from *Leishmania donovani* revealed the presence of very long chain polyunsaturated fatty acids, and of a relatively high proportion of the negatively charged phospholipids phosphatidyl glycerol and phosphatidyl inositol. These phospholipids appeared to be essential for a proper membrane attachment of LdPEX14. Domain mapping showed that the hydrophobic region of LdPEX14, comprised between the amino acids 149 and 179, was essential for proper binding to liposomes mimicking the glycosomal membrane phospholipid composition. Bioinformatics analysis revealed that this region also contains a important cluster of basic residues which we hypothesize could be involved in the initial membrane attachment.

A further investigation using fluorescence spectroscopy showed that this region was sufficient for binding to liposomes mimicking the glycosomal and was penetrating the lipid bilayer. By performing a dye leakage assay from the liposomes, we observed that ldpex14 (120-200), a peptide comprising the hydrophobic region of LdPEX14, was capable of perforating the liposomes and cause a leakage of the encapsulated dye. Finally, using density flotation assays with liposomes, we demonstrated that not only LdPEX14 was recruting LdPEX5 at the surface of the bilayer, but that LdPEX5, upon attachment, was adopting a conformation similar to integral membrane proteins. This observation tends to suggest that LdPEX5 could also be implicated in the pore formation for the translocation of glycosomal enzymes.

The results presented in this PhD thesis shed the light on some of the mechanistic details of the glycosomal translocation machinery in *Leishmania donovani*, including the assembly of LdPEX14, the conformational changes that take place upon attachment to LdPEX5, the membrane binding capability of LdPEX14 and the role of its hydrophobic region in attachment and possibly in the formation of a pore through the glycosomal membrane.

Abrégé

Le peroxysome est un organite des cellules eucaryotes où plusieurs procédés oxydatifs prennent place. Les enzymes destinés au peroxysome contiennent un signal peptidique soit à leur terminal C ou N, nommés PTS1 ou PTS2 respectivement. Ce signal est reconnu dans le cytoplasme par son récepteur correspondant (PEX5-PTS1, PEX7-PTS2) avant d'être dirigé vers la surface du peroxysome où le complexe PTS1-PEX5/PEX7-PTS2 s'arrime à PEX14. Ensuite, les enzymes sont transportés sous leur forme native à l'intérieur de l'organite où ils sont relâchés de leur récepteur. Ce mécanisme existe chez le parasite protozoaire *Leishmania* et chez les autres trypanosomatides (incluant les parasites *Trypanosoma brucei* et *Trypanosoma cruzi*). Cependant, chez ces micro-organismes, plusieurs voies métaboliques, incluant la glycolyse, sont ségrégées dans l'organite (renommé le glycosome pour refléter la présence d'enzymes glycolytiques). Puisque ces parasites causent de graves maladies chez l'humain, cet aspect se présente comme une cible thérapeutique potentielle.

Afin d'obtenir une meilleure compréhension à propos du transport et de la machinerie de translocation existant chez *Leishmania donovani*, nous avons d'abord investigué l'implication des différents domaines de la protéine d'arrimage LdPEX14 sur sa structure quaternaire, en utilisant diverses techniques biochimiques et biophysiques. En faisant usage de la chromatographie d'exclusion stérique, de l'ultracentrifugation analytique et de la réticulation chimique, nous avons observé que LdPEX14 forme de larges structures oligomériques de taille variable en solution et dans le parasite. En excluant différents domaines sur LdPEX14, nous avons identifiés trois domaines impliqués dans la formation de ces oligomères: (i) le site d'attachement à LdPEX5, (ii) une région hydrophobe et (iii) une glissière à leucine. De plus, nous avons recherché quels étaient les changements structuraux prenant place sur LdPEX14 lors de l'attachement à LdPEX5.

En utilisant la calorimétrie isotherme à titration et la spectroscopie de fluorescence, nous avons découvert que la région hydrophobe de LdPEX14 devenait exposée au solvant lorsque LdPEX5 s'y attachait. De plus, des études de dichroïsme circulaire ont démontré qu'une portion de LdPEX14 perdait une partie de sa structure secondaire lors de l'attachement à LdPEX5, ce qui fut aussi relié à la formation d'un complexe LdPEX14-LdPEX5 plus compact, tel qu'illustré par ultracentrifugation analytique. Nous avons donc supposé que ces propriétés structurelles et ces changements de conformation seraient liés à la formation d'un pore qui faciliterait la translocation des protéines dirigées vers le glycosome.

La structure oligomérique de LdPEX14 fut aussi confirmée par microscopie immuno-électronique en utilisant des sections de parasite. La protéine LdPEX14 s'assemblait en grappes formant des rosettes d'environ 30-40 nm de diamètre, principalement à la surface extérieure de la membrane du glycosome. Une étude lipidomique de la membrane du glycosome de *Leishmania donovani* a révélé la présence élevée d'acides gras poly-insaturés, ainsi qu'une proportion importante de phosphatidyl glycérol et de phosphatidyl inositol, des phospholipides chargés négativement. Ces derniers sembleraient essentiels pour l'attachement de LdPEX14 à la membrane du glycosome. Nous avons ensuite démontré que la région hydrophobe de LdPEX14, comprise entre les acides aminés 149 et 179, était essentielle pour l'attachement à des liposomes imitant la membrane du glycosome. Une analyse bio-informatique a d'ailleurs révélé la présence de grappes d'acides aminés basiques que nous croyons impliqués dans l'attachement initial à la membrane.

Une investigation plus poussée, utilisant la spectroscopie de fluorescence, a montré que cette région était suffisante pour l'attachement à des liposomes imitant la membrane du glycosome, et était de plus capable de pénétrer cette membrane bicouche. En effectuant un test d'écoulement de colorant fluorescent encapsulé dans les liposomes, nous avons observé que ldpex14(120-200), un peptide comprenant la région hydrophobe de LdPEX14, était capable de perforer les liposomes et causer un écoulement du colorant fluorescent. Finalement, en utilisant des tests de flottaison par densité différentielle avec des liposomes, nous avons démontré que non seulement LdPEX14 est capable de recruter LdPEX5 à la surface des liposomes, mais que LdPEX5 finissaient par adopter une conformation semblable à une protéine membranaire intégrale. Cette observation tend à suggérer que LdPEX5 pour elle aussi être impliquée dans la formation d'un pore pour la translocation d'enzymes destinés pour le glycosome.

Les résultats présentés dans cette thèse de doctorat portent un regard sur plusieurs détails mécaniques de la machinerie de translocation des protéines glycosomales chez *Leishmania donovani*, incluant l'assemblage de LdPEX14, les changements conformationnels prenant place lors de l'attachement à LdPEX5, la capacité de LdPEX14 à s'attacher à la membrane du glycosome, ainsi que le rôle de sa région hydrophobe dans l'attachement et dans la formation d'un pore à travers la membrane du glycosome.

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All this would not have been possible without the amazing mentorship and friendship from my thesis supervisor, Dr. Armando Jardim. I still think he took a great risk six years ago by accepting a biochemical engineer into his laboratory to pursue a PhD. He deserves my gratitude for challenging me and transmitting his passion for science. Every day in the lab has been a learning experience.

Also, I would like to thank all the former and current members of the Jardim lab who, each in their own way, have carved the working ambiance in the lab in a way that encouraged me to collaborate, share, challenge and be challenged. Special thanks to Rona (especially), Jamie, Louis-Philippe, Hamed, Dayal, Manon, Celia, Mike, Line, and Ana for their support throughout my PhD work. Moreover, I had the chance to work closely with Amanda and Anwer, who have offered me some mentoring opportunities: thanks for your patience. Other friends from Parasitology also have to be acknowledged for making my working and social life simply great: *la conexión latina* (Yovany, Elizabeth, Pablo and Rodrigo), Vanessa, Rémi, Nick, Kevin and Virginie. Let'd keep in touch!

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Abbreviations

ADP	**A**denosine-5'-**D**i**P**hosphate
AIDS	**A**cquired **I**mmuno**D**eficient **S**yndrome
AMP	**A**denosine-5'-**M**ono**P**hosphate
APRT	**A**denine **P**hospho**R**ibosyl **T**ransferase
ATP	**A**denosine-5'-**T**ri**P**hosphate
BSA	**B**ovine **S**erum **A**lbumin
CD	**C**ircular **D**ichroism
CHAPS	3-[(3-**CH**olamidopropyl)dimethyl**A**mmonio]-1-**P**ropane**S**ulfonate
Chl	**Ch**o**l**esterol
CF	5(6)-**C**arboxy**F**luorescein
CL	**C**utaneous **L**eishmaniasis
CR3	**C**omplement **R**eceptor type **3**
DME-L	**D**ulbecco **M**odified **E**agles-***L****eishmania*** medium
DNA	**D**eoxy**R**ibo**N**ucleic **A**cid
DOPA	**D**i**O**leyl**P**hosphatidic **A**cid
DOPC	**D**i**O**leyl**P**hosphatidyl **C**holine
DOPG	**D**i**O**leyl**P**hosphatidyl **G**lycerol
DOPS	**D**i**O**leyl**P**hosphatidyl **S**erine
DTT	**D**i**T**hio**T**hreitol
EDTA	**E**thylene**D**iamine**T**etra**A**cetate
FAME	**F**atty **Acid** **M**ethyl **E**ster
FBS	**F**etal **B**ovine **S**erum
gp63	**G**lyco**P**rotein **63**
GroEL	Heat shock 60kDa protein 1 from *Escherichia coli*
HGPRT	**H**ypoxanthine-**G**uanine **P**hospho**R**ibosyl **T**ransferase
HRP	**H**orse**R**adish **P**eroxidase
HSP70	**H**eat **S**hock **P**rotein **70**
I-TASSER	**I**terative-**T**hreading/**ASS**embly/**R**enement modelling server
IEM	**I**mmuno**E**lectron **M**icroscopy
IgG	**I**mmuno**G**lobulin **G**
IMP	**I**nosine **M**ono**P**hosphate
IMPDH	**I**nosine **M**ono**P**hosphate **D**e**H**ydrogenase
IPTG	**I**so**P**ropyl**T**hio**G**alactoside
ITC	**I**sothermal **T**itration **C**alorimetry

LUV	**L**arge **U**nilamellar **V**esicles
LPG	**L**ipo**P**hospho**G**lycan
MCL	**M**uco**C**utaneous **L**eishmaniasis
mDNA	**M**itochondrial **D**eoxy**R**ibo**N**ucleic **A**cid
mRNA	**M**essenger **R**ibo**N**ucleic **A**cid
MSP	**M**ajor **S**urface **P**rotease
NAD^+/NADH	**N**icotinamide **A**denine **D**inucleotide
NMR	**N**uclear **M**agnetic **R**esonance
NMWL	**N**ominal **M**olecular **W**eight **L**imit
NTA	**N**itrilo**T**ri**A**cetate
NTD	**N**egelected **T**ropical **D**isease
OG	n-**O**ctyl-b-D-**G**lucoside
ORF	**O**pen **R**eading **F**rame
PAGE	**P**oly**A**crylamide **G**el **E**lectrophoresis
PBS	**P**hosphate **B**uffered **S**aline
PCR	**P**olymerase **C**hain **R**eaction
PDB	**P**rotein **D**ata**B**ase
PEX	**PE**ro**X**in protein
PI	**P**hosphatidyl **I**nositol
PRT	**P**hospho**R**ibosyl**T**ransferase
PTS	**P**eroxisomal **T**argeting **S**ignal
PVDF	**P**oly**V**inyli**D**ene **F**luoride
RNA	**R**ibo**N**ucleic **A**cid
RNAi	**R**ibo**N**ucleic **A**cid **I**nterference
SDS	**S**odium **D**odecyl **Sulfate**
SEC	**S**ize-**E**xclusion **C**hromatography
SELDI	**S**urface-**E**nhanced **L**aser **D**esorption **I**onization
SH3	**Src H**omology **3**
SUV	**S**mall **U**nilamellar **V**esicles
TBS	**T**ris **B**uffered **S**aline
TCA	**T**ri**C**arboxylic **A**cid
TDOC	**T**auro**D**e**O**xy**C**holate
TLC	**T**hin **L**ayer **C**hromatography
ToF	**T**ime **O**f **F**light
TPR	**T**etratrico**P**eptide **R**epeat
Tris	**Tris**(hydroxymethyl)aminomethane
VL	**V**isceral **L**eishmaniasis
VSP	**V**ariant **S**urface **G**lycoproteins
WD40	40 amino acids tryptophan-aspartate repeat
WHO	**W**orld **H**ealth **O**rganisation
XMP	**X**anthosine **M**ono**P**hosphate
XPRT	**X**anthine **P**hospho**R**ibosyl **T**ransferase

Statement of Originality

Manuscript 1

Normand Cyr, Kleber P. Mardrid, Rona Strasser, Mark Aurousseau, Ron Finn, Juan Ausio and Armando Jardim (2008)

The *Leishmania donovani* Peroxin 14 Undergoes a Marked Conformational Change Following Association with Peroxin 5.

Journal of Biological Chemistry 283(46):31488-99.

In this manuscript, a structural characterization of the glycosomal membrane receptor PEX14 from *Leishmania donovani* and of its interaction with the PTS1 receptor PEX5 was assessed by various biophysical and biochemical means. Previously, Furuya *et al.* (2002) and Galland *et al.* (2007) have demonstrated, using RNA interference in the evolutionary related parasite *Trypanosoma brucei* that TbPEX14 and TbPEX5 are proteins essential components for the viability of the parasite and therefore the capability to interfere with the interaction between these two proteins present an attractive drug target. Our work expands on this and describes the quaternary structure of LdPEX14 and identify the domains on the protein that are involved in such assembly. We also investigates on the secondary and tertiary structure changes that take place upon LdPEX14-LdPEX5 complex formation and hypothesizes on the capability for an hydrophobic region on LdPEX14 to be implicated in the formation of a pore to facilitate the translocation of enzymes inside the glycosome.

Manuscript 2

Normand Cyr, Terry K. Smith, Isabelle Rouiller, Isabelle Coppens and Armando Jardim (2012)

The Hydrophobic Region of the *Leishmania donovani* Peroxin 14 is Required for Glycosome Membrane Anchoring and Mediating Membrane Insertion of the Peroxin 5-PTS1 Trafficking Complex.

Ready for submission.

In this manuscript, we continue our investigations on the biophysical and biochemical characterization of the glycosomal transport machinery by describing the components involved in the LdPEX14-glycosomal membrane interaction. This work takes ground from our previous work where we observed the solvation of a predicted hydrophobic region on LdPEX14 upon attachment with LdPEX5 (Cyr *et al.*, 2008). Hypothesizing that this region (comprised between amino acids 149 and 179 of LdPEX14) could be important for attachment to the glycosomal phospholipid bilayer, we show by immunoelectron microscopy that LdPEX14 is found at the surface of the glycosomal membrane. Furthermore, using purified glycosomes, our work takes a lipidomics approach to describe for the first time the phospholipid and fatty acid composition of the *L. donovani* glycosomal membrane. By using liposomes mimicking the glycosomal membrane of *L. donovani*, this manuscript shows that the hydrophobic region of LdPEX14 is indeed essential for binding to the liposomes. Moreover, this region alone appears to be capable of disturbing the lipid bilayer and cause leakage of an encapsulated dye. Finally, we demonstrate that LdPEX14 bound to liposomes is capable of recruiting LdPEX5 and makes the latter acting as an integral membrane protein. It is thus tempting to propose that this membrane-inserted LdPEX5 is also actively participating in a pore formation at the glycosomal surface.

Contribution of Authors

Experiments presented in this thesis have been designed and executed by the author under the supervision of Prof. Armando Jardim.

In the first manuscript, Dr. Kleber P. Madrid and Prof. Armando Jardim performed the ITC experiments. Rona Strasser performed the cross-linking experiments with digitonin-permeabilized *Leishmania donovani* parasites. Mark Aurousseau kindly cloned the ldpex5(203-391)3WF (triple tryptophan-to-phenylalanine mutant) gene. Additionally, Ron Finn and Prof. Juan Ausio (U. Victoria) performed the analytical ultracentrifugation experiments.

In the second manuscript, Prof. Terry K. Smith (U. St. Andrews) performed the lipidomics analysis of the glycosomal membrane, Prof. Isabelle Coppens (Johns Hopkins U.) conducted the immunoelectron microscopy experiments and Prof. Isabelle Rouiller (McGill U.) provided access to the Facility for Electron Microscopy Research (FEMR) at McGill University. Cynthia Santamaria and Prof. Momar Ndao did the SELDI-ToF experiments.

Chapter 1

General introduction

1.1 Parasitology and the glycosome of trypanosomatids

The parasitic lifestyle of various organisms is a good example of the great variability in forms of life on the planet. These organisms tend to co-evolve with their respective host and adopt specialized cellular organizations favoured by their parasitic nature.

Such evolution can be illustrated in trypanosomatids where part of the glycolytic pathway is compartmentalized in the peroxisome, also known as the glycosome in trypanosomatids for this specific reason (Opperdoes *et al.*, 1977b, 1984). This organelle is home to a variety of metabolic pathways, which include enzymes implicated in the oxidative metabolism of carbohydrates, lipids, amino acids and purines, as well as synthesis of certain lipids (Gualdrón-López *et al.*, 2012).

The unique nature of the glycosome in certain parasitic protozoa which include *Leishmania* sp. and *Trypanosoma* sp. offer a great starting point to develop therapeutic molecules which could fight the often deadly diseases they infer, namely leishmaniasis, sleeping sickness and Chagas' disease (Renslo and McKerrow, 2006).

On a broader perspective, the biophysical approach taken here to the study of the glycosomal targeting and translocation mechanisms will permit not only a better understanding of how folded proteins cross the glycosomal membrane, but also a better knowledge about conformational changes in large pore-forming proteins, their interactions with membrane bilayers and with other protein binding partners involved in protein transport across phospholipid bilayers.

1.2 Thesis objectives

The aim of this thesis is to biophysically characterize the role of the *Leishmania donovani* peroxin 14 (LdPEX14) in the glycosomal targeting and translocation mechanism, throught the following objectives:

- Investigate the quaternary structure of LdPEX14, the membrane receptor.
- Describe the conformational changes taking place on LdPEX14 upon binding to *Leishmania donovani* peroxin 5 (LdPEX5).
- Identify the membrane binding region(s) of LdPEX14.
- Characterize the role of the membrane binding region(s) of LdPEX14 in the translocation of proteins across the membrane of the glycosome.

Chapter 2

Literature Review

2.1 Neglected tropical diseases

As of 2002, the World Health Organization (WHO), has estimated that one billion people suffer from one or more neglected tropical diseases (NTD) (Hotez *et al.*, 2009) and about 534,000 deaths yearly are attributed to these illnesses (Moran, 2005; Molyneux *et al.*, 2005). These infections fall into a category of diseases that receive much less media and governmental attention than the *Big Three Diseases* (*i.e.* AIDS, tuberculosis and malaria).

NTDs are geographically endemic in developing areas of the world that include Africa, Asia and Latin America (Figure 2.1) where these diseases spread particularly in rural and poor urban areas. Major diseases in the NTD group include sleeping sickness, leishmaniasis, Chagas disease, schistosomiasis, lymphatic filariasis, onchocerciasis, ascariasis, trachoma, and leprosy (WHO Expert Committee on the Control of *Leishmania*ses, 2010).

Such diseases are also found to be poverty-promoting by impairing child growth, intellectual development and by reducing the productivity of the affected populations. Despite the socio-economic impact, pharmaceutical corporations have devoted little research effort to help treat NTD. From 1975 to 1996, of the 1223 new medicines launched on the market, less than 1% targeted tropical diseases[1] (Pecoul *et al.*, 1999; Trouiller and Olliaro, 1999).

[1] As a research consortium from Médecins Sans Frontière argues "tropical diseases have become progressively neglected because of the insufficient return on investment that such drug development and disease research financial efforts offer. "(Trouiller *et al.*, 2002)

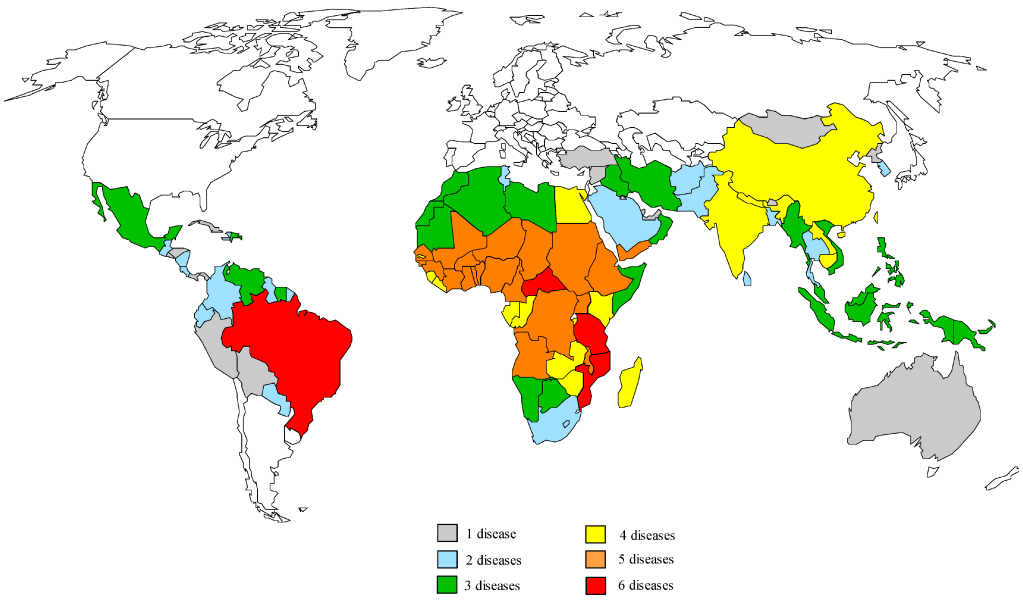

FIGURE 2.1: **Geographic distribution and overlap of neglected tropical diseases. Different colours represent the number of NTD present in given countries.** (Hotez *et al.*, 2009; Molyneux *et al.*, 2005)

Although several public-private partnerships for drug development have started in recent years, governments remain hesitant to finance such initiatives, due to the precarious nature of these partnerships (Moran, 2005; Hotez *et al.*, 2009; Hotez and Aksoy, 2011). Indeed, the drug development process is risky and young projects may lack appropriate experience, resulting in spending more public funds than necessary.

Consequently, there is an obvious and imperative requirement for the input of further efforts in fundamental research for a better understanding of the NTD biology, which will lead to the development of more effective diagnostic tools, identification of potential drug targets and later development of effective drugs.

2.2 Leishmaniasis

Leishmaniasis, one of the diseases categorized as an NTD by the WHO, is a disease caused by *Leishmania* sp., a protozoan parasite that is transmitted to the mammalian host during feeding of the arthropod vector *Phlebotomus* or *Lutzomyia* sp. (sand fly). The general disease was named after the Scottish pathologist William Boog Leishman, who identified the parasite in 1901 in a human spleen sample from a British soldier serving in India (Leishman, 1903, 1904)[2]. Approximately at the same time, Charles Donovan, another medical doctor working in India, observed similar parasites (Ross, 1903). His name was used to designate the *Leishmania* species involved in the visceral form of the disease.

Leishmaniasis is currently endemic in 88 countries, where 350 million people are at risk. From a recent meta-analysis, it is estimated that between 0.9 to 1.6 million new cases of leishmaniasis occur each year (Alvar *et al.*, 2012). Moreover, it is estimated that about 59,000 people died from this disease in 2001, mainly from visceral leishmaniasis (Bern *et al.*, 2008; Desjeux, 2004). However, as authors of these studies insist, theses figures tend to be largely underestimated, and that the numbers of cases of this disease may be ∼4-40 times higher (Ostyn *et al.*, 2011; Alvar *et al.*, 2012; Vogel, 2012) depending on the manifestation of the diseases and geographic location (Alvar *et al.*, 2012).

[2]The reader is invited to read the original 1903 and 1904 reports by Leishman in the *British Medical Journal* where he provides the first description and illustration of *Leishmania* protozoa, biopsized from the spleen of a British soldier deseased allegedly from an infection by the parasite.

2.2.1 Disease manifestations

Three distinct forms of leishmaniasis have been described based on the clinical symptoms and the *Leishmania* species involved. Among the *Leishmania* genus, several species have been identified and characterized based on their tropism (dermo-, muco- or viscero-) and the disease manifestations they cause (Table 2.1). A phylogenetic analysis, based on HSP70 sequences (Fraga *et al.*, 2010), is illustrated in Figure 2.2. The *Viannia* subgenus, which tend to develop mucocutaneous leishmaniasis clearly cluster in a distinct clade from the *Leishmania* subgenus, of which two groups diverge in both their HSP70 genomic sequence as well as in the disease manifestations they cause. Likewise, the lizard-infecting *Leishmania (Sauroleishmania) tarentolae* diverges from the latter. Sequences from *Trypanosoma* sp. were used as outgroups.

Clinical manifestations of leishmaniasis have been characterized into three

Subgenus:	*L.* (*Leishmania*)	*L.* (*Leishmania*)	*L.* (*Vianna*)	*L.* (*Vianna*)
Old World	*L. donovani* *L. infantum*	*L. major* *L. tropica* *L. aethiopica* *L. infantum*		
New World	*L. infantum*	*L. infantum* *L. mexicana* *L. amazonensis* *L. venezuelensis* *L. pifanoi* *L. garnhami*	*L. braziliensis* *L. guyanensis* *L. panamensis* *L. shawi* *L. naffi* *L. lainsoni* *L. lindenbergi* *L. peruviana* *L. colombiensis*	*L. braziliensis* *L. panamensis*
Principal tropism:	Viscerotropic	Dermotropic	Dermotropic	Mucotropic

TABLE 2.1: ***Leishmania* species of medical importance** (Magill, 1995; WHO Expert Committee on the Control of *Leishmania*ses, 2010; Schonian *et al.*, 2010; Fraga *et al.*, 2010)

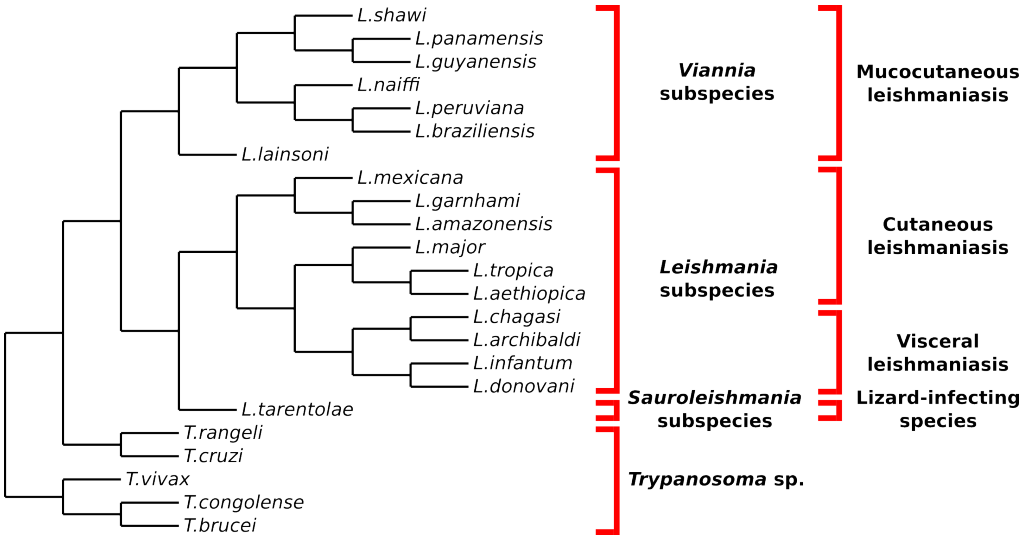

FIGURE 2.2: **Phylogenetic analysis of *Leishmania* species.** The HSP70 gene from the different recognized *Leishmania* species was amplified, sequenced, and phylogenetically analyzed. The HSP70 sequences from *Trypanosoma* sp. were used as outgroups. (Fraga *et al.*, 2010; Schonian *et al.*, 2010)

categories. Firstly, cutaneous leishmaniasis (CL) is represented by a skin reaction following a sand fly bite, is caused mainly by the dermotropic *L. major* and *L. mexicana* species. It is predominantly present mainly in parts of Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria (Hepburn, 2000; Piscopo and Mallia, 2006; Murray *et al.*, 2005).

Secondly, mucocutaneous leishmaniasis (MCL) is characterized by a degeneration of mucosal membranes, primarily in the nose and mouth region. This form of the disease can be fatal if untreated, as secondary infections by opportunistic bacteria aggravate cases. This form of leishmaniasis is prominently observed in Latin America where infections with the mucotropic *L. Viannia* subgenus are common (Vergel *et al.*, 2006; Weigle and Saravia, 1996; Amato *et al.*, 2008). Interestingly, a recent study by Ives *et al.* have observed that occurrence of RNA virus (*Leishmania*

RNA virus-1) infected *Leishmania guyanensis* parasites caused increased metastasis and dissemination of the parasite to mucosa (Ives *et al.*, 2011).

Thirdly, visceral leishmaniasis (VL) is the most aggressive form of leishmaniasis (accounts for 70% of all deaths associated with leishmaniasis) and is characterized by acute fever and swelling of the liver and the spleen. *L. donovani* and *L. infantum* are the two main species, known to be viscerotropic, involved in the disease (Table 2.1) which in India is known as Kala azar, Dum-dum fever or black fever (Matlashewski *et al.*, 2011; Guerin *et al.*, 2002). Over 90% of cases of this disease occur in rural regions of India, Bangladesh, Nepal, Sudan and Brazil (Alvar *et al.*, 2012; WHO Expert Committee on the Control of *Leishmania*ses, 2010).

Initial symptoms of leishmaniasis are skin sores localized at the site where the infected female sand fly took its blood meal, followed by fever, night sweats and general fatigue. The parasite may later, depending on the species, invades various macrophage-rich tissues (lymph nodes, bone marrow, spleen, liver) and cause local, or systemic inflammation characterized by splenomegaly, anaemia and liver malfunction. Such outcomes further result in severe growth retardation in children, and rapid deterioration in general health resulting in immuno-compromised individuals (Murray, 2002; Murray *et al.*, 2005).

2.2.2 Life cycle of *Leishamania* sp.

Leishmania has two definitive hosts: the sand fly and mammals. In the gut of the sand fly, promastigotes (flagellated form, elongated cells) reproduce and transform from a procyclic non-infectious stage to a metacyclic

infectious stage. The metacyclic promastigotes form migrates from the sand fly midgut to the pharyngeal valve and are regurgitated to a mammalian host when the sand fly pierces the skin while taking a blood meal (Kamhawi, 2006).

Then, metacyclic promastigotes invade macrophages by receptor-mediated phagocytosis. Indeed, *Leishmania* promastigotes display high abundance the surface molecules, glycoprotein 63 (gp63) and the lipophosphoglycan (LPG) (Chakrabarty *et al.*, 1996; Russell and Talamas-Rohana, 1989) are known to be involved in attachment to the complement receptor type 3 (CR3) (Wilson and Pearson, 1988), to mannose/fucose receptors (Wilson and Pearson, 1986) and to fibronectin receptors (Rizvi *et al.*, 1988) on macrophages. Such attachment triggers the phagocytosis of the parasite and formation of a parasitophorous vacuole. Drastic changes in pH and temperature then triggers the transformation of *Leishmania* into its amastigote form (round cells with reduced, or absent flagella) which later reproduces inside the parasitized cells. Further proliferation of the parasite occurs by the release of amastigotes via cell burst followed by another phagocytosis event in uninfected macrophages. In that case, several low-affinity interactions that are species-dependant are responsible for the attachment (Peters *et al.*, 1995). Finally, the sand fly will take a blood meal from an infected person, thus ingesting *Leishmania* amastigotes which will retransform into promastigotes in the sand fly midgut (Kamhawi, 2006; Gossage *et al.*, 2003), and consequently restarting the cycle (Figure 2.3).

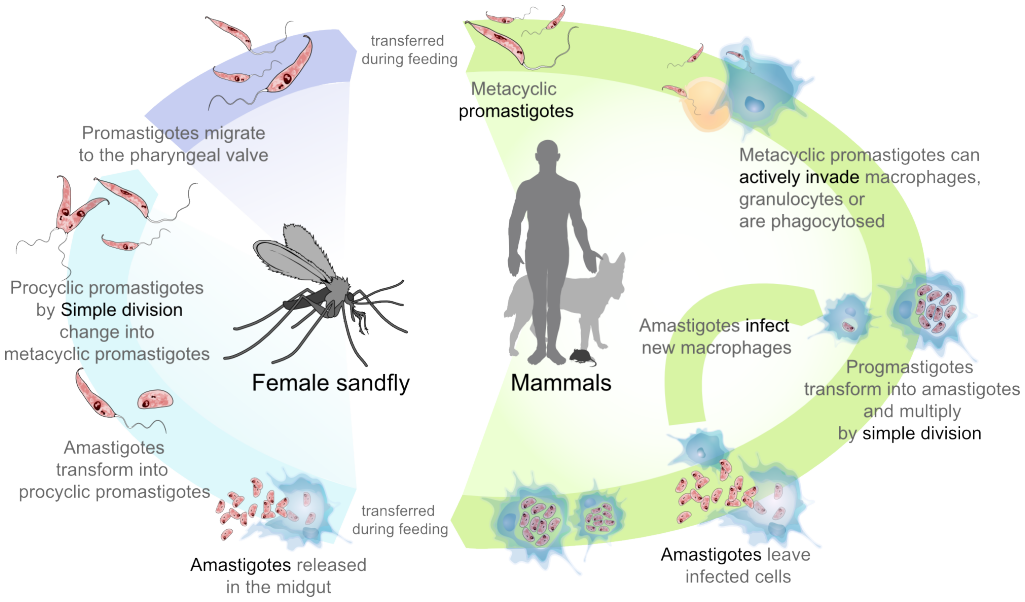

FIGURE 2.3: **Life-cycle of *Leishmania* sp.** The digenetic lifecycle of *Leishmania* sp. involves both a female sand fly host and a mammalian host. Transmission occurs when the female sand fly takes a blood meal from a mammal (Ruiz Villarrea, 2009; WHO Expert Committee on the Control of *Leishmania*ses, 2010).

2.2.3 Chemotherapeutic treatments

Current treatments available for visceral leishmaniasis involve the use of drugs that are often toxic to the patient, require parenteral administration and are frequently cost prohibitive for use in developing countries (Matlashewski *et al.*, 2011; Croft and Olliaro, 2011). Moreover, resistance to the first line drugs such as pentavalent antimonial compounds exists and alternatives are required (Ouellette *et al.*, 2004; Croft *et al.*, 2006; Stuart *et al.*, 2008). Chemotherapeutic agents currently used clinically for treatments for visceral leishmaniasis and some of the side effects associated with these drugs are summarized in Table 2.2.

More recently, it has become evident that visceral leishmaniasis is frequently associated with human immunodeficient virus (HIV) infections. Indeed, in

Drug	Trade names	Known issues
Pentavalent antimonials	Pentostam, Glucantime	Resistance, toxicity, quality of generics
Amphotericin B	Fungizone	Intravenous infusion, toxicity
Lipid-associated amphotericin B	Ambisome	Less toxic, cost prohibitive
Pentamidine	Pentacrinate	Second line treatment, toxicity, intravenous infusion
Miltefosine	Impavido, Miltex	Teratogenicity, resistance
Paromomycin	Humatin	Resistance, may be good for topical treatment
Sitamaquine	(WR-6026)	May cause methemoglobinaemia

TABLE 2.2: **Current drug treatments commercially available for visceral leishmaniasis** (Croft *et al.*, 2006; Guerin *et al.*, 2002; Murray, 2002; Piscopo and Mallia, 2006; Singh *et al.*, 2012)

Ethiopia and Sudan, co-infections are prevalent in 15 to 40% of VL cases (Hailu *et al.*, 2005; Alvar *et al.*, 2008). Moreover, this situation is spreading a more severe form of leishmaniasis to new geographical areas, mainly in Southern Europe, where it is associated with intravenous drug usage (Garg *et al.*, 2007; Cruz *et al.*, 2002). Leishmaniasis and acquired immunodeficient syndrome (AIDS) work synergistically since they both infect and reproduce in common cell targets (macrophages), and induce a depression of the immune system, which in turn stimulates the replication of the AIDS virus (Berhe *et al.*, 1999; Piscopo and Mallia, 2006). Hopefully, according to a study by Berhe *et al.* (Berhe *et al.*, 1999), treatment of VL with adequate medication result in lower viral load, and proper retroviral treatment tend to prevent relapse of leishmaniasis (Berhe *et al.*, 1999; Murray *et al.*, 2005).

Another issue is the emergence of leishmaniasis in new areas, probably due to drug resistance, global warming and immigration of people from endemic regions. Moreover, cases of leishmaniasis in soldiers coming back from military missions in the Middle East have been reported (Plourde *et al.*, 2012; Weina *et al.*, 2004; van Thiel *et al.*, 2010). Detection, diagnostic and treatment of these cases is of paramount important in order to prevent the spread of the disease, particularly through blood transfusions.

2.3 Overview of kinetoplastid biology

Kinetoplastids are a group of protists that are believed to have diverged early in evolution from the main eukaryotic lineage. They are flagellated protozoa, of which many are obligate parasites to humans and animals. Two subgroups have been identified: the *Bodonidae* and the *Trypanosomatidae* (Simpson *et al.*, 2006; Simpson and Roger, 2004; Simpson *et al.*, 2004). The first cluster of organisms contains both free-living species, feeding on bacteria, and parasites of fish and snails; whereas the *Trypanosomatidae* are all obligate parasites of invertebrates, vertebrates or plants (Figure 2.4).

Kinetoplastids are characterized by several biological traits: these include the presence of kinetoplast organelles, unusual RNA editing, transcription of long multicistronic mRNA molecules, and compartmentation of glycolysis and other metabolic pathways (Figure 2.5) (de Souza, 2002; Hannaert *et al.*, 2003a; Simpson *et al.*, 2003; Docampo *et al.*, 2005; Clayton *et al.*, 1995).

Kinetoplasts are part of the mitochondrion structure and consist of DNA (kDNA) that is organized in a unique disk-shape fashion (Figure 2.5). The network of kDNA is comprised of two patterns: a dozen of maxicircle plasmids (20 to 40 kb) and a thousand of minicircle plasmids (0.7 to 2.5 kb) (Liu *et al.*, 2005; Lukes *et al.*, 2005). Maxicircles function similarly to mitochondrial genomic DNA (mDNA) in higher eukaryotes, where most genes responsible for respiration are encoded. However, these transcripts are considered cryptic and require RNA editing guided by fragments of the genes encoded in the minicircles (Liu *et al.*, 2005; Pérez-Morga and Englund, 1993).

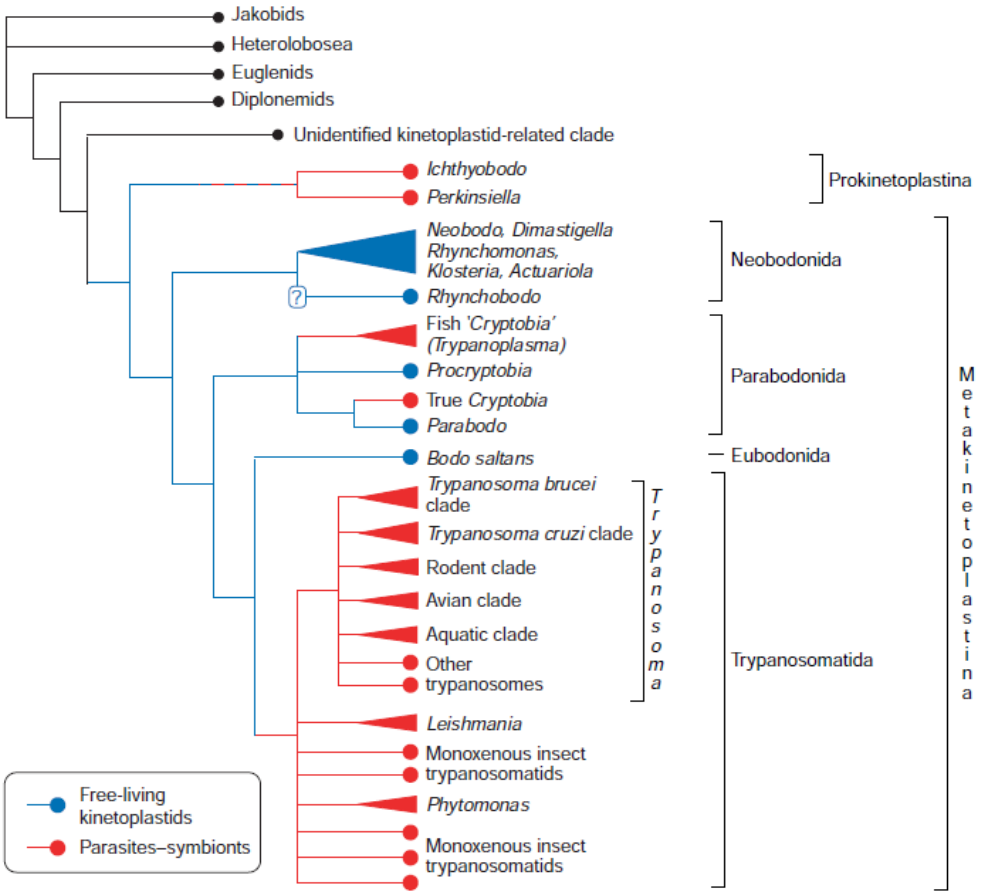

FIGURE 2.4: **Evolutionary relationship analysis of kinetoplastids species based on rRNA and protein phylogenies.** (Simpson *et al.*, 2006)

The atypical RNA editing, a feature characteristic of kinetoplastids, involves the insertion, or deletion, of uridine residues at specific sites of coding regions. These create new initiation codons, new open reading frames, stop codons and even frame shifts (Estévez and Simpson, 1999; Hajduk *et al.*, 1993; Simpson *et al.*, 2003) from long multicistronic RNA molecules (Alfonzo and Thiemann, 1997; Lukes *et al.*, 2005). The other peculiarity of kinetoplastids resides in the compartmentation of several metabolic pathways in organelles called glycosomes, similar to peroxisomes in other eukaryotes.

Kinetoplastids also contain a small invagination in the plasma membrane, located at the base of the flagellum (Figure 2.5), named the flagellar pocket. This is currently the only documented site for exocytosis and endocytosis in *Trypanosoma* sp. and *Leishmania* sp. (Field and Carrington, 2009). The flagellar pocket appears to also be involved in cell division and immune evasion via excretion and recycling of; variant surface glycoproteins (VSG) in *Trypanosoma brucei* or major surface protease (MSP, also known as gp63) in *Leishmania* (Overath *et al.*, 1997).

Another particular physiological feature of kinetoplastids resides in the presence of acidocalcisomes. As the name implies, these electron-dense, acidic and calcium-rich organelles are implicated in storage of various chemicals including the basic amino acids arginine and lysine, calcium, polyphosphate, magnesium, zinc, potassium, inorganic pyrophosphate sodium and iron (Docampo *et al.*, 2005). Several ion transporters have been localized to acidocalcisomes which have strengthen the hypothesis that these organelles play a homeostasis role for the various molecules they store (Docampo *et al.*, 2005). Such homeostasis appears to play a role in parasite virulence (reviewed by Docampo and Moreno (2011)).

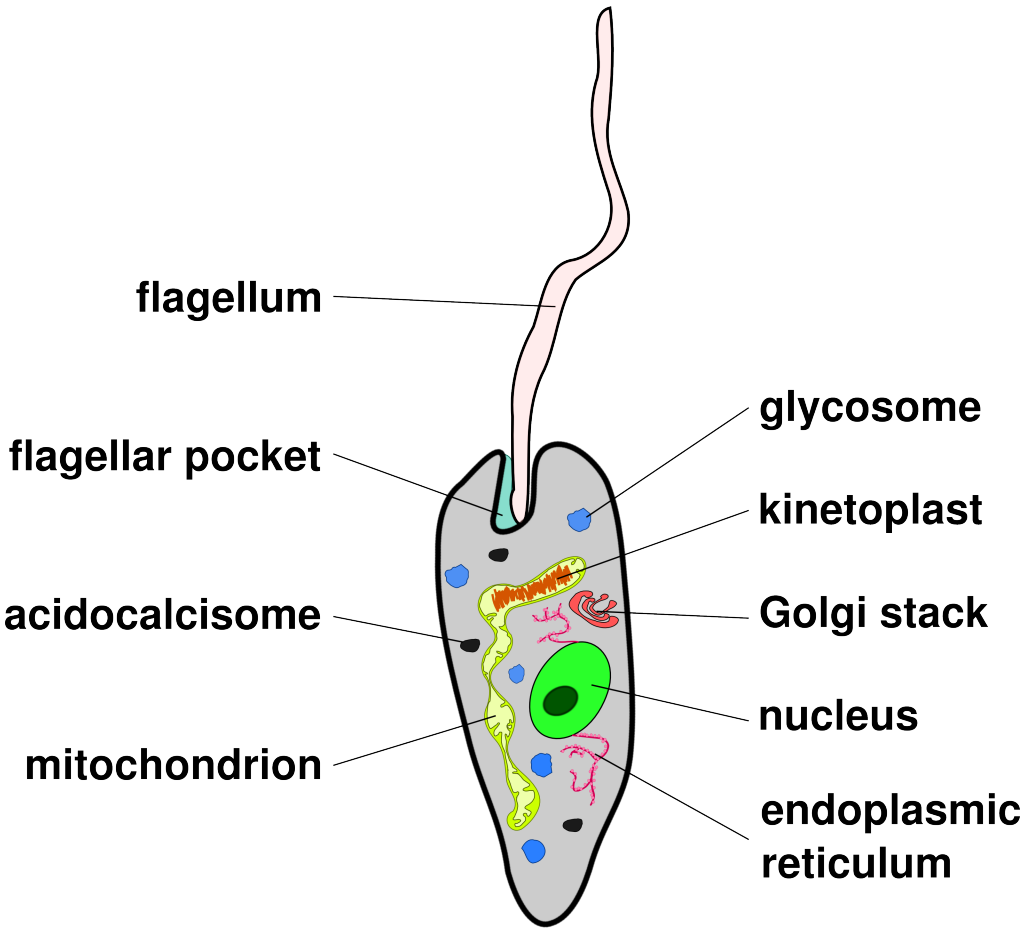

FIGURE 2.5: **General diagram of a kinetoplastid** (inspired from Docampo *et al.* (2005) and Clayton *et al.* (1995))

2.4 Role of glycosomes in trypanosomes

It was first discovered by Opperdoes and Borst, in 1977 (Opperdoes *et al.*, 1977b,a), that trypanosomes compartmentalize several glycolytic enzymes in highly specialised microbodies, named glycosomes. These organelles, present in all kinetoplastids, are evolutionarily related to the peroxisomes of fungi and mammalian cells, and glyoxysomes of plants (Gualdrón-López *et al.*, 2012). These microbodies are globular organelles with a diameter of 0.2 to 0.3 μm, are bound by a single phospholipids bilayer, do not contain DNA material, and have a proteinaceous electron-dense matrix (Sommer and Wang, 1994; Opperdoes *et al.*, 1984). The estimated number of glycosomes range from 10 (*L. mexicana*) to >200 in *T. brucei* (Tetley and Vickerman, 1991; Opperdoes *et al.*, 1984).

The glycosome compartmentalizes several essential metabolic functions that include: glycolysis (Opperdoes, 1987; Opperdoes *et al.*, 1977b), pentose-phosphate pathway (Maugeri, 2003; Maugeri and Cazzulo, 2004), purine salvage (Landfear *et al.*, 2004; Hammond and Gutteridge, 1984), pyrimidine biosynthesis (Hammond and Gutteridge, 1984), β-oxidation of fatty acids (Lee *et al.*, 2007), ether-lipid biosynthesis (Heise and Opperdoes, 1997; Lux *et al.*, 2000) and oxidant defence mechanisms (Dey and Datta, 1994). Interestingly, the medically important kinetoplastids that include *Leishmania* and African and South American trypanosomes lack catalase, a hallmark enzyme found in peroxisomes of most other eukaryotic species (Adak and Datta, 2005; Opperdoes *et al.*, 1984). Instead, a thiol-dependent peroxidase activity involved in hydrogen peroxide metabolism is present in these organisms (Müller *et al.*, 2003). It should be noted that not all kinetoplastids are devoid of the enzyme catalase, as this activity has been

detected in the insect parasite *Crithidia* (Soares and De Souza, 1988), the plant parasite *Phytomonas* (Sanchez-Moreno *et al.*, 1992) and in the fish parasites *Cryptobia* (Ardelli *et al.*, 2000) and *Trypanoplasma* (Opperdoes *et al.*, 1988).

2.4.1 Evolutionary aspects of the glycosome

It is now well accepted that glycosomes are the trypanosome analogues to the peroxisome of other eukaryotes (Parsons, 2004). However, on the origin of the organelle, scientists have not come to a consensus yet. On one hand, it has been recently demonstrated by Hannaert *et al.* (Hannaert *et al.*, 2003b) that several peroxisomal targeting signal bearing enzymes involved in glycolysis were more closely related to plant homologues than to similar enzymes in other lower eukaryotes. It has been proposed that photosynthesis may have been a metabolic function of earlier kinetoplastids. In that sense, ancestors of trypanosomes and euglenoids, the *Euglenozoa*, would have had plastid organelles (chloroplasts in plants) that have been lost in time by trypanosomes (scenario A in Figure 2.6).

On the other hand, it has been suggested that plant and prokaryote-related genes may have been incorporated into the genome of earlier trypanosomes by phagocytosis of bacteria, hence resulting in a lateral gene transfer (Waller *et al.*, 2004) (scenario B in Figure 2.6) and explaining the presence of plant homologous enzymes. This would corroborate the theory of Doolittle (Doolittle, 1998) who described a ratchet mechanism by which genes from bacteria used as food could replace archaeal homologues in the nuclear genome. Such a mechanism, however, is lacking the explanation for

plastid traces found in glycosome of numerous trypanosomatids (Parsons, 2004). A combination of the two models is more likely to have happen (Ginger *et al.*, 2010a).

Very recently, Gualdrón-López *et al.* (2012) nicely summarized the current hypothesis on how glycosomes evolved from peroxisomes. It is now believed that a common eukaryotic ancestor to *Kinetoplastea* and *Diplonemida* was facing large nutritional changes which put pressure on glycolysis and/or gluconeogenesis metabolic activities (Figure 2.7). Compartmentalization inside an organelle therefore constituted the evolutionary driving force and facilitated the adaptation to altering conditions. Furthermore, the authors nicely conclude by proposing that "glycosomes played a facilitating role in the development of parasitism in *Kinetoplastea* ". This remains to be verified using a technically challenging experimental setup: trypanosomatids mutants lacking functional glycosomes (Gualdrón-López *et al.*, 2012).

2.4.2 Energy metabolism and compartmentation of glycolysis in the glycosome

Although several metabolic pathways are sequestered in the glycosome of trypanosomes, the membrane of this organelle is believed to be poorly permeable to most metabolites (Michels *et al.*, 2000; Opperdoes, 1987) and requires specialized transporter to move solutes across (Gualdron-López *et al.*, 2012). Consequently, appropriate balance of both ADP/ATP and NAD^+/NADH are required. This has been illustrated by Hannaert *et al.* (Hannaert *et al.*, 2003a) using bloodstream form *T. brucei*. In that

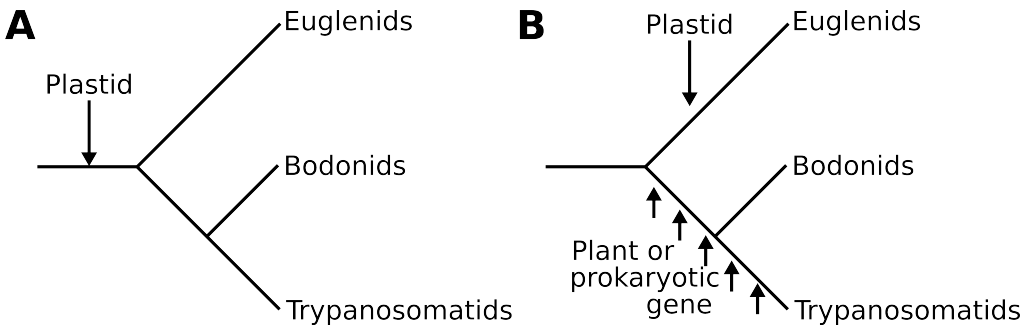

FIGURE 2.6: **Evolutionary hypothesis for the appearance of glycosomes in trypanosomes** (from Waller *et al.* (2004))

case, glycolysis is virtually the only metabolic activity taking place in the glycosome and other important processes are suppressed (tricarboxylic acid (TCA) cycle, electron transport chain). The enzymes converting glucose to 3-phosphoglycerate are all residing inside the glycosome, whereas phosphoglycerate mutase, enolase and pyruvate kinase are cytosolic. The former enzyme activities lead to the consumption of 2 ATP molecules, which are balanced by the activities of two kinases: phosphoglycerate kinase and glycerol kinase (Figure 2.8). In a similar fashion, the NADH produced by the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate is reoxidized by molecular oxygen, via a redox shuttle involving the mitochondrion and glycerol-3-phosphate oxidase (Bakker *et al.*, 1999; Blum, 1994; Guerra *et al.*, 2006; Michels *et al.*, 2006; Moyersoen *et al.*, 2004; Opperdoes, 1987).

On the other hand, procyclic form of *T. brucei* (insect stage) and other trypanosomes, including *Leishmania* sp. and *T. cruzi*, have a more complex energy production and carbohydrate metabolism, where the mitochondrion is extensively involved. Here, phosphoglycerate kinase is located in the

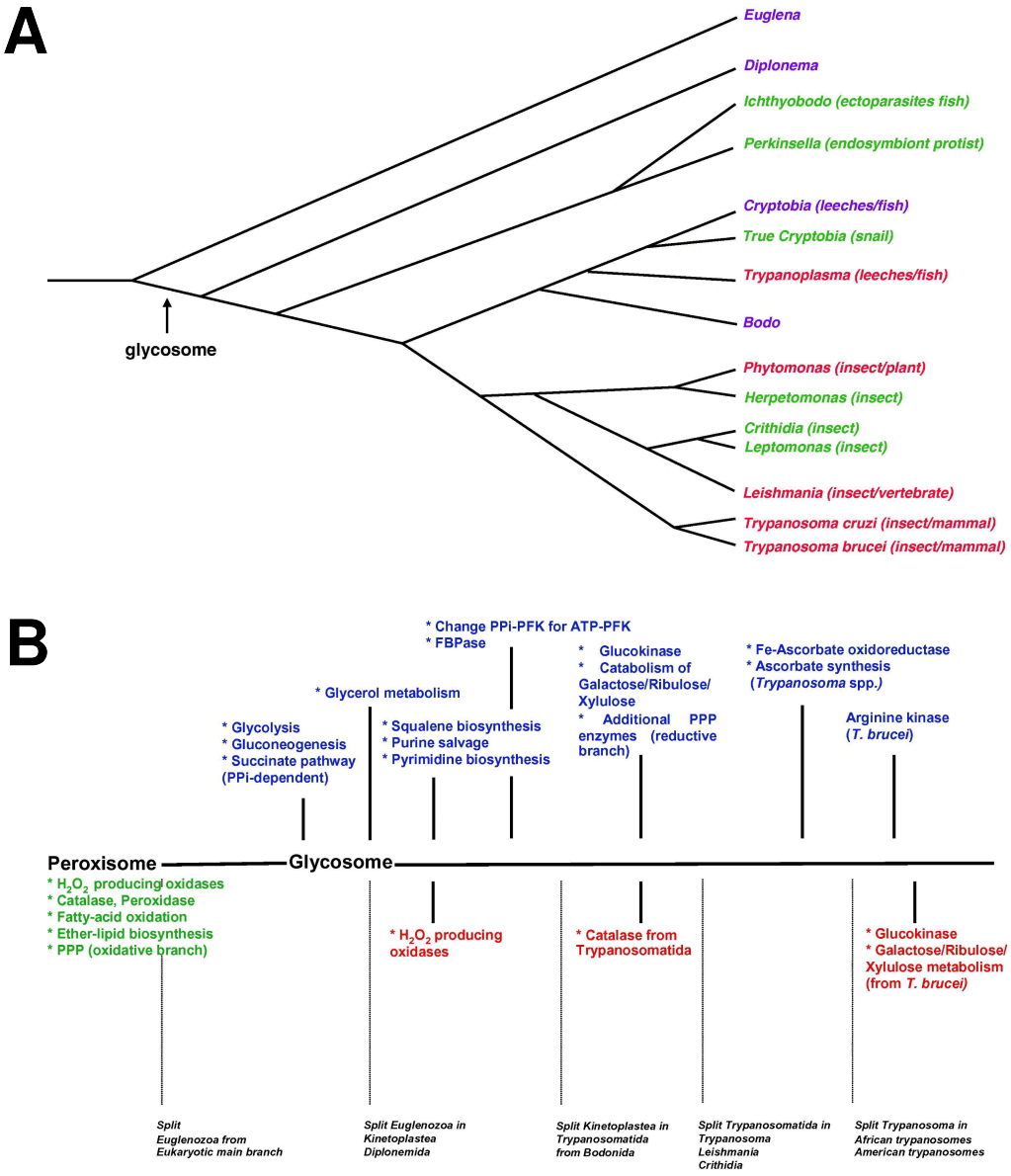

FIGURE 2.7: **Evolution of parasitism and of the glycosome within *Euglenozoa*** A) Origins of parasitism. Phylogenetic relationships of the major *Euglenozoa*. Free-living organisms are labelled in mauve, monogenetic parasites in green and digenetic parasites in red. B) Glycosome evolution. Acquisions and losses of various enzymes and pathways in glycosomes. Ancestral enzymes are marked in green, acquired enzymes and pathways in blue and lost enzymes and pathways in red. (from Gualdrón-López *et al.* (2012))

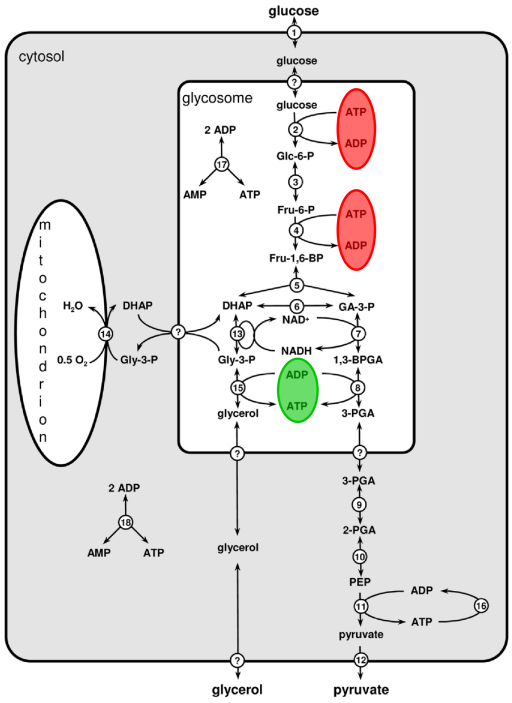

FIGURE 2.8: **Energy metabolism involved in glycolysis taking place in the glycosome of the bloodstream form of *T. brucei*.** Red ellipses indicate ATP consumption, whereas green ellipse indicates ATP formation. (1) glucose transport; (2) hexokinase; (3) phosphoglucose isomerase (4) phosphofructokinase; (5) aldolase (6) triosephosphate isomerase; (7) glyceraldehyde-3-phosphate dehydrogenase; (8) phosphoglycerate kinase; (9) phosphoglycerate mutase; (10) enolase; (11) pyruvate kinase; (12) pyruvate transport; (13) glycerol-3-phosphate dehydrogenase; (14) combined process of mitochondrial glycerol-3-phosphate dehydrogenase and trypanosome alterative oxidases; (15) glycerol kinase; (16) combined ATP utilization; (17) glycosomal adenylate kinase; (18) cytosolic adenylate kinase. Abbreviations: Glc-6-P, glucose-6-phosphate; Fru-6-P, fructose-6-phosphate; Fru-1,6-P, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; Glyc-3-P, glycerol-3-phosphate; GA-3-P, glyceraldehyde-3-phosphate; 1,3-BPGA, 1,3-bisphosphoglycerate; 3-PGA, 3-phosphoglycerate; 2-PGA, 2-phosphoglycerate; PEP, phosphoenolpyruvate. (adapted from Bakker *et al.* (2010) and Gualdrón-López *et al.* (2012))

cytosol[3]. As a consequence, in order to maintain a proper energy balance, phosphoenolpyruvate carboxykinase and pyruvate phosphate dikinase are localised inside the glycosome, which lead to the formation of succinate. Likewise, the transformation of phosphoenol pyruvate inside the glycosome takes advantage of the NAD^+-dependent enzyme malate dehydrogenase and fumarate reductase, a NADH-dependent enzyme, in order to maintain the balance (Bakker *et al.*, 1999; Blum, 1994; Michels *et al.*, 2006; Moyersoen *et al.*, 2004; Opperdoes, 1987). In this case, this situation leads to a significant drop on the activity of glycerol-3-phosphate dehydrogenase, and thus glycerol formation.

A similar mechanism is believed to take place in *Leishmania* and such organization is illustrated in Figure 2.9 where the localization of the different enzymes involved in glycolysis in *Leishmania major*, as determined experimentally and by *in silico* genome analysis (Opperdoes and Szikora, 2006), are mapped.

2.4.3 Evolutionary advantage for compartmentation of glycolysis in Trypanosomatids

From these observations, one may ask the following question: What is the evolutionary advantage of compartmentalizing glycolysis in an organelle? It was initially believed that segregation was necessary to sustain a high glycolytic flux (Opperdoes, 1987), since enzymes in the glycosome are present in relatively high concentrations (up to 340 mg/mL) (Sommer and Wang, 1994). Therefore, this segregation within a microbody would

[3]Although a glycosomal isoform has been identified and recently partially characterized (Adjé *et al.*, 1997; Kaushik *et al.*, 2012). The exact location of the enzyme remains to be determined

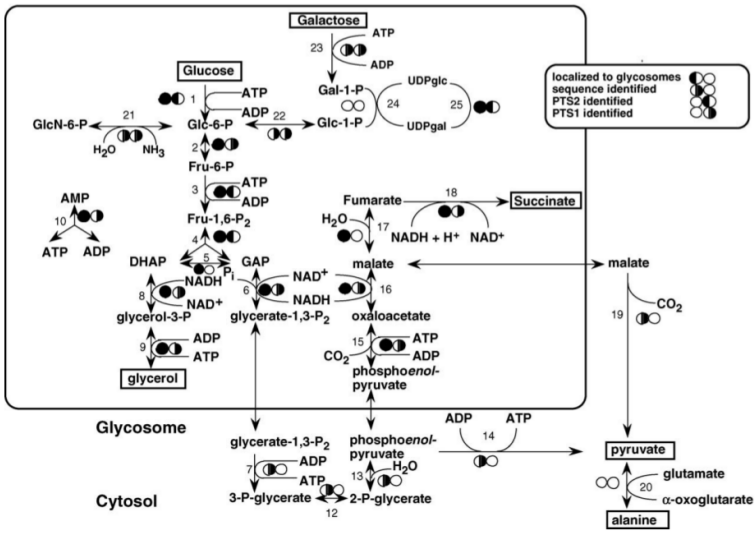

FIGURE 2.9: **Glycolysis in *Leishmania major* and localization of involved enzymes by *in silico* genome analysis** (see Opperdoes and Szikora (2006)). Boxed metabolites are nutrients or end-products of metabolism. Enzymes: (1) hexokinase; (2) glucose-6-phosphate isomerase; (3) phosphofructokinase; (4) aldolase; (5) triosephosphate isomerase; (6) glyceraldehyde-3-phosphate dehydrogenase; (7) phosphoglycerate kinase; (8) glycerol-3-phosphate dehydrogenase; (9) glycerol kinase; (10) adenylate kinase; (12) phosphoglycerate mutase; (13) enolase; (14) pyruvate kinase; (15) phosphoenolpyruvate carboxykinase; (16) malate dehydrogenase; (17) fumarate hydratase (see text for explanation); (18) NADH-dependent fumarate reductase; (19) malic enzyme; (20) alanine aminotransferase; (21) glucosamine-6-phosphate isomerase; (22) phosphomannomutase/phosphoglucomutase; (23) galactokinase; (24) galactose-1-phosphate uridylyl transferase; (25) UDP-galactose-4-epimerase. Glc-6-P, glucose-6-phosphate; Fru-6-P, fructose-6-phosphate; Fru-1,6-P, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3-phosphate; GlcN-6-P, glucosamine-6-phosphate; Glc-1-P, glucose-1-phosphate; Gal-1-P, galactose-1-phosphate.

overcome the diffusion of enzymes and substrates throughout the cell, where a lower concentration would be found. This initial hypothesis is now considered unlikely since: (a) high glycolytic fluxes are only found in trypanosomatids that live in a high glucose milieu (e.g. *T. brucei* in the bloodstream) (Michels and Hannaert, 1994), (b) yeast cells have a two-fold higher glycolytic flux and do not depend on the compartmentation of enzymes nor substrates (Bakker *et al.*, 1995, 2000), (c) rate limiting step in the glycolytic flux in the bloodstream form of *T. brucei* was determined to be due to catalytic activity of the individual enzymes (Hannaert *et al.*, 2003a; Bakker *et al.*, 2010).

Therefore, another more plausible hypothesis was proposed. It was first discovered by Nwagwu and Opperdoes (1982) that there is an important lack of regulation during glycolysis in trypanosomes. Indeed, the authors found that hexokinase and phosphofructokinase, enzymes that catalyze the first reactions of glycolysis, are not inhibited by a feedback mechanism via the reaction products (Kosow and Rose, 1970) nor by allosteric effectors (Steitz *et al.*, 1977) as observed in other eukaryotic organisms such as yeast. Another type of regulation mechanism, the compartmentation of glycolysis, was proven to be essential for survival of trypanosomes, since accumulation of glycolytic intermediates would become deleterious (Moyersoen *et al.*, 2004; Teusink *et al.*, 1998; Kessler and Parsons, 2005). A system biology approach based on known metabolic flux data was built to understand the role of compartmentalizing glycolysis in *T. brucei* (Bakker *et al.*, 2000, 1999, 2010). This experiment suggested that in the absence of compartmentation, toxic level of hexose phosphates would accumulate in parasites grown in glucose. These models also predicted that such a build up would

be prevented by distinct pools of adenine nucleotides in the cytosol and glycosome.

Moreover, the ATP/ADP ratio in the glycosome, which is apparently low (more ADP than ATP appear to be present), would control the activity of hexokinase and phosphofructokinase in the glycosome. Therefore, the lack of ATP decreases the turnover rate for these two enzymes which leads to the maintenance of hexose phosphates concentrations within a narrow range not toxic to the cell, thus avoiding the "Turbo"effect (Figure 2.10).

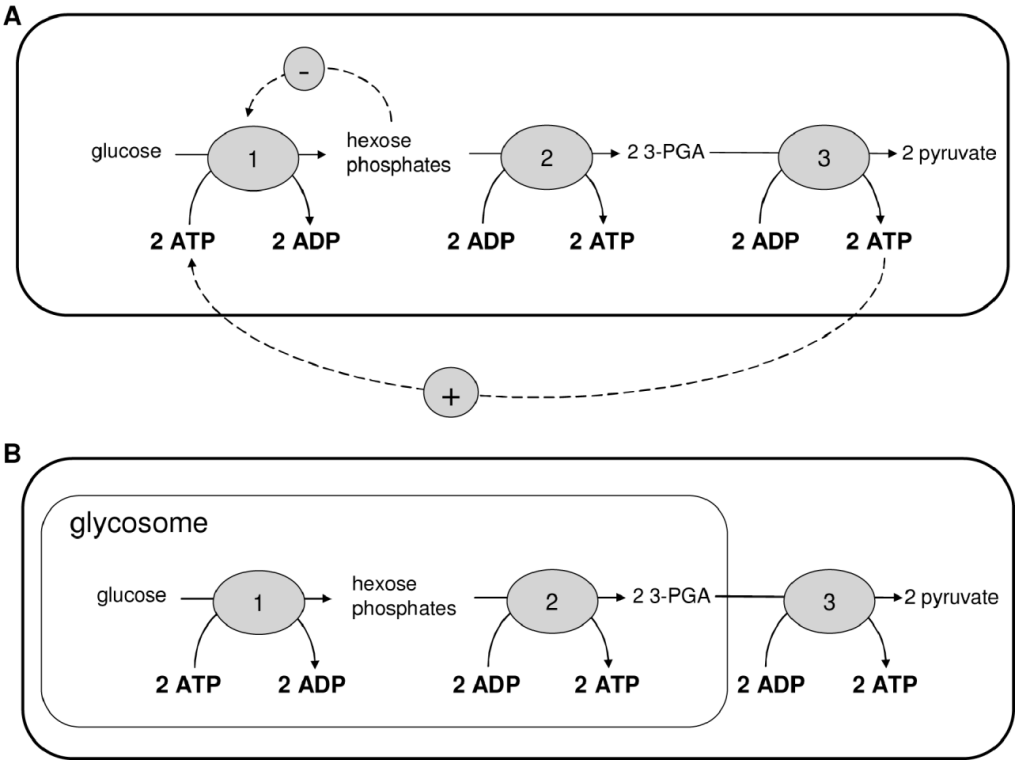

FIGURE 2.10: **"Turbo"design implication in glycolysis.** (A) Steps involving hexokinase and phosphofructose kinase result in a constant reinvestment of ATP (from surplus), leading to accumulation of intermediates, which do not act as feedback inhibitors in trypanosomes. (B) Compartmentalization of adequate steps in glycolysis enables proper control of ATP/ADP ratio, hence avoiding accumulation of intermediates (Hannaert *et al.*, 2003a; Bakker *et al.*, 2010).

All this may therefore explain the compartmentation of glycolysis in the glycosome. Indeed, the sequestration of specific metabolic enzymes, in addition to the impermeability of the glycosomal membrane, facilitates the maintenance of appropriate balanced ATP/ADP and NAD^+/NADH ratios that will prevent the overproduction of hexose phosphates and fructose-6-phosphate to toxic levels. The compartmentation enables the regulation of glycolysis within an organelle which its membrane is essentially impermeable for solutes (Bakker *et al.*, 2000; Moyersoen *et al.*, 2004; Bakker *et al.*, 2010; Ginger *et al.*, 2010b). In contrast, such compartmentation is not necessary in yeast and mammalian cells since both hexokinase and phosphofructokinase are regulated by feedback inhibition mechanisms (Teusink *et al.*, 1998). Nevertheless, it remains unclear whether a lack of regulation led to compartmentation or that compartmentation led to a lack of regulation. The latter is likely to have happened (Gualdrón-López *et al.*, 2012).

2.4.4 Compartmentation of the purine salvage pathway to the glycosome

One of the most striking differences between trypanosomes and the insect and mammalian hosts is the way organisms synthesize purines: the hosts are capable of *de novo* synthesis, whereas the parasite is completely reliant on the extracellular salvage of such compounds (Looker *et al.*, 1983; Opperdoes, 1987; Carter *et al.*, 2008; Boitz *et al.*, 2012). Via specific transporters, nucleobases and nucleosides are imported inside the parasite cells (Carter *et al.*, 2001; Landfear, 2011; Landfear *et al.*, 2004). From there, phosphoribosyltransferase (PRT) enzymes react

with the purine bases to catalyze the addition of 5-phosphoribosyl-1-pyrophosphate from the corresponding nucleotides (Michels *et al.*, 2000). The latter enzymes involved in this pathway have been extensively characterized (hypoxanthine-guanine phosphoribosyl transferase (HGPRT) (Allen *et al.*, 1995b; Zarella-Boitz *et al.*, 2004; Boitz and Ullman, 2006), adenine phosphoribosyl transferase (APRT) (Allen *et al.*, 1995a), inosine monophosphate dehydrogenase (IMPDH) (Dobie *et al.*, 2007) and xanthine phosphoribosyl transferase (XPRT) (Jardim *et al.*, 1999; Ullman *et al.*, 2010)). Many purine salvage enzymes (*e.g.* HGPRT and XPRT), typically located in the cytosol of eukaryotes, have been localize in the glycosome of trypanosomes (Figure 2.11) (Parsons *et al.*, 2001; Boitz and Ullman, 2006; Boitz *et al.*, 2012). Similarly, inosine monophosphate dehydrogenase (IMPDH), which catalyze the addition of a hydroxyl group on the C-2 of IMP to yield XMP, was localized in the glycosome of *Leishmania* by immunofluorescence (Dobie *et al.*, 2007). AMP deaminase has also been proposed to be glycosomal since it displays a C-terminal (type 1) peroxisomal targeting signal (Opperdoes and Szikora, 2006). It however remains unknown why trypanosomes compartmentalize such enzymes in their glycosomes.

A recent study by Boitz and Ullman (Boitz and Ullman, 2006) shed further light on the global mechanism involved in purine acquisition in *Leishmania*. They constructed a conditional Δhgprt/Δxprt double knock-out mutant that required both adenine and deoxycoformycin (inhibitor of adenine aminohydrolase) for survival. Through this biochemical approach, it has been possible to demonstrate that both HGPRT and XPRT were essential for the proper functioning of the parasite's purine salvage system,

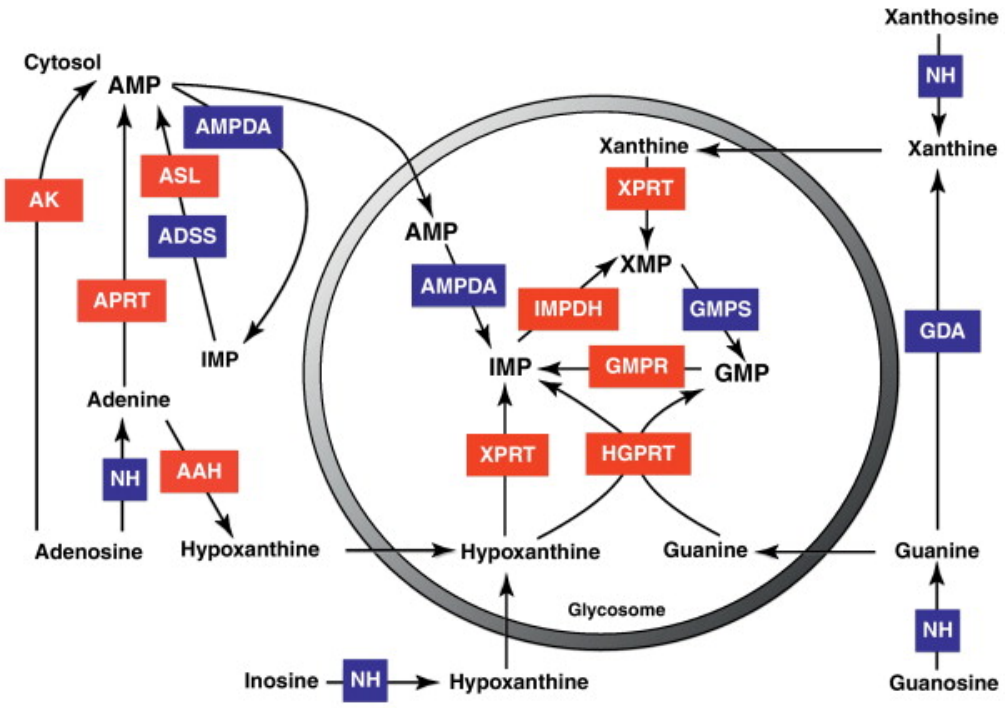

FIGURE 2.11: **Compartmentation of the purine salvage pathway.** The gray circle represents the glycosomal membrane. Enzymes have been localized based on experimental (red) or predicted (blue) data. (HGPRT, hypoxanthine-guanine phosphoribosyltransferase; XPRT, xanthine phosphoribosyltransferase; IMPDH, inosine monophosphate dehydrogenase; GMPR, GMP reductase; GMPS, GMP synthase; APRT, adenine phosphoribosyltransferase; AAH, adenine aminohydrolase; AK, adenosine kinase; ADSS, adenylosuccinate synthetase; ASL, adenylosuccinate lyase; AMPDA, AMP deaminase; GDA, guanine deaminase; NH, nucleoside hydrolase) (from Boitz *et al.* (2012)).

for viability in culture media and for infectivity of macrophages. This also showed that most purines are biochemically transformed to hypoxanthine or xanthine (not to AMP by APRT) since absence of deoxycoformycin in growth media resulted in a rapid arrest of growth. Therefore, this preference by the parasite to convert adenine to hypoxanthine or xanthine over AMP opens a novel route for vaccine development through usage of attenuated *Leishmania* strains exhibiting this genotype. A further initial metabolic flux evaluation has been recently elaborated by Boitz *et al.* (2012).

2.5 Targeting and import of proteins to the glycosome

The compartmentation of several vital enzymes in the glycosome, an organelle lacking DNA material and protein translational mechanism requires an effective protein transport system. In trypanosomes, like the transport of peroxisomal proteins in other eukaryote cells, involves two distinct targeting signals known as peroxisomal targeting signals (PTS) (Holroyd and Erdmann, 2001). PTS1 is characterized by a C-terminal degenerative tripeptide: [STAGCN]-[RKH]-[LIVMAFY][4] (Gould *et al.*, 1989; Opperdoes and Szikora, 2006), whereas PTS2 is located close to the N-terminal region and bears a more complex, yet evolutionary conserved, signal: [RK]-[LVIQ]-X_2-[LVIHQ]-[LSGAK]-X-[HQ]-[LAF], where X denotes any amino acid (Lazarow, 2006; Subramani *et al.*, 2000; Kunze *et al.*, 2011; Petriv *et al.*, 2004).

These PTS targeting sequences are recognized by two cytosolic receptors, the peroxisomal targeting signal-1 receptor PEX5 which binds PTS1 proteins and the peroxisomal targeting signal-2 (PEX7) which recognizes the PTS2 topogenic signal. From there, the two cytosolic receptors dock to peroxin 14 (PEX14), at the surface of the glycosome (Jardim *et al.*, 2002), in order to translocate their cargo inside the glycosome, as depicted in the model below (Figure 2.12). The mechanism by which the cargoes penetrate the glycosomal membrane remains to be elucidated.

[4]However, some researchers argue that better predictive results are obtained when a full dodecamer is considered. This takes into account other amino acids found to interact with the TPRs of PEX5 (reviewed by Brocard and Hartig (Brocard and Hartig, 2006))

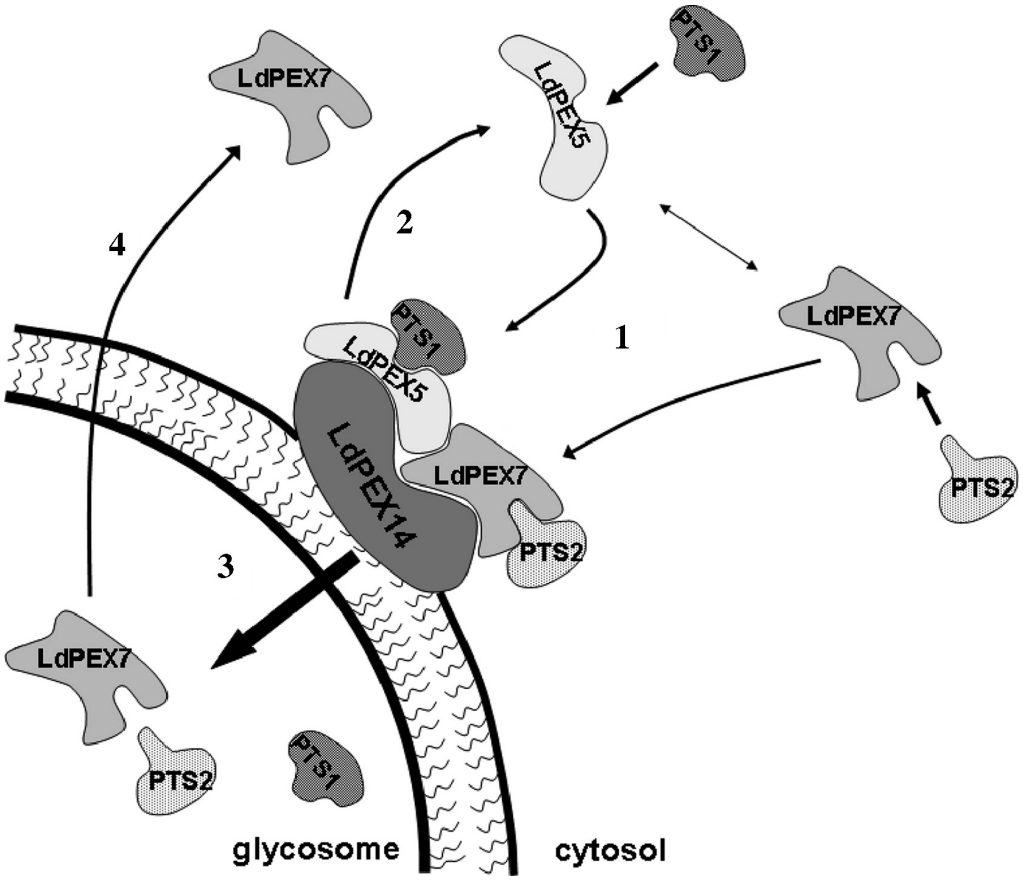

FIGURE 2.12: **General model for the import of PTS1 and PTS2 proteins in the glycosome of trypanosomes.** (1) PTS1 and PTS2 proteins are recruited by their respective cytosolic receptors; (2) the complexes dock on LdPEX14; (3) the cargo proteins are translocated through the glycosomal membrane into the lumen of the organelle; (4) LdPEX5 and LdPEX7 are recycled back to the cytosol of the parasite. (Jardim *et al.*, 2000, 2002; Madrid and Jardim, 2005; Pilar *et al.*, 2008).

2.5.1 PTS1 proteins transport in the glycosome of trypanosomes

In *Leishmania* and other trypanosomatids, PTS1 proteins are synthesized in the cytosol and bound as cargos to the receptor PEX5. This protein-protein interaction is believed to be mediated by six to seven tetratricopeptide repeats (TPR) (D'Andrea, 2003; Blatch and Lässle, 1999; Kajander *et al.*,

2007; Hirano *et al.*, 1990), located at the C-terminal portion of PEX5. This domain is localized between the amino acids 324 and 588 in *Leishmania donovani* (Jardim *et al.*, 2000; Madrid *et al.*, 2004).

Similarly, using crystallography data from the human PEX5 bound to a five amino acids PTS1 peptide (YQSKL), Gatto *et al.* (2000) demonstrated that six of these TPR were actually involved in the process while the seventh was acting as an hinge to the clam-shaped structure. They also illustrated that each TPR is forming a helix-turn-helix structure which exhibits, at its surface, the asparagine residues responsible for the interaction with the PTS1 signal (Asn378 with leucine, Asn524 with lysine and Asn497 with serine) (Figure 2.13).

Recently, Sampathkumar *et al.* (2008), following an initial crystallographic characterization of PEX5 from *Trypanosoma brucei* by Kumar *et al.* (2001), were able to solve a high resolution structure of the complete PTS1 binding region of TbPEX5 in complex with various PTS1 peptides derived from glycolytic enzymes localized to the glycosome of the parasite (Sampathkumar *et al.*, 2008). These solved structures were compared with their human counterparts previously published (see Gatto *et al.* (2000); Stanley and Wilmanns (2006); Stanley *et al.* (2007, 2006))

Additionally to the similar positioning of the atoms from the different PTS1 signals (Figure 2.14, panel B), the authors identified conserved asparagines on TbPEX5 (N429, N538, N546, N573 and N580) that were implicated in hydrogen bonding with the amino acids -1 and -2 of the PTS1 signal[5] (see Figure 2.14, panel A for detailed molecular interactions). They also noted

[5]For ease of understanding, the PTS1 tripeptide sequence positions will be numbered from the C-terminus of the signal.

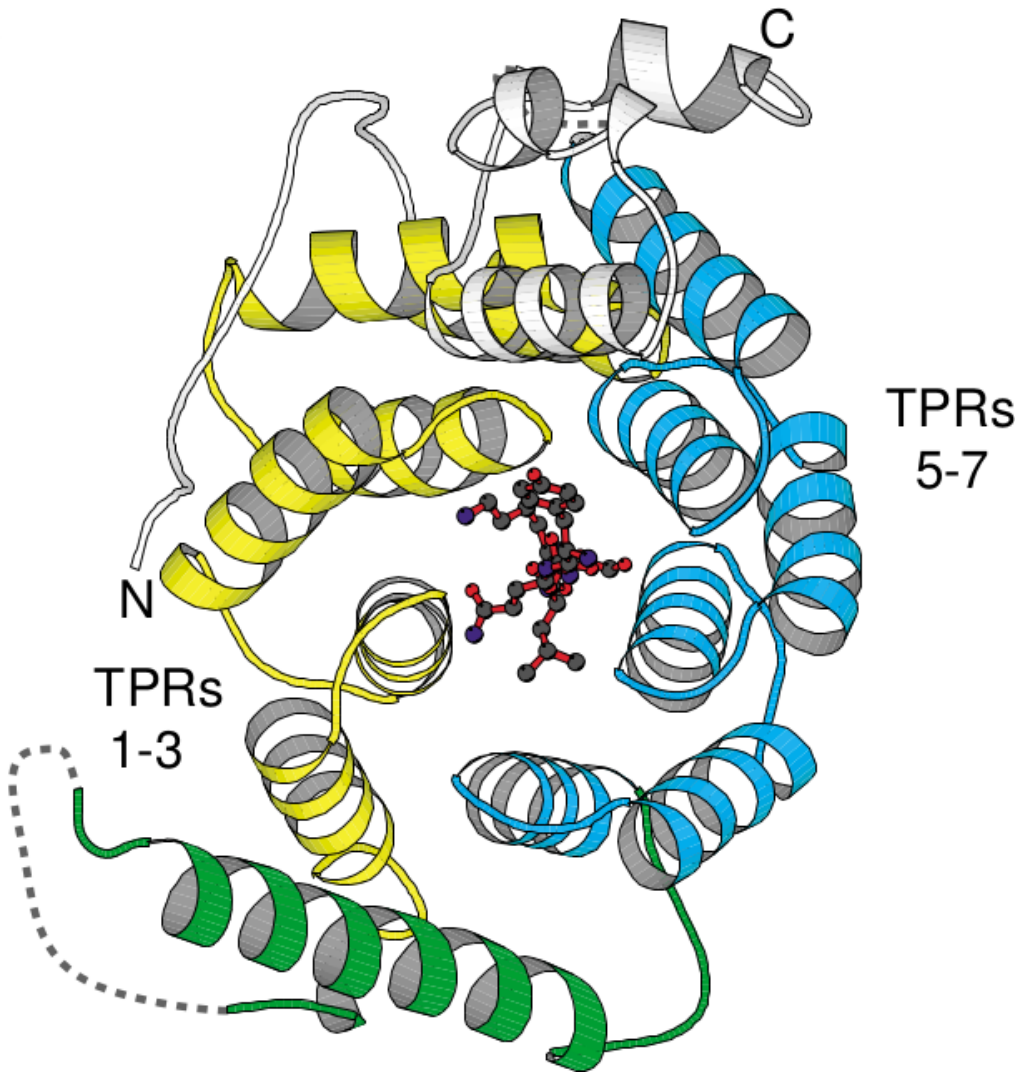

FIGURE 2.13: **First PEX5-PTS1 crystal structure based on the human protein.** Schematic view of the PEX5-PTS1 complex based on a crystal structure of the human PEX5 interacting with the pentapeptide YQSKL (Gatto *et al.*, 2000).

that the side chain from the different amino acids present at position -3 of the PTS1 signal were only involved in protein-peptide interactions via water molecules.

With all this in mind, one can summarize that the PTS1 binding mode involves first the recognition of the carboxylate from the amino acid -1; second, an hydrophobic interaction with the aliphatic chain from the side

chain of the C-terminal residue; and third, several interactions, mainly hydrogen bonds, between the conserved asparagine residues on PEX5 with the side chains of the PTS1 signal (Sampathkumar *et al.*, 2008).

Another feature noted from these studies was the presence of a hydrophobic pocket at the entrance of the TbPEX5 PTS1 binding domain, named the "double-Ala pocket"(A421 and A504), absent in the human PEX5 (the two alanines in the human PEX5 are substituted with a methionine and a cysteine respectively) (Sampathkumar *et al.*, 2008). It was then hypothesized that this feature could be exploited for a rational drug design which could inhibit a PEX5-PTS1 interaction *Trypanosoma brucei*[6].

The quaternary structure of PEX5 remains a subject of debate as several studies using phygenetically different PEX5 have suggested that this protein may adopt a monomeric (Carvalho *et al.*, 2006; Costa-Rodrigues *et al.*, 2005) or tetrameric form (Jardim *et al.*, 2000; Madrid *et al.*, 2004; Schliebs *et al.*, 1999) in solution. It appeared to be mediated by pH in the case of *Hansenula polymorpha* (Wang *et al.*, 2003) whereas for the *Leishmania donovani* PEX5, upon binding its cargo, the protein transitions from a tetrameric to a dimeric form, a structural characteristic which is believed to enhance the affinity to the docking protein PEX14 present on the surface of the glycosome (Figure 2.15) (Madrid *et al.*, 2004).

Oppositely, PEX5 from yeast is known to interact with an intraperoxisomal protein, PEX8, which is believed to cause a dissociation of the oligomer and facilitate release of the PTS1 cargo (Wang *et al.*, 2003). Evidence for the

[6]Only A421 is conserved in *Leishmania donovani*; a threonine is present at the second alanine position. Therefore it is unlikely that a similar hydrophobic pocket be present in LdPEX5.

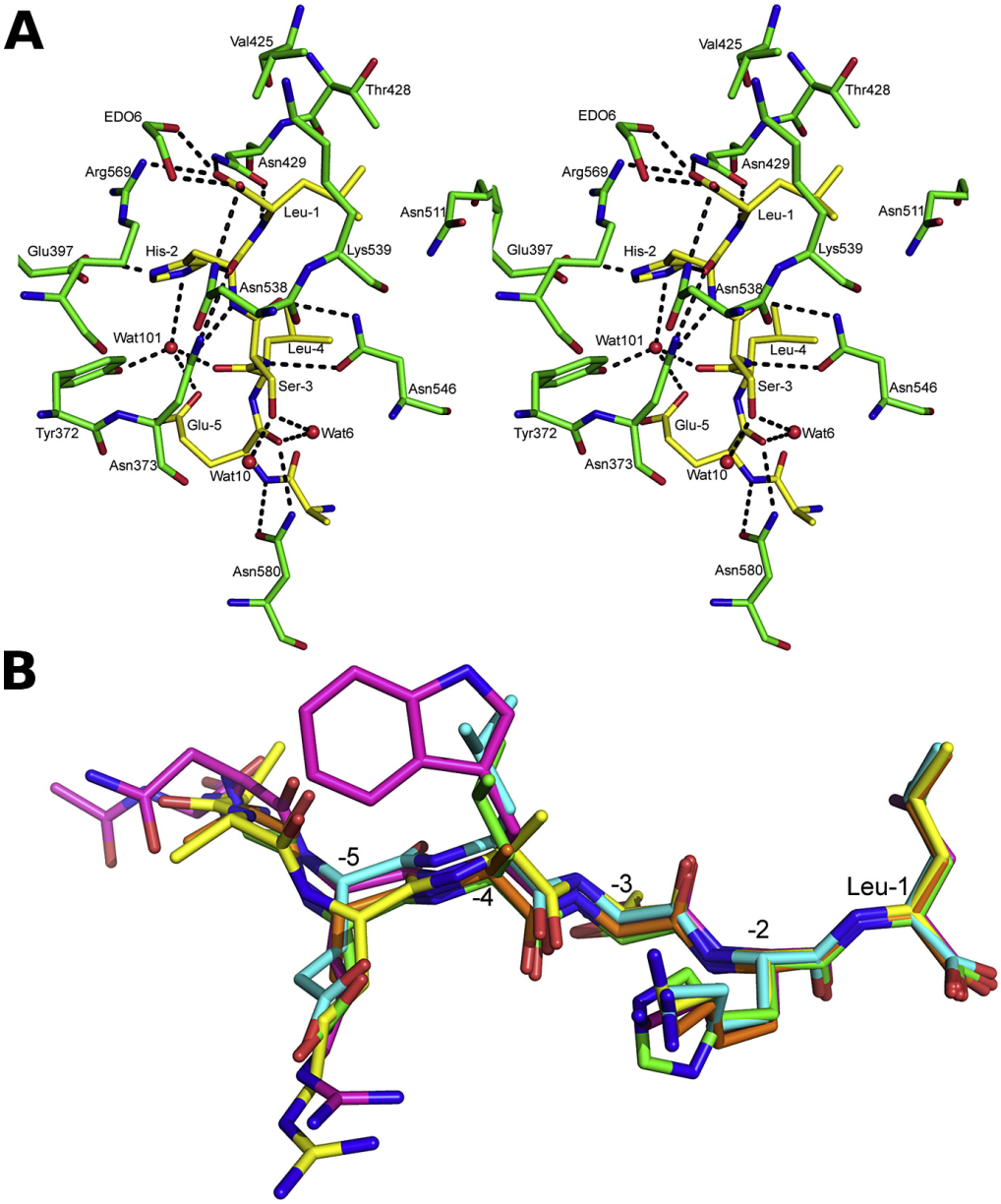

FIGURE 2.14: **TbPEX5-PTS1 molecular interactions.** A) Stereoview of the interactions between TbPEX5 and a representative PTS1 peptide. TbPEX5 and the peptide residues are shown in green and yellow carbon atoms, respectively, with oxygen atoms in red and nitrogen atoms in blue. B) Superposition of the alpha-carbons from the five PTS1 peptides bound to TbPEX5, with different colouring for the carbon atoms. (Sampathkumar *et al.*, 2008)

presence of such protein in trypanosomatids is still lacking (Galland and Michels, 2010).

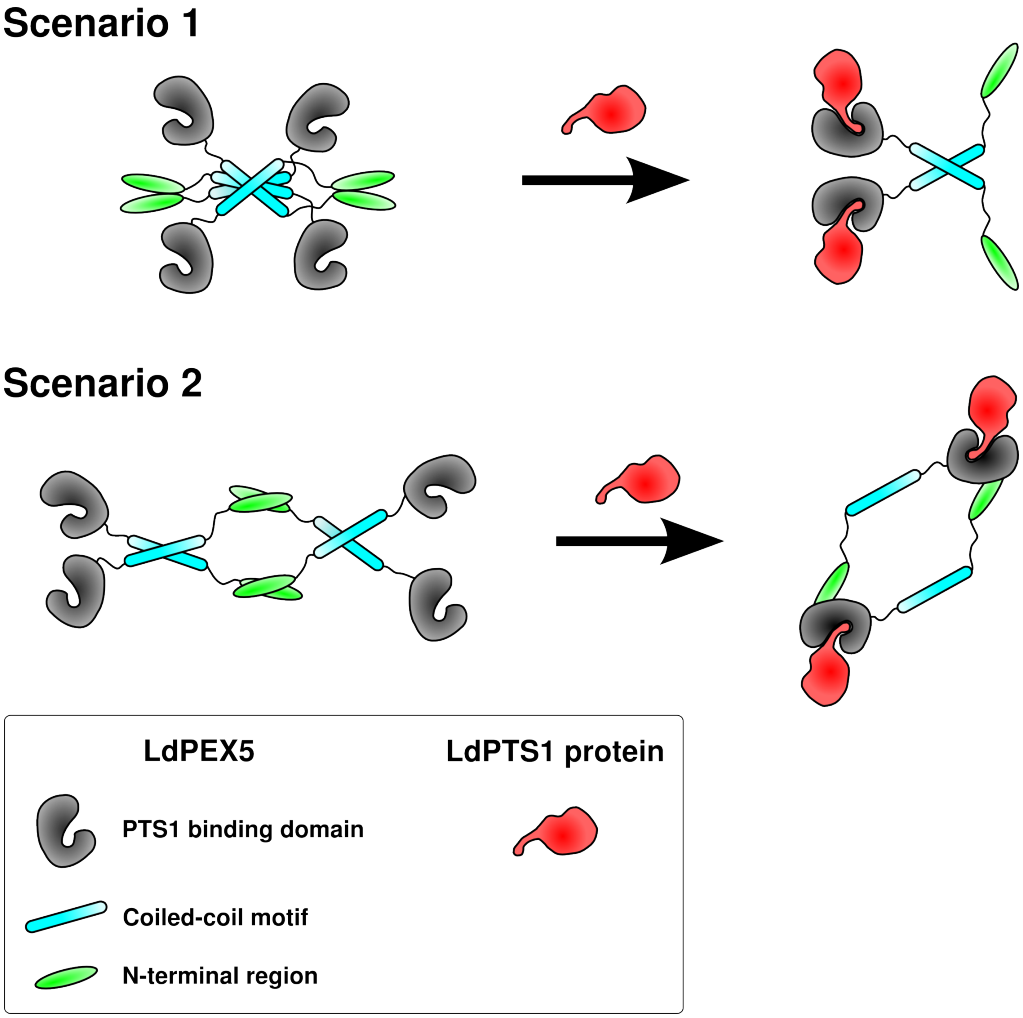

FIGURE 2.15: **Proposed models for the quaternary structure of LdPEX5.** Two possible scenarios have been proposed for the assembly of the tetrameric form of LdPEX5, where a dimer of dimers would assemble and be stabilized by either (1) a coiled-coil motif located between amino acids 269 and 291 or by (2) an N-terminal region (amino acids 1-202). Addition of a PTS1 protein causes the complex to separate into dimers (Madrid *et al.*, 2004). These quaternary structure changes are hypothesized to modulate the affinity for LdPEX14 on the surface of the glycosome.

2.5.2 PTS2 protein transport to peroxisomes and the glycosomes of trypanosomes

Similarly to PTS1 proteins, several proteins containing a PTS2 signal for proper trafficking to the glycosome of trypanosomes. This signal is recognized by the cytosolic transporter PEX7. In *Leishmania*, approximately 25% of the glycosomal proteins bear a PTS2 sequence, while the remaining proteins exhibit a PTS1 signal[7] (Opperdoes and Szikora, 2006).

Structurally, PEX7 consists of six or seven 40 amino acid long tryptophan-aspartate (WD40) repeats, that are likely responsible for making up the binding pocket which recognizes the PTS2 motif on glycosomal proteins (Figure 2.16, panels A and C) (Heiland and Erdmann, 2005; Moyersoen *et al.*, 2004; Schliebs and Kunau, 2006). These WD40 repeats form typically four antiparallel β-sheets that assemble into a circularized β-propeller (Figure 2.16). Proteins containing WD40 repeats are implicated in a wide variety of crucial cellular functions (transduction and transcription regulation, cell cycle control, autophagy, apoptosis) that involve protein-protein interactions (Smith *et al.*, 1999; Xu and Min, 2011).

PEX7 from *Leishmania* sp. contains numerous hydrophobic amino acids which are predicted to be at the surface of the protein (Figure 2.16, panels B and D) despite a clear localization of the majority of the protein to the cytosol of the parasite. This has presented a great challenge where initial attempts to purify recombinant *Leishmania* PEX7 yielded in co-purification with the bacterial chaperone GroEL (Pilar *et al.*, 2008). Also,

[7] A few exceptions have been observed where proteins contained either both PTS1 and PTS2 signals, or appeared to be transported via an internal peroxisomal targeting signal.

it is only recently that our laboratory elaborated a protocol which was able to circumvent this issue (Pilar *et al.*, 2012).

It is now well accepted that PEX7 also associates tightly with both PEX5 and PEX14 in *Leishmania* (Pilar *et al.*, 2008) and *Trypanosoma* (Galland *et al.*, 2007), and that PEX5 is required for proper trafficking of the PEX7-PTS2 complex to the glycosomal membrane. In contrast, in yeast and fungal species, additional co-receptors PEX18/PEX20 or PEX21, respectively are required (Sichting *et al.*, 2003; Einwächter *et al.*, 2001; Schliebs and Kunau, 2006; Dodt *et al.*, 2001; Heiland and Erdmann, 2005) as PEX5 lacks a PEX7 binding domain. These co-receptors act as linkers (for more details, see Schliebs and Kunau (2006)).

In a recent protein mapping study, Pilar *et al.* (2008) found that *Leishmania* PEX7 associates with LdPEX5 through an interacting domain encompassing amino acids 111 to 181, which contrast with previously predicted binding region in *Trypanosoma brucei* and conserved domain in *Leishmania* sp.[8]. Likewise, in the case of PEX14 from *Leishmania donovani*, the region between amino acids 120 to 148 in was also shown to be essential for binding PEX7 (Pilar *et al.*, 2008).

Another feature of trypanosomatid PEX7 proteins is the presence of a C-terminal proline-rich extension that is absent in other eukaryotes (Galland *et al.*, 2007; Pilar *et al.*, 2008). These segments may favour the formation of an helical structure known as a poly-proline type II helix which is typically

[8]Schliebs *et al.* (Schliebs and Kunau, 2006) have used a PEX7 binding motif [KRH]-[YVMIFLA]-[QSAKETNRDG]-[QSAKETNRDG]-S-[ETKRN]-F-[YVMIFL]-[QSAKETNRDG]-[YVMIFL]-[YVMIFL]-[QSAKETNRDG]-[QSAKETNRDG]-[YVMIFL] which is found in all known binding peroxins to PEX7 (except PEX14), including *Trypanosoma brucei*. A search in the genome database of *Leishmania infantum* and *Leishmania major* (http://www.tritrypdb.org) highlighted the same conserved region, which was comprised between amino acids 198-211 and 204-217 respectively. Such discrepancy was also noted in a review by Galland *et al.* (Galland and Michels, 2010).

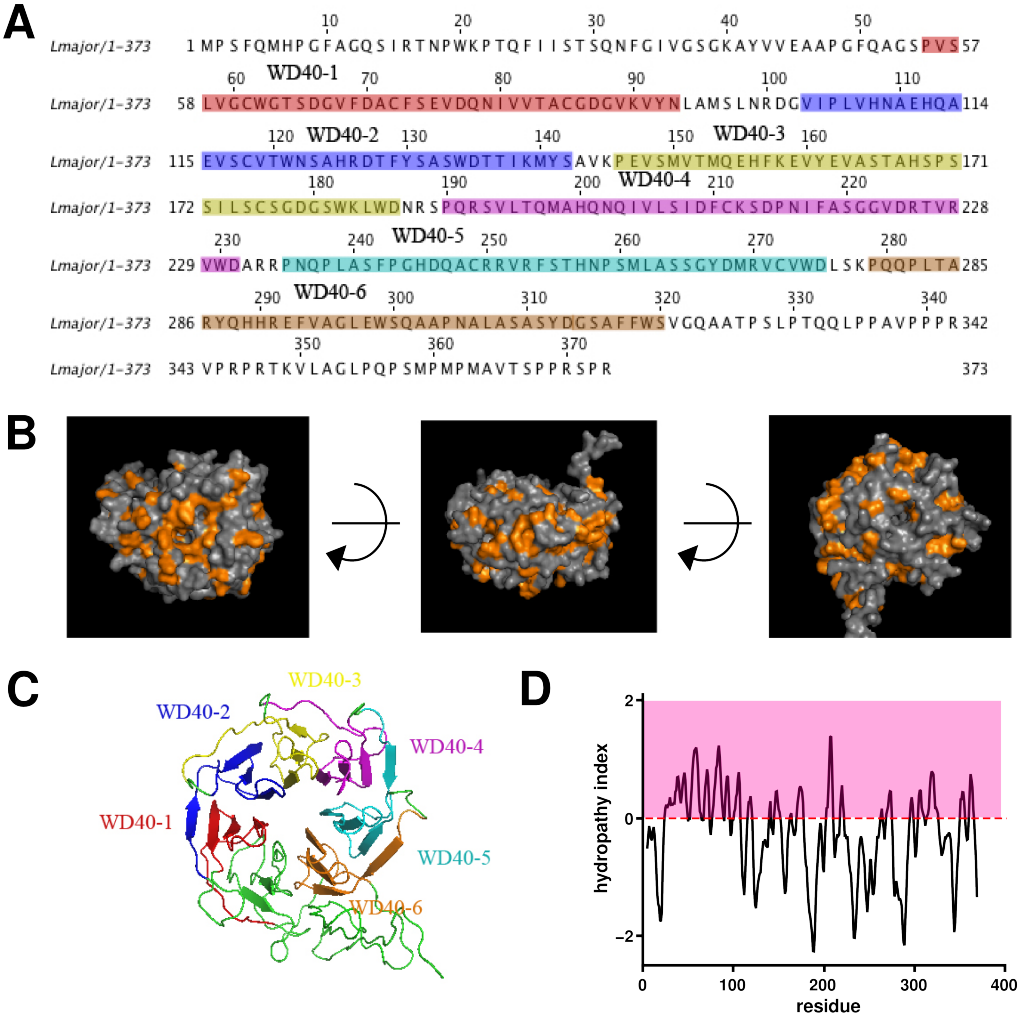

FIGURE 2.16: **Topology of *Leishmania major* PEX7.** A) Color highlights represent the WD40 repeat sequences. B) Surface representation of an I-TASSER *in silico* model (Zhang *et al.*, 2008; Roy *et al.*, 2010; Zhang, 2009; Wu *et al.*, 2007) of *Leishmania major* PEX7. The orange color represent the hydrophobic residues present at the surface of the modelled protein (Pilar *et al.*, 2012). C) Ribbon representation of the above I-TASSER model depicting the β-propeller tertiary structure and the position of the different WD40 repeats. D) Hydropathy plot based of the Kyte-Doolittle scale (Kyte and Doolittle, 1982) illustrating the multiple hydrophobic peaks (in the pink highlighted region).

recognized by an SH3 class II domain found in Src and Grb2 proteins[9] (Sparks *et al.*, 1996; Cohen *et al.*, 1995). One may hypothesize here that this motif will likely be involved in the interaction with SH3 domains.

Recently, PEX13, a peroxisomal membrane protein which plays a membrane-anchoring role for PEX14 and a cargo-release facilitator for PEX5 and PEX7 (Gould *et al.*, 1996; Mukai and Fujiki, 2006), has been identified in trypanosomatids (Verplaetse *et al.*, 2009; Brennand *et al.*, 2012). It has been shown by RNAi to be essential for the viability of *Trypanosoma brucei in vitro* (Verplaetse *et al.*, 2009). Moreover, this protein contains a predicted SH3 domain which could be implicated in an interaction with PEX7 at the glycosomal surface. Such feature has not been observed in plants (Mano *et al.*, 2006) nor in yeast (Girzalsky *et al.*, 1999).

2.5.3 Interaction of PEX14 with PEX5 and PEX7

The current model for translocation of proteins into the glycosome of *Leishmania* minimally involves an interaction between PEX7 and PEX5, and PEX14. A PTS-bearing proteins are predicted to first bind to their respective receptors in the cytosol following their release from free ribosomes. The complexes are thought to bind to the other PTS-receptor complex to forms a PTS1-PEX5-PEX7-PTS2 quaternary complex that then moves to the surface of the glycosome where it docks to PEX14 (Strasser *et al.*, 2012). Then, PTS-bearing proteins are translocated across the glycosomal membrane to the glycosomal lumen. Finally, PEX5 and PEX7

[9] A good structural example can be visualized with the crystal structure 1SEM from the Protein Database (Lim *et al.*, 1994).

are recycled back to the cytosol (Pilar *et al.*, 2008; Strasser *et al.*, 2012) by a mechanism that remains to be elucidated (Figure 2.12). The exact localization of PEX5 and PEX7 is still debated. In *Leishmania donovani*, only trace amounts of LdPEX5 was detected inside glycosomes (Jardim *et al.*, 2000), whereas LdPEX7 was found both in the cytosol and associated with the glycosome (Pilar *et al.*, 2008).

The role of PEX14 is vital to trypanosomes and was demonstrated by Furuya *et al.* (2002) in a study showing that a lethal phenotype was created by RNA silencing of *Trypanosoma brucei* PEX14. Interestingly, the lethal phenotype appeared to be conditional and was toxic only when parasites were cultivated in media containing glucose. The lack of TbPEX14 resulted in mislocalization of the glycosomal phosphoglycerate kinase enzyme which requires compartmentation for proper functioning.

A similar hypothesis emerged from our laboratory where several attempts at creating PEX14 null mutants by deletion of the two *Leishmania donovani* PEX14 alleles did not yield viable knock out strains, presumably due to the lethal phenotype.

Likewise, peroxisomal malfunctioning mediated by knocking out the PEX14 gene in yeast did not cause such drastic phenotype, unless the micro-organism was grown using fatty acids as the sole carbon source (lipid metabolism takes place in the peroxisome of *Saccharomyces cerevisiea*). A similar behaviour was observed in cultured mammalian cells, although it is well accepted that peroxisomal disorders are lethal after several years in multicellular organisms (Gould and Valle, 2000).

Previously, it has been found that the N-terminal region of PEX14, comprising the amino acids 23 to 63 (which includes a conserved PEX14 signature sequence across all eukaryotes: F-L-X_{15}-F-L-X_2-K-G-[ILV]-X_3-[DEQ]-[ILV]) is essential for binding to PEX5 (Madrid and Jardim, 2005; Jardim *et al.*, 2002). On the other hand, a central portion of PEX5 (amino acids 290 to 323) was identified to be critical for association with PEX14 (Madrid and Jardim, 2005). This observation contrasts with previous biochemical and structural studies showing that in other organisms the PEX5-PEX14 interaction was mediated by one of three aromatic W-X_3-[FY] pentapeptide repeats present in PEX5 (Figure 2.17, panel B) (Otera *et al.*, 2002; Urquhart *et al.*, 2000; Neufeld *et al.*, 2009; Su *et al.*, 2009).

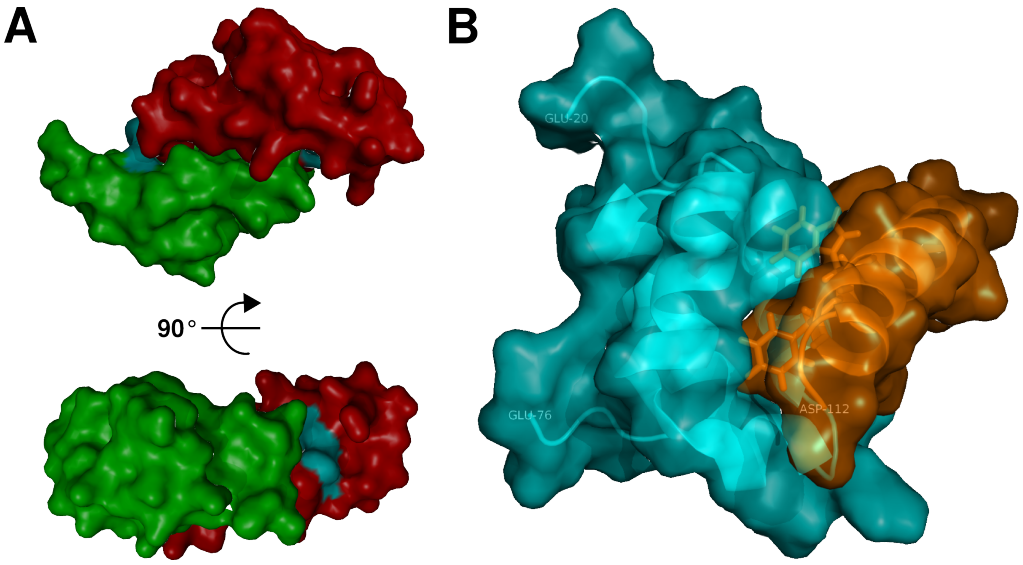

FIGURE 2.17: **Structure of the PEX14 conserved domain.** A) Crystal structure of the rat PEX14 (PDB code 3FF5) where the protein forms a dimer close to the interface where PEX5 is predicted to interact. In blue are the conserved phenylalanines and leucines believed to be part of the PEX5 binding site (Su *et al.*, 2009). B) NMR solution structure of the human N-terminal portion of PEX14 (cyan) in complex with a peptide comprising the first W-X_3-F pentapeptide from PEX5 (orange) (PDB code 2W84) (Neufeld *et al.*, 2009).

Madrid and Jardim (2005) demonstrated, by site-directed mutagenesis of the three pentapeptide motifs, that comparable affinities with *Leishmania donovani* PEX14 could be retained when compared to the wild-type LdPEX5. Moreover, these W-X_3-[FY] pentapeptide repeats from *Leishmania donovani* poorly align with the similar repeats present on PEX5 from yeast (*Saccharomyces cerevisiae*), nematode (*Caenorhabditis elegans*) and mammal (*Homo sapiens*) (Figure 2.18).

Moreover, we have recently investigated the capability of LdPEX14 to bind directly PEX7 *in vitro* (Pilar *et al.*, 2008, 2012) and *in vivo* (Strasser *et al.*, 2012). LmPEX7 bound LdPEX14 with an apparent dissociation constant of 43 nM (Strasser *et al.*, 2012) and the interaction was mediated by a

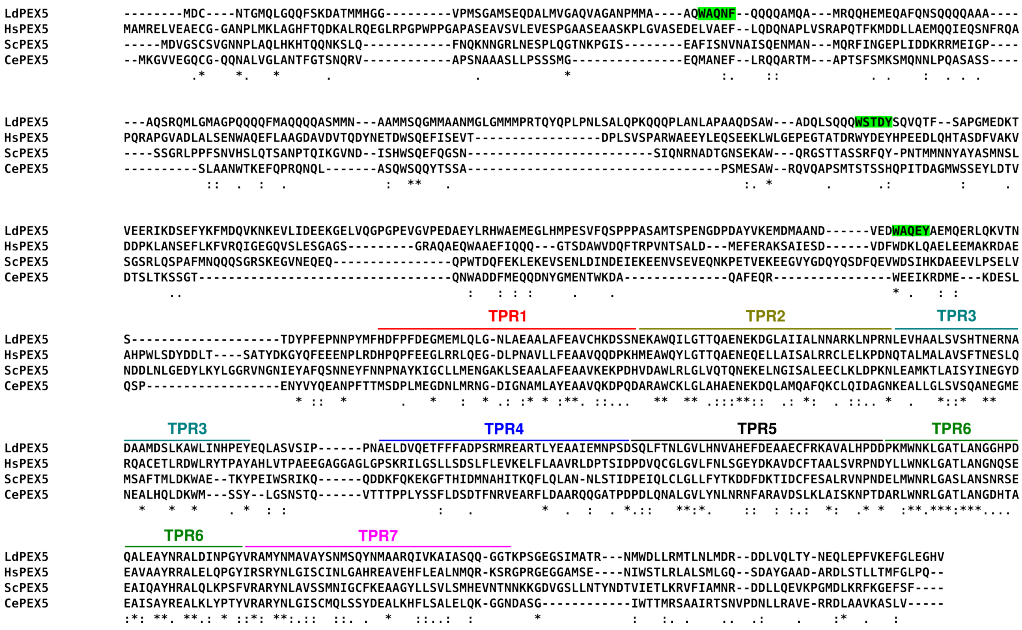

FIGURE 2.18: **Protein sequence alignment of PEX5.** The protein sequences of PEX5 from *Saccharomyces cerevisiae* (ScPEX5), *Caenorhabditis elegans* (CePEX5) and *Homo sapiens* (HsPEX5) were aligned with PEX5 from *Leishmania donovani* (LdPEX5) using MUSCLE sequence alignment software (Edgar, 2004). The three W-X_3-[FY] pentapeptide repeats from *Leishmania donovani* are highlighted in green (Madrid *et al.*, 2004).

domain located between the amino acids 120 and 148 of LdPEX14 (Pilar *et al.*, 2008; Strasser *et al.*, 2012). Interestingly, no PEX14-PEX7 direct interactions have been reported in mammals, yeast and plants (Mukai and Fujiki, 2006; Nito *et al.*, 2002; Dodt *et al.*, 2001).

2.5.4 LdPEX5-LdPEX7-LdPEX14 complex as a potential drug target

Findings enumerated above illustrate significant differences between the peroxisomal import machinery in yeast, plants and mammals, and the glycosomal protein translocation system currently being investigated in *Leishmania*, thus opening the door to the identification of potential drug targets to fight leishmaniasis and other trypanosome-associated diseases (Spithill *et al.*, 2002; de Souza, 2002). Such differences include: (i) the compartmentation of glycolysis, purine salvage and other essential metabolic pathways in the glycosome (Galland and Michels, 2010; Boitz *et al.*, 2012), (ii) the poor protein sequence conservation between *Leishmania* and human PEX5, PEX7 and PEX14 (Jardim *et al.*, 2002, 2000; Pilar *et al.*, 2008; Moyersoen *et al.*, 2003), (iii) the unique LdPEX5-LdPEX14 interacting interface which does not require the W-X_3-[FY] pentapeptide repeats essential in other species (Madrid and Jardim, 2005) and (iv) the requirement for functional glycosomal import machinery in the cell (Furuya *et al.*, 2002; Galland *et al.*, 2007; Verplaetse *et al.*, 2009).

2.6 Connecting statement

In addition to the therapeutic potential that can be developed by further characterizing the glycosomal protein translocation machinery, it offers a unique opportunity to study the biophysical fundamentals implicated in the transport of folded proteins across membrane bilayers.

In the following chapter, a more in depth analysis of the quaternary structure of PEX14 from *Leishmania donovani* will be investigated, with respect to the importance of various domains present on the protein and the role they play in the assembly of PEX14 oligomeric structures. Moreover, structural changes taking place on PEX14 upon binding to PEX5 will be described, using various biophysical methods including circular dichroism, isothermal titration calorimetry and fluorescence spectroscopy.

This will enable a better comprehension of the central role that LdPEX14 plays in the glycosomal transport machinery and its implication in the actual formation of a translocation pore through the glycosomal membrane.

Chapter 3

The *Leishmania donovani* Peroxin 14 undergoes a marked conformational change following association with Peroxin 5

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3.1 Abstract

The import of PTS1 proteins into the glycosome or peroxisome requires binding of a PTS1 laden PEX5 receptor to the membrane associated protein PEX14 to facilitate translocation of PTS1 proteins into the lumen of these organelles. Quaternary structure analysis of protozoan parasite *Leishmania donovani* PEX14 (LdPEX14) revealed that this protein forms a homomeric complex with a size >670 kDa. Moreover, deletion mapping indicated that disruption of LdPEX14 oligomerization correlated with the elimination of the hydrophobic region and coiled-coil motif present in LdPEX14. Analysis of the LdPEX5:LdPEX14 interaction by isothermal titration calorimetry revealed a molar binding stoichiometry of 1:4 (LdPEX5:LdPEX14) and an in solution dissociation constant (K_d) of ∼74 nM. Calorimetry, intrinsic fluorescence and, analytical ultracentrifugation experiments showed that binding of LdPEX5 resulted in a dramatic conformational change in the LdPEX14 oligomeric complex that involved the reorganization of the hydrophobic segment in LdPEX14. Finally, limited tryptic proteolysis assays established that in the presence of LdPEX5, LdPEX14 became more susceptible to proteolytic degradation consistent with this protein interaction triggering a significant conformational change in the recombinant and native LdPEX14 structures. These structural changes provide essential clues to how LdPEX14 functions in the translocation of folded proteins across the glycosomal membrane

3.2 Introduction

Leishmania and *Trypanosoma* protozoan parasites represent organisms that branched off early from the eukaryotic cell lineage (Dacks and Doolittle, 2001; Simpson *et al.*, 2006; Douzery *et al.*, 2004). Consequently, these organisms have retained a myriad of unique metabolic, biochemical, and structural features that are distinctive from other eukaryotic cells. Prominent among these features is the glycosome, an organelle that is distantly related to the peroxisomes in mammalian, yeast, fungi, and plant cells (Michels *et al.*, 2005; Opperdoes *et al.*, 1977b; Opperdoes and Michels, 1993). The glycosome compartmentalizes a multitude of indispensable metabolic and biosynthetic pathways that include glycolysis, purine salvage, pyrimidine and ether-lipid biosynthesis, and β-oxidation of fatty acids (Opperdoes and Michels, 1993; Fairlamb, 1989). Glycosomal function is essential for parasite viability as mis-targeting of glycolytic enzymes to the cytosol or disruption of glycosome biogenesis leads to a lethal phenotype (Bakker *et al.*, 2000; Furuya *et al.*, 2002; Galland *et al.*, 2007; Guerra-Giraldez *et al.*, 2002; Kessler and Parsons, 2005) making the glycosome and glycosome biogenesis machinery attractive chemotherapeutic targets (Parsons *et al.*, 2001; Sommer and Wang, 1994).

Glycosomal and peroxisomal matrix proteins are post-translationally trafficked from cytosolic ribosomes to these microbodies by utilizing primarily one of two topogenic signals termed peroxisomal targeting signal 1 and 2 (PTS1 and PTS2) located at the C- or N-termini proteins, respectively (Blattner *et al.*, 1992; Gould *et al.*, 1989; Lametschwandtner *et al.*, 1998; Purdue and Lazarow, 1994, 2001; Swinkels *et al.*, 1991). In *Leishmania*, newly synthesized proteins containing PTS1 or PTS2 signals

are bound by the receptors peroxin 5 (LdPEX5) and peroxin 7 (LdPEX7), respectively and these cargo-laden receptors traffic to the glycosome surface where they bind to the membrane associated protein peroxin 14 (LdPEX14). This latter protein-protein interaction is paramount for the translocation of proteins across the glycosomal and peroxisomal membrane and for the biogenesis of these organelles. In Δ*pex14* yeast and mammalian mutant cell lines that lack a functional PEX14, the matrix PTS1 and PTS2 proteins are mis-targeted into the cytosol (Albertini *et al.*, 1997; Komori *et al.*, 1997; Salomons *et al.*, 2000; Shimizu *et al.*, 1999). In trypanosomes knockdown of PEX14 using RNA interference caused mis-targeting of glycosomal matrix proteins to the cytosol and resulted in a lethal phenotype when parasites were cultivated in media containing glucose or glycerol (Furuya *et al.*, 2002; Kessler and Parsons, 2005; Moyersoen *et al.*, 2003).

PEX14 in fungi and mammalian interacts with PEX13 forming a subcomplex known as the importomer (Agne *et al.*, 2003; Azevedo and Schliebs, 2006; Erdmann and Schliebs, 2005; Girzalsky *et al.*, 1999; Rayapuram and Subramani, 2006; Reguenga *et al.*, 2001); whether a comparable importomer complex exists in the protozoa *Leishmania* is not clear since a PEX13 homolog has not yet been identified in this group of organisms. Numerous studies in phylogenetically diverse organisms have demonstrated that PEX14 is a membrane-associated protein, however the nature of this interaction and the topology of PEX14 in the peroxisomal and glycosomal membranes, as assessed by physiochemical techniques, seem to vary. In mammalian cells, *Hansenula polymorpha*, *Pichia pastoris*, and *Trypanosoma brucei* (Shimizu *et al.*, 1999; Moyersoen *et al.*, 2003;

Fransen *et al.*, 1998; Johnson *et al.*, 2001; Komori *et al.*, 1999; Oliveira *et al.*, 2002; Will *et al.*, 1999) PEX14 is reported to be an integral protein, whereas in *Saccharomyces cerevisiae* PEX14 association with the peroxisomal membrane is more plastic and has been reported to behave either as a peripheral or integral membrane protein (Albertini *et al.*, 1997; Brocard *et al.*, 1997; Niederhoff *et al.*, 2005). Using similar biochemical approaches, the *Leishmania* PEX14 has been shown to be peripheral membrane protein that associates tightly with the cytosolic surface of the glycosomal membrane (Jardim *et al.*, 2002).

Interestingly, despite the fact that PEX14 proteins exhibit <10% sequence conservation across phylogeny (Jardim *et al.*, 2002), this family of proteins has retained three structural elements. These include an N-terminal 33 amino acid signature motif A-X_2-F-L-X_8-P-X_6-F-L-X-K-G-X_5-I-X_2-A that contains a PEX5 binding motif (Purdue and Lazarow, 2001; Choe *et al.*, 2003), a hydrophobic region, and a coiled-coil motif (Albertini *et al.*, 1997; Will *et al.*, 1999; Brocard *et al.*, 1997; Jardim *et al.*, 2002; Choe *et al.*, 2003). The hydrophobic region and the coiled-coil motif are believed to be involved in the formation of PEX14 homomeric structures (Shimizu *et al.*, 1999; Oliveira *et al.*, 2002; Fransen *et al.*, 2002). However, little is known about the structural changes induced in PEX14 upon binding its receptor, PEX5. Here we reported the use of a number of biophysical techniques that include size exclusion chromatography, analytical ultracentrifugation, isothermal titration calorimetry, and limited proteolysis to examine the LdPEX14 structural and conformational changes triggered in the LdPEX14 complex following LdPEX5 binding.

3.3 Experimental procedures

3.3.1 Material

All restriction endonucleases and DNA modifying enzymes were purchased from Invitrogen Life Technologies Inc. (Grand Island, NY) or New England Biolabs (Beverly, MA). Horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit IgG were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were of the highest quality commercially available.

3.3.2 Cell culture

L. donovani promastigotes were cultured in Dulbeccos modified Eagles-*Leishmania* medium (DME-*L*) supplemented with hemin, xanthine, and fetal bovine serum as described (Pilar *et al.*, 2008).

3.3.3 LdPEX5 and LdPEX14 expression and purification

The ORFs encoding the full length LdPEX14 or mutant proteins encompassing residues 1-321 (ldpex14 1-321), 1-254 (ldpex14 1-254), 1-200 (ldpex14 1-200), 1-148 (ldpex14 1-148), or 1-120 (ldpex14 1-120) were amplified by PCR and cloned into the NcoI/BamHI sites of the pET30b(+) expression vector as previously described (Jardim *et al.*, 2002; Madrid and Jardim, 2005). The ORF for the ldpex14 fragment encompassing residues 1-75 (ldpex14 1-75) was amplified by PCR and cloned into the NdeI/XhoI

sites of pET15b(+). The internal deletion mutants ldpex14 Δ149-179 and ldpex14 Δ270-321 were generated by PCR mutagenesis using the template pET30b(+)-His_6/S-LdPEX14 and the primer pairs listed in Table 3.1.

Deletion	Primer sequence
Δ149-179	5-CTGGCTAATGAGCGGACGCAGACGACAGCG-3
	5-CGCTGTCGTCTGCGTCCGCTCATTAGCCAG-3
Δ270-321	5-GAGCGGCAGACGAAGGAATTTCGTCGCAAG-3
	5-CTTGCGACGAAATTCCTTCGTCTGCCGCTC-3

TABLE 3.1: Primers used to generate internal deletions in LdPEX14 gene

The LdPEX14 N-terminal truncations were generated as previously described (Madrid and Jardim, 2005). Recombinant LdPEX14 proteins expressed using pET30b(+) contained an N-terminal hexahistidine and S-tag. Proteins expressed using the pET15b(+) only contain a hexahistidine affinity tag. LdPEX5 and ldpex5 203-391 were expressed in *E. coli* using the pTYB12 expression vector (Madrid *et al.*, 2004).

E. coli ER2566 cells transformed with pTYB12-LdPEX5 or pTYB12-ldpex 203-391 (Madrid *et al.*, 2004) were grown to an OD_{600nm} of 1.2 at 37°C then shifted to 20°C for protein expression. Protein expression was induced for 4 h with 0.5 mM isopropylthiogalactoside (IPTG). Cell pellets were re-suspended in 20 ml of 40 mM Tris-HCl pH 8.0 containing an EDTA-free protease inhibitor cocktail (Roche Applied Science, Laval, QC) and lysed by French press. Lysates were clarified by centrifugation and the supernatant was loaded onto a chitin column (1 x 3 cm) and the column was washed with 100 ml of 0.5 M NaCl in TB buffer. LdPEX5 and ldpex5 203-391 were cleaved by incubating the column matrix with 5 ml 50 mM DTT in

TB buffer for 40 h at 4°C. Recombinant LdPEX14/ldpex14 proteins were over-expressed in *E. coli* ER2566 strain and purified as previously described (Jardim *et al.*, 2002).

For analytical centrifugation and tryptic digest experiments the LdPEX5-LdPEX14 complex was isolated from *E. coli* ER2566 cells co-transformed with the pTYB12-LdPEX5 and pET30b-His_6/S-LdPEX14. Clarified cells lysates were applied onto a Ni^{2+}-NTA column (1 x 5 cm) and the bound proteins were eluted with 250 mM imidazole in 50 mM phosphate pH 7.5, 150 mM NaCl (PBS). The eluates were then applied to a chitin column (1 x 3 cm) to capture complexes containing the chitin-LdPEX5 fusion protein. The column was washed with 50 ml of 40 mM Tris-HCl pH 8.0, 0.5 M NaCl and the LdPEX5-LdPEX14 complex was eluted by incubating the column matrix with 5.0 ml of 50 mM DTT in 40 mM Tris-HCl pH 8.0 for 40 h at 4°C. All recombinant proteins were concentrated and the buffer exchanged for 40 mM Tris-HCl pH 8.0, 150 mM NaCl using a Biomax 5K NMWL centrifugal filter unit (Millipore, Bedford, MA). Protein concentrations were determined spectrophotometrically (Pace *et al.*, 1995).

3.3.4 Site-directed mutagenesis

The three tryptophan residues (W246, W293, and W361) in ldpex5 203-391 were sequentially mutated to phenylalanine residues using PCR QuikChange protocol to generated the ldpex5 203-391 W3F. The sequence of this tryptophanless mutant was validated by DNA sequence analysis. Intrinsic fluorescence analysis of ldpex5 203-391 W3F excited at 295 nm showed no notable fluorescence emission between 310 and 400 nm,

diagnostic of this protein being devoid of a tryptophan residue. Binding studies performed with ldpex5 203-391 W3F using either pull down or ELISA assays (Schliebs *et al.*, 1999; Gouveia *et al.*, 2000) showed that this mutant ldpex5 fragment exhibited LdPEX14 binding characteristics similar to ldpex5 203-391 (data not shown).

3.3.5 Tryptic analysis of LdPEX14 and LdPEX14-LdPEX5 complex

LdPEX14 (∼100 μg) or LdPEX5-LdPEX14 (125 μg) complex in 100 μl of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl (TBS) were incubated with 0.3 μg of sequencing grade trypsin (Promega, Madison, WI) at 20°C. Alternatively, purified glycosomes (50 μg of total protein) from wild type *L. donovani* promastigotes (43) were incubated with recombinant LdPEX5 or bovine serum albumen (15 μg) and LdXPRT (50 μg) for 20 min at 20°C prior to the addition of 0.3 μg of sequencing trypsin in 150 μl of TBS. Aliquots (10 μl) were removed at 0, 2, 5, 10, 15, 60, and 180 min and mixed with 4 μl of protease inhibitor cocktail (1 mini tablet/ml dH_2O) to inactivate the trypsin prior to addition SDS-PAGE sample buffer. Samples were resolved on a 10% SDS-PAGE, transferred to PVDF membrane and stained with Coomassie blue R250. Membranes were blocked with 3% skimmed milk powder in PBS then probed with anti-Ni^{2+}NTA-HRP in PBS (1:2,000, Qiagen, Mississauga, ON) and anti-LdPEX14 (1:16,000) and anti-rabbit IgG-HRP (1:10,000).

3.3.6 Quaternary Structure Analysis

Size exclusion chromatography (SEC) was performed on a Beckman-Coulter system Gold equipped with a Superdex 200 column (GE HealthCare, Baie dUrfé, QC) equilibrated with 50 mM phosphate pH 7.5, 150 mM NaCl, 5 mM β-mercaptoethanol. Samples containing 50-200 μ protein in buffer containing 10 mM β-mercaptoethanol were injected and the column was developed at 0.5 ml/min while monitoring the column effluent at 280 nm. Fractions (0.5 ml) were collected and proteins precipitated with trichloroacetic acid for SDS-PAGE analysis. The Superdex 200 column was calibrated using thryoglobulin (670 kDa), bovine IgG (158 kDa), ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B12 (1.3 kDa) (Bio-Rad, Hercules, CA).

3.3.7 Isothermal titration calorimetry (ITC)

ITC experiments were performed on a MicroCal VP-ITC calorimeter (MicroCal, Northampton, MA). For the forward reaction a 292 μM solution of LdPEX14 was loaded into the syringe and titrated into an 8 μM solution of LdPEX5 present in the ITC cell (1.42 ml). Alternatively the syringe was loaded with a 1.0 mM solution of ldpex14 1-120 which was titrated into a 29 μM solution of ldpex5 203-391 in the ITC cell. For the reverse titrations the ITC syringe was loaded with a 149 μM LdPEX5 solution and the ITC cell was charged with a 40 μM LdPEX14 solution. Alternatively, the syringe was loaded with 397 μM solution of ldpex5 203-391 was titrated into a 145 μM ldpex14 1-120 solution in the ITC cell. All reactions were performed at a constant temperature of 303 K and protein solutions were

dialyzed against the same batch of 40 mM sodium phosphate, 120 mM NaCl pH7.5, 2 mM β-mercaptoethanol buffer to minimize heat of dilution effects. For all experiment, the contents of the cell were mixed at 300 rpm and an equilibration time of 6 min between injections was used. The first injection used a 2 μl aliquot and the 20-30 subsequent injections were performed using 10 μl volumes over a duration of 10 s. The experimental titration curves were corrected for the heat of dilution, the initial 2 μl injections were typically omitted from the data set, and the curve fitting was performed using the MicroCal Origin software 7.0 assuming a one site model. The binding constants ($K_d = 1/K_a$) and the enthalpy (ΔH) was determined from the isotherm and the Gibbs free energy (ΔG) and entropy (ΔS) was calculated using the equation $\Delta G = \Delta H - T\Delta S = - RT \ln K_a$.

3.3.8 Analytical ultracentrifugation

Sedimentation velocity experiments were performed in 40 mM Tris pH 7.5 at 20°C in a Beckman Optima XL-I (Fullerton, CA) analytical ultracentrifuge using an An 55 AL aluminum rotor. Samples containing 250 μg/ml LdPEX14 or LdPEX5-LdPEX14 (molar ratio of 1:4) were loaded into double-sector cell with aluminum-filled Epon centerpieces. LdPEX14 and LdPEX5-LdPEX14 complex were analyzed at rotor speeds of 26,000 and 30,000 rpm, respectively. UV scans were obtained at 230/280 nm and analyzed by the van Holde-Weischet method (Van Holde and Weischet, 1978). The G/g (S) integral distribution attained with this method were determined using the XL-I UltraScan II version 9.7 sedimentation data analysis software (B. Demeler, The University of Texas Health Science Center, San Antonio, TX).

3.3.9 Fluorescence spectroscopy

Fluorescent measurements were performed on a Varian Cary Eclipse spectrofluorometer (Palo Alto, CA) at 25°C using an excitation wavelength of 295 nm. Emission spectra were recorded from 305 to 400 nm using a scan rate of 120 nm/min with an excitation and emission slit widths of 5 nm. A solution of recombinant LdPEX14 (40 μg) in 40 mM Tris pH 8.0 was titrated with ldpex5 203-391 W3F to a final concentration of 0-40 μM. Dilution effects were corrected by titrating LdPEX14 with buffer alone. For the chemical denaturation experiment, guanidine hydrochloride (Gnd-HCl) was added to a final concentration of 4.5 M in 20 μM of LdPEX14 in 40 mM Tris pH 8.0. The mixture was then incubated at 4°C for 18 h. Fluorescence spectra were collected under similar conditions than mentioned above, except that the detector sensitivity was increased by ∼15 % to compensate for the lower protein concentration. For quenching experiments a 5 M acrylamide solution was titrated into a mixture containing 40 μM LdPEX14, or LdPEX14-ldpex5 203-391 W3F mixture (40:10 μM) in 75 mM phosphate pH 7.1.

3.3.10 Circular Dichroism (CD)

Purified proteins were exhaustively dialyzed at 4°C against 10 mM phosphate buffer pH 7.6 and the protein concentration measured by the method of Pace et al. (Pace *et al.*, 1995). CD measurements were performed on a Jasco 810 spectropolarimeter at 20°C, using a cuvette with 0.1 cm pathlength at a scan rate of 50 nm/min. Five spectra were collected and averaged. For all samples, data were collected at wavelengths between 250

nm and 185 nm. LdPEX14 was diluted in dialysis buffer to a concentration of 3.1 μM, and ldpex5 203-391 was added to a final concentration of 0.8 μM to obtain a 4:1 molar ratio of LdPEX14:ldpex5 203-391.

3.3.11 Crosslinking studies

L. donovani promastigotes (5 x 10^8 cells/ml PBS) were permeabilized for 5 min at 20°C with 15 μg/ml of digitonin and aliquots (100 μl) were incubated with increasing concentrations of glutaraldehyde (0-1.5 mM) for 20 min at 20°C. Cells were washed with 3 x 1.0 ml PBS to remove excess crosslinking agent and the cell pellet was re-suspended in 100 μl of 2X SDS-PAGE sample buffer containing 6 M urea. Purified recombinant LdPEX14 (2 μg/50 μl PBS) was subjected to glutaraldehyde crosslinking using the above conditions. Reaction mixtures were resolved on a 5-10% gradient SDS-PAGE and the proteins were transferred to a PVDF membrane. Western blots were probed with rabbit anti-LdPEX14 (1:10,000) (Jardim *et al.*, 2002) or rabbit anti-*L. donovani* adenine phosphoribosyltransferase (LdAPRT) (1:1,000) antibodies. For crosslinking reactions with ldpex5 203-391, ldpex14 1-148, and ldpex14 1-120, proteins were reduced for 3 h at 20°C with 5 mM tris 2-carboxyethyl phosphine to reduce potential disulfide bonds prior to crosslinking with glutaraldehyde (0-3.2 mM). Crosslinked complexes were characterized by Western blots probed with anti-LdPEX5 and anti-LdPEX14 antisera.

3.4 Results

3.4.1 LdPEX14 quaternary structure

Native LdPEX14 extracted from glycosomes was previously demonstrated to migrate on a sucrose density gradient predominantly as a macromolecular structure of ∼800 kDa[1]. To further validate oligomeric structure of LdPEX14 on the glycosome surface, chemical cross-linking was used to trap these complexes. Western blot analysis of digitonin-permeabilized *L. donovani* promastigotes treated with glutaraldehyde showed a concentration-dependent accumulation of a cross-linked complex that SDS-PAGE was estimated to have an apparent mass of >250 kDa (Figure 3.1). A comparable complex was also observed when the cross-linking reaction was performed with zero length cross-linking agent ethyl dimethylaminopropyl carbodiimide (data not shown). Western blots of glutaraldehyde cross-linked reactions probed with antisera against adenine phosphoribosyltransferase, a 26 kDa cytosolic protein (Allen *et al.*, 1995b; Zarella-Boitz *et al.*, 2004), revealed a single immunoreactive band indicating that nonspecific cross-linking was minimal (Figure 3.1 panel B). Similar complexes were also detected with purified glycosomes (Jardim *et al.*, 2002) or recombinant LdPEX14 treated with glutaraldehyde (Figure 3.1 panel C).

Analysis of the LdPEX14 primary sequence showed three structural motifs that are conserved among the PEX14 protein family. These include the PEX5 binding domain (residues 23-70), a hydrophobic region (residues 149-179), and a coiled-coil motif (residues 270-321) (Albertini *et al.*, 1997; Komori *et al.*, 1997; Brocard *et al.*, 1997; Jardim *et al.*, 2002; Itoh

[1]Madrid *et al.*, in preparation

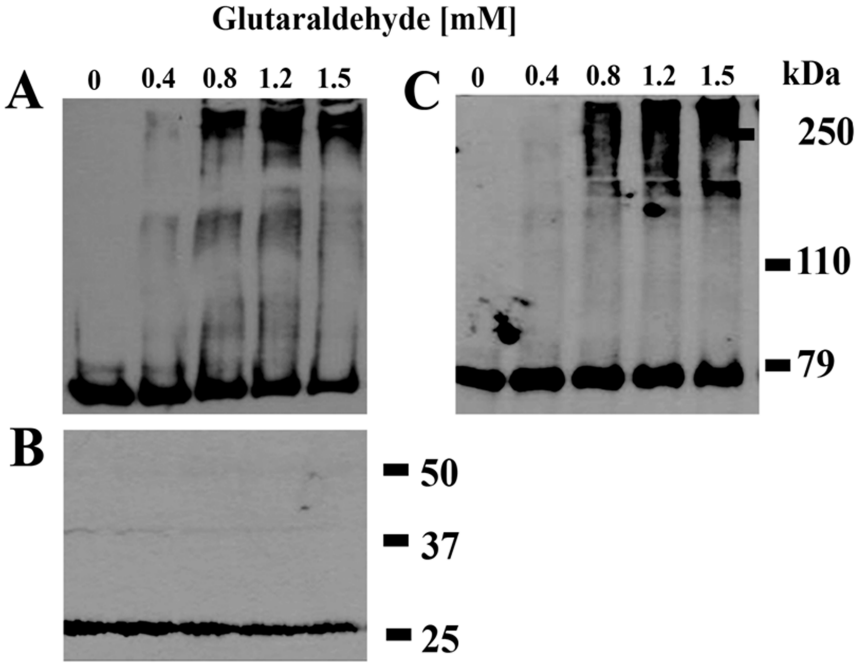

FIGURE 3.1: **LdPEX14 cross-linking.** Digitonin permeabilized *L. donovani* promastigotes re-suspended in PBS were treated with increasing concentrations of glutaraldehyde (0-1.5 mM) for 20 min at 20°C and the crosslinking reactions were resolved on a 5-10% gradient SDS polyacrylamide gel. Proteins were transferred to a PVDF membrane and probed with rabbit anti-LdPEX14 (1:10,000) (panel A) and rabbit anti-*L. donovani* APRT antibodies (panel B) to identify crosslinked forms of these protein. Recombinant LdPEX14 (2 μg/lane) purified from *E. coli* was also treated with 0-1.5 mM glutaraldehyde and the crosslinked products were examined by Western blot using rabbit anti-LdPEX14 antibodies (panel C).

and Fujiki, 2006; Shimozawa *et al.*, 2004). To elucidate the elements required for LdPEX14 oligomerization, a panel of ldpex14 truncation and internal deletion mutants was expressed in *E. coli* (Figure 3.2 panel A) and the quaternary structure examined by SEC. All of the ldpex14 proteins were readily purified from *E. coli* lysates using Ni^{2+}-NTA affinity chromatography as soluble proteins. No precipitation was observed even after prolonged storage at 4°C or -80°C. Interestingly, SEC analysis of

freshly purified full-length recombinant LdPEX14, ldpex14 Δ1-63, ldpex14 Δ270-321, ldpex14 Δ149-179, ldpex14 1-321, ldpex14-1-254, and ldpex14-1-200 revealed that these proteins all eluted in the void volume of a Superdex 200 column suggesting the formation of oligomeric complexes with a size of >670 kDa (Figure 3.3 panel A). Similar results were also observed when a 5-10-fold more dilute concentration of these proteins was injected onto the gel permeation column.

Analysis of several of these mutant proteins by analytical ultracentrifugation confirmed the formation of large complexes of differing sizes (Figure 3.3 panel D). In low ionic strength buffers LdPEX14 assembled into structures that varied in size from ∼10 to 70 S, whereas ldpex14 Δ149-179 appeared to form smaller structures ranging in size from 10 to 30 S (Figure 3.3 panel D). Surprisingly, in low ionic strength buffer ldpex14 1-254 migrated as a relatively homogeneous complex of ∼2-10 S. However, when the sedimentation analysis of this protein was performed in buffer containing 150 mM NaCl, a marked increase in oligomerization occurred resulting in structures with a size distribution of 2-90 S (data not shown). Similarly, sedimentation velocity analysis of ldpex14 1-200 in low ionic strength buffers showed that this protein was also heterodisperse and sedimented with S values ranging from 10 to 170 S (Figure 3.3 panel D).

In contrast, truncation mutants lacking the hydrophobic region, which include ldpex14 1-148, ldpex14 1-120, and ldpex14 1-75, migrated on the Superdex 200 column with masses of ∼50, 42, and 20 kDa, respectively, consistent with these proteins forming a dimeric structure (Figure 3.2 and 3.3 panel B). For ldpex14 1-75, the dimer appears to be particularly stable as indicated by the significant population of the dimeric species (∼22

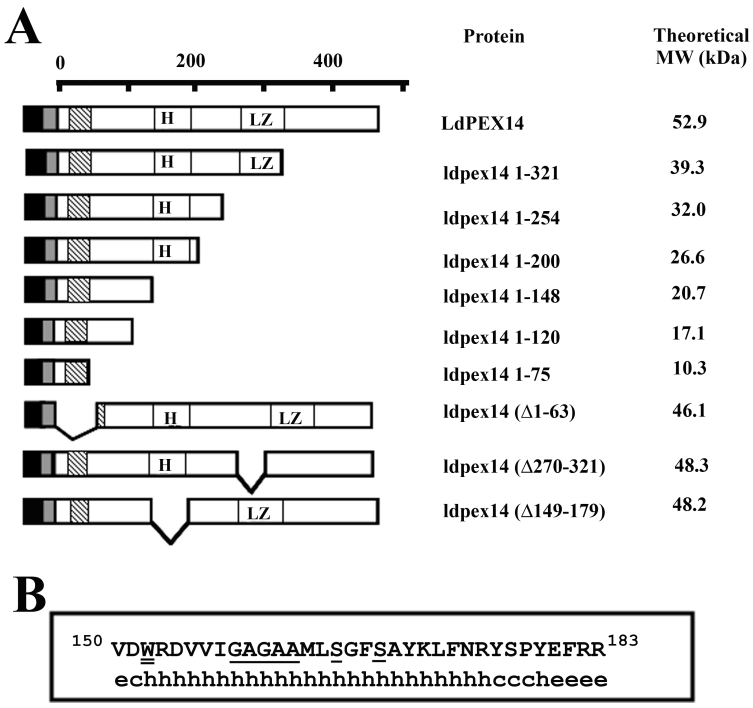

FIGURE 3.2: **LdPEX14 primary structure.** (A) The diagram illustrates recombinant LdPEX14 constructs used in this study. The black and gray shaded boxes represents the hexahistidine and S-protein tags, respectively, that were derived from the pET30b(+) expression vector. These tags were used for the affinity purification of the recombinant proteins on Ni^{2+}-NTA or S-protein affinity resins. The boxes label denoted with an H or LZ correspond to the predicted hydrophobic domain (residues 149-179) and the coiled-coiled motif (residues 270-321) present in LdPEX14 (Jardim *et al.*, 2002). Finally, the hatched box corresponds to the PEX14 signature motif that is highly conserved among PEX14 homologues. The theoretical molecular weights for the constructs are also indicated. (B) The sequence encompasses residues 149-173, the predicted hydrophobic region for LdPEX14. The underlined sequences GXXXA and SXXS represent putative helix-helix interaction motifs (Dawson *et al.*, 2002; Kleiger *et al.*, 2002). Lowercase letters correspond to the secondary structure prediction using the Hierarchical Neural Network algorithm (www.expasy.org), e denotes residues in an extended strand, h denotes residues favoring helix structure, and c represents residues favoring a random coil structure. The double underline denotes the single tryptophan residue present in LdPEX14.

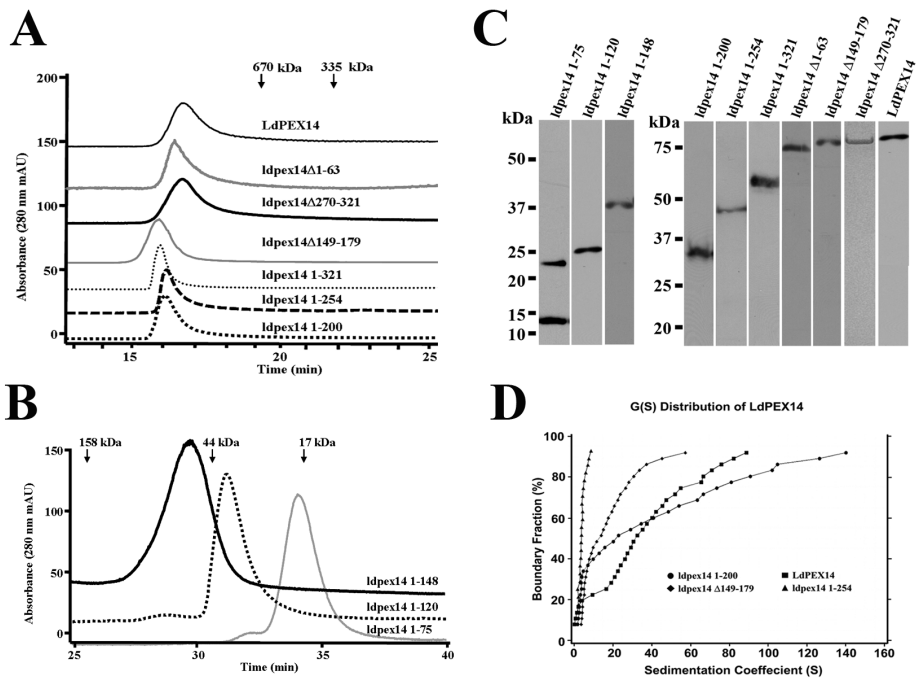

FIGURE 3.3: **Quaternary structure of LdPEX14.** (A) The quaternary structure of the full length His_6/S-LdPEX14, His_6/S-ldpex14 Δ-63, His_6/S ldpex14 Δ270-321, His_6/S ldpex14 Δ149-179, His_6/S ldpex14 1-321, His_6/S ldpex14 1-254, and His_6/S ldpex14 1-200 was analyzed by injecting a 20 μl aliquot of a 5-10 mg/ml solution of freshly purified protein onto a Superdex 200 size exclusion chromatography column. The column was developed with 50 mM sodium phosphate, 150 mM NaCl, 5 mM β-mercaptoethanol, pH 7.6 at a flowrate of 0.5 ml/min and proteins were detected by monitoring the column effluent at 280 nm to detect proteins. (B) The quaternary structure of His_6/S-ldpex14 1-148, His_6/S-ldpex14 1-120 and His_6-ldpex14 1-75 was determined by using a Superdex 200 column as described above. The Superdex 200 column was calibrated using a standard protein mixture containing thryoglobulin (670 kDa), bovine IgG (158 kDa), ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B12 (1.3 kDa). (C) SDS-PAGE analysis of affinity purified LdPEX14 and ldpex14 proteins used for gel permeation analysis. (D) To further examine the quaternary structures, His_6/S-LdPEX14 and select group of the deletion mutants (His_6/S ldpex14 Δ149-179, His_6/S ldpex14 1-254, and His_6/S ldpex14 1-200) were analyzed by sedimentation velocity on analytical ultracentrifugation at 20°C in 40 mM Tris pH 7.5 in a Beckman Optima XL-I ultracentrifuge at a rotor speed of 26,000 rpm.

kDa) by SDS-PAGE (Figure 3.3 panel C). It should be noted that the wild type and mutant LdPEX14 proteins all migrated with anomalously higher molecular weight than theoretically predicted (Jardim *et al.*, 2002). Stabilization of the LdPEX14 homomeric structure probably involves multiple protein-protein contacts because the mutants ldpex14 Δ149-179 and ldpex14 Δ270-321, which lack the hydrophobic domain or the coiled-coil motif, still formed large complexes that were not disrupted by singly deleting either of the elements (Figure 3.3 panel A and 3.3 panel D).

3.4.2 Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) was employed to determine the binding stoichiometry (N), binding affinity (K_d), enthalpy (ΔH), entropy ($T\Delta S$), and the Gibbs free energy (ΔG) for the LdPEX5-LdPEX14 and ldpex5 203-391-ldpex14 1-120 interactions (Leavitt and Freire, 2001; Velazquez-Campoy *et al.*, 2000). Figure 3.4 illustrates the isothermograms for representative reactions. Titration of LdPEX14 into a solution of LdPEX5 showed that the LdPEX5-LdPEX14 interaction resulted in an exothermic heat release that diminished as the level of free LdPEX5 in the ITC cell decreased (Figure 3.4 panel A). Integration heat release for each injection was fit using the Microcal Origin 7.0 nonlinear regression software to a one-site model that had a binding stoichiometry of 4.2:1 (LdPEX14:LdPEX5). The K_d for the LdPEX5-LdPEX14 interaction was 512 nM (or 128 nM per LdPEX14 subunit). Similar titration experiments performed with ldpex14 1-120 and ldpex5 203-391, fragments known to a form stable interactions (Madrid and Jardim, 2005), gave a binding stoichiometry of 4.1:1 (ldpex14 1-120:ldpex5 203-391) and a K_d of 625

nM (156 nM per ldpex14 1-120 subunit) (Figure 3.4 panel B). The ΔG ($\sim$-36kJ/mol) for the binding reaction was derived from favorable negative ΔH and positive TΔS components (Table 3.2). In ligand binding reactions, hydrogen bonding and van der Waal interactions are proposed to be the major sources contributing to a negative ΔH, whereas desolvation of hydrophobic surfaces, an event that increases the randomness of the system, favours a positive ΔS (Leavitt and Freire, 2001; Sturtevant, 1977; Connelly *et al.*, 1994). Collectively, this suggests that the LdPEX5-LdPEX14 interaction is stabilized by a combination of hydrogen bonding and hydrophobic interactions.

Reverse titration experiments in which LdPEX5 or ldpex5 203-391 was titrated into a solution of LdPEX14 or ldpex14-(1-120), respectively, in both cases gave a binding stoichiometry $\sim$0.25:1 (LdPEX5:LdPEX14 or ldpex5 203-391:ldpex14 1-120) (Figure 3.4 panel C and 3.4 panel D; Table 3.2). These titrations, like the forward reaction, were exothermic throughout and had a ΔG of approximately -40 kJ/mol (Table 3.2). However, the favorable ΔG was primarily derived from a large negative ΔH term (-90 to -99 kJ/mol), which offset the negative entropy change (TΔS) (Table 3.2). The unfavorable TΔS suggests that interaction of LdPEX5 or ldpex5 203-391 with LdPEX14 or ldpex14 1-120 likely leads to the solvation of a hydrophobic surface or a conformational change that decreases the degrees of freedom in the system (Leavitt and Freire, 2001; Sturtevant, 1977). Comparable K_d values for the LdPEX5-LdPEX14 and ldpex5 203-391-ldpex14 1-120 interactions were also obtained for these reactions (Table 3.2). The solution phase binding affinities for the LdPEX5-LdPEX14 interactions, measured by ITC, are somewhat higher than values

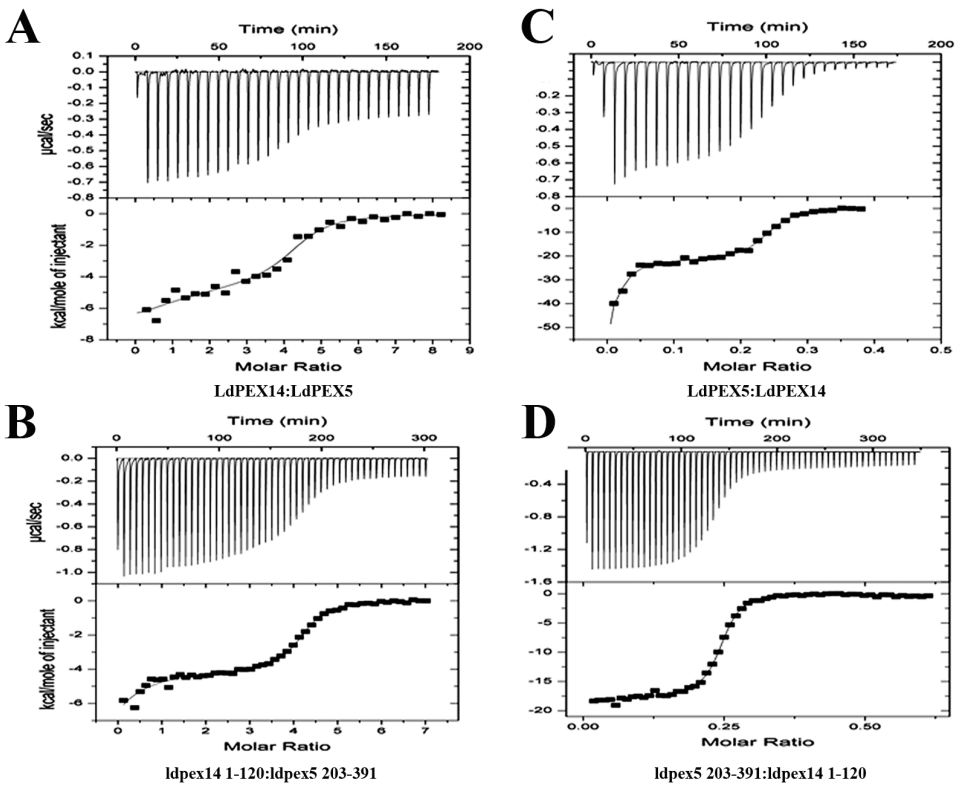

FIGURE 3.4: **Isothermal titration calorimetry.** The thermodynamic parameters, dissociation binding constants, and the binding stoichiometries for the LdPEX5-LdPEX14 interaction were determined by titrating; (A) a 292 μM solution of LdPEX14 was titrated into an 8 μM solution of LdPEX5; (B) a 1.0 mM solution of ldpex14 1-120 was titrated into a 29 μM solution of ldpex5 203-391; (C) a 149 μM LdPEX5 solution was titrated into a 40 μM LdPEX14; (D) a 397 μM solution of ldpex5 203-391 was titrated into 145 μM ldpex14 1-120. All reactions were performed at 30°C in 40 mM sodium phosphate, 120 mM NaCl pH7.5, 2 mM β-mercaptoethanol buffer. In each panel the top portion of the figure represent the heat of reaction after each 10 μl injection and the lower portion represents the integrated heat of for each injection plotted as a function of the molar ratio of LdPEX14:LdPEX5, LdPEX5:LdPEX14, ldpex14 1-120:ldpex5 203-391, or ldpex5 203:ldpex14 1-120. The solid line represents the best fit of the data assuming a one site model.

previously reported using an ELISA-based assay (Madrid and Jardim, 2005). This discrepancy is likely because of the cooperative nature or avidity effects associated with interactions that may occur at liquid-solid interphase (Shinohara *et al.*, 1997).

SEC analysis of the ITC reaction mixtures revealed that the LdPEX5-LdPEX14 complex eluted in the column void volume suggesting that LdPEX5 was recruited to the LdPEX14 complex (Figure 3.5 panel A). The presence of LdPEX5 in the void volume was confirmed by SDS-PAGE (Figure 3.5 panel A). Previous studies demonstrated that LdPEX5 alone migrated on an SEC column with an apparent molecular mass of ∼270 kDa (Madrid *et al.*, 2004), and indeed a small population of LdPEX5 alone was detected in the ITC reaction mixtures (fractions 31-36 (Figure 3.5 panel A)). SEC analysis of the ldpex5 203-391-ldpex14 1-120 ITC reaction mixture revealed a peak eluting with a mass of ∼95 kDa that by SDS-PAGE was found to contain both ldpex5 203-391 and ldpex14 1-120 (Figure 3.5 panel B) and is consistent with a hetero-oligomeric complex containing one ldpex5 203-391 and four ldpex14 1-120, as predicted by ITC.

To validate the structure of ldpex5 203-391-ldpex14 1-120 complex, a

Protein in the syringe	Protein in the cell	K_d nM	Stoichiometry N	ΔH kJ/mol	$T\Delta S$ kJ/mol	ΔG kJ/mol
LdPEX14	LdPEX5	512	4.2:1	-17.6	26.4	-36.4
ldpex14 1-120	ldpex5 203-391	625	4.1:1	-16.7	19.3	-36.0
LdPEX5	LdPEX14	74	0.23:1	-90.0	-48.1	-41.9
ldpex5 203-391	ldpex14 1-120	166	0.23:1	-99.6	-60.3	-39.3

TABLE 3.2: Thermodynamic parameters for the LdPEX5-LdPEX14 interaction

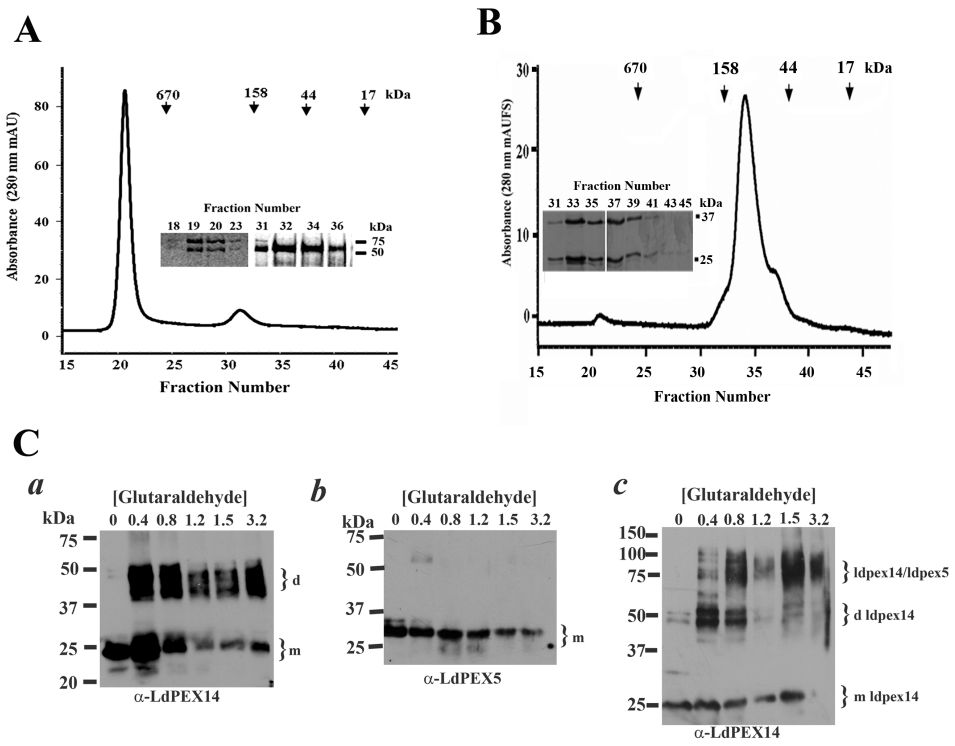

FIGURE 3.5: **Analysis of the isothermal titration calorimetry complexes.** Complexes form in the ITC cell were analyzed by size exclusion chromatography on Superdex 200 column equilibrated with 50 mM phosphate pH 7.5, 150 mM NaCl, 5 mM β-mercaptoethanol and developed at a flowrate of 0.5 ml/min. A 20 μl aliquot from the (A) LdPEX5:LdPEX14 or (B) ldpex5 203-391:ldpex14 1-120 titration reactions was injected onto the column and 1.0 min fractions were collected and analyzed by Coomassie blue or silver stained SDS-PAGE (inset) to validate the protein composition of each peak. (C) To confirm the quaternary structure of the ldpex5 203-391-ldpex14 1-120 complex by crosslinking experiments. Ldpex14 1-120 (panel a), ldpex5 23-391 (panel b) or ldpex5 203-391-ldpex14 1-120 mixture (1:4 mole ratio) (panel c) was treated with 0-3.2 mM glutaraldehyde for 20 min at 20°C. Brackets designate the monomer (m) and dimer (d) forms of ldpex14 1-120 or the ldpex14 1-120:ldpex5 203-391 complex. Reaction mixtures were analyzed by Western blot using anti-LdPEX14 or anti-LdPEX5 antibodies.

glutaraldehyde cross-linking was performed. Treatment of ldpex14 1-120 with increasing concentrations glutaraldehyde revealed a substantial accumulation of an ∼50 kDa species that confirmed previous SEC analysis showing that the N-terminal region of LdPEX14 contained a dimerization domain (Figure 3.3 panel B and Figure 3.5 panel C, panel a). In contrast, only a monomeric species was detected for ldpex5 203-391 (Figure 3.5 panel C, panel b). This was rather surprising, because by SEC ldpex5 203-391 eluted with an apparent molecular mass of ∼55 kDa suggesting that this protein behaved as a dimer (data not shown). Western blot analysis of ldpex5 203-391:ldpex14 1-120 (1:4) mixtures show that at low glutaraldehyde concentrations the ldpex14 1-120 dimer is primarily detected. However, at a glutaraldehyde concentration of 0.8 -3.2 mM, an ∼100-kDa hetero-oligomeric complex is detected (Figure 3.5 panel C, panel c). The presence of the ∼80- and 100-kDa doublets in the reactions containing 0.4 and 0.8 mM of glutaraldehyde suggests that the ldpex14 1-120 dimers may bind sequentially to ldpex5 203-391 (Figure 3.5 panel C, panel c).

3.4.3 Fluorescence Measurements

A striking feature of the LdPEX5-LdPEX14 ITC isotherm (Figure 3.4 panel C) was the large ΔH change that occurred with low levels of LdPEX5. The absence of this dramatic heat loss when LdPEX14 was added to LdPEX5 (Figure 3.4 panel A) suggested that this heat loss may be associated with a conformational change in the LdPEX14 oligomeric complex triggered by binding of LdPEX5. Secondary structure analysis of LdPEX14 using the HNN algorithm revealed that the single tryptophan

residue (Trp152) in LdPEX14 mapped to the N-terminus of a putative α-helix formed by the hydrophobic domain (Figure 3.2 panel B). We exploited the intrinsic fluorescence of Trp152 to follow structural changes induced in LdPEX14 on binding LdPEX5. To eliminate fluorescence contributions from LdPEX5, ldpex5 203-391 W3F, a mutant fragment in which the three tryptophan residues found within this region were mutated to phenylalanines was used in these studies (Jardim *et al.*, 2000). Pull-down assays indicated that ldpex5 203-391 and ldpex5 203-391 W3F exhibited similar LdPEX14 binding characteristics. Excitation of LdPEX14 at 295 nm revealed that Trp152 had an emission maximum (λ_{max}) at 332 nm, a wavelength diagnostic of a tryptophan located in a nonpolar environment (Figure 3.6 panel A). Addition of ldpex5 203-391 W3F to LdPEX14, however, induced a concentration-dependent increase in the fluorescence intensity and a red-shift in the emission λ_{max} to 341 nm, an alteration consistent with Trp152 shifting to a more exposed polar environment (Figure 3.6 panel A). Correlating the wavelength change with the molar ratio of ldpex5 203-391 W3F revealed a plateau in the shift of the Trp152 emission λ_{max} at an ldpex5 203-391 W3F:LdPEX14 mole ratio of 0.25:1. This binding stoichiometry is in agreement with the results obtained by ITC. Denaturation of LdPEX14 with 4.5 M guanidinium hydrochloride resulted in a shift in the 152 λ_{max} from 332 to 360 nm, indicating that this tryptophan residue in native LdPEX14 was located in nonpolar environment (Figure 3.6 panel B) (Eftink and Ghiron, 1981). Stern-Volmer plots revealed a linear response with Stern-Volmer constant of 5.4 M^{-1} for LdPEX14-ldpex5 203-391 W3F indicating that Trp152 underwent dynamic quenching with acrylamide (Figure 3.6 panel C). A lower Stern-Volmer constant of 3.8 M^{-1} was measured for LdPEX14 alone,

indicating that in the absence of ldpex5 203-391 W3F, Trp152 was less accessible to the quenching agent (Figure 3.6 panel C) (Eftink and Ghiron, 1981).

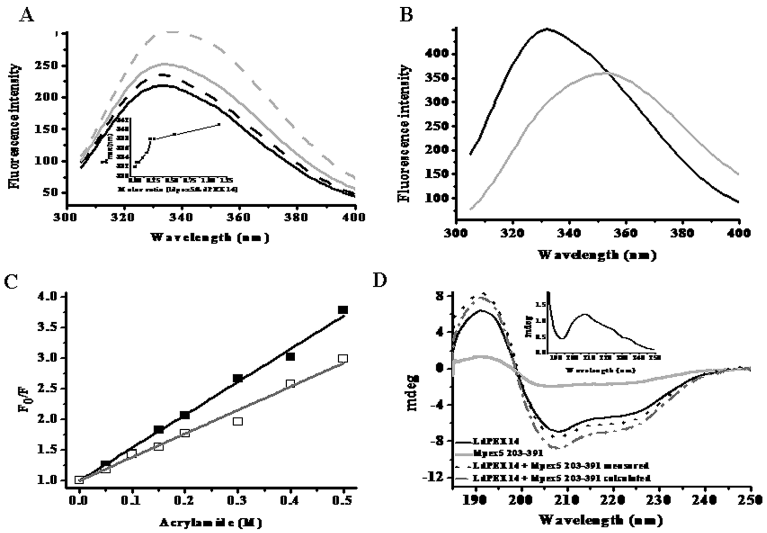

FIGURE 3.6: **Spectroscopy analysis of LdPEX14.** (A) LdPEX14 structural changes induced by the binding of ldpex5 203-391 W3F were examined by intrinsic fluorescence using an excitation wavelength of 295 nm and the emission spectra for LdPEX14 (40 μM) (solid black line) and ldpex5 203-391 W3F:LdPEX14 with molar ratios of 0.05:1 (dashed black line), 0.25:1 (solid grey line) or 1.15:1 (dashed grey line) were recorded from 305 to 400 nm. No significant fluorescence was observed with ldpex5 203-391 W3F alone. All spectra were recorded in 75 mM phosphate pH 7.1. The inset show change in the emission λ_{max} of Trp152 as a result of titrating resulting in ldpex5 203-391 W3F (0-40 μM) into a solution of LdPEX14 (40 μM). (B) The emission λ_{max} for Trp152 in native LdPEX14 (black line) was centered at 331 nm, however denaturation of LdPEX14 (grey line) with 4.5 M guanidinium hydrochloride resulted in a marked red shift in the emission λ_{max} to 360 consistent with Trp152 being fully exposed. (C) Acrylamide quenching of the tryptophan residue in LdPEX14 alone (open squares, grey line) or ldpex5 203-391 W3F:LdPEX14 complex (10:40 μM) (filled squares, black line) was analyzed by a Stern-Volmer plot. The plot was generated using an excitation of 295 nm and monitoring the change in fluorescence intensity at 340 nm. (D) To evaluate the secondary structure changes induced in LdPEX14 on binding ldpex5, far-UV CD were recorded for LdPEX14 alone (3.1 μM), ldpex5 203-391 alone (0.8 μM), a mixture containing a 4:1 mole ratio of LdPEX14:ldpex5 203-391 (3.1:0.8 μM). To estimate the conformation change associated with the protein-protein interaction the spectra for LdPEX14 alone and ldpex5 203-391 alone were summed and compared to the recorded spectrum of the LdPEX14:ldpex5 203-391 complex (Greenfield, 2004). The difference CD spectrum generated from the measured and calculated spectra for the LdPEX14:ldpex5 203-391 complex is shown as an inset.

3.4.4 CD Analysis

The effect of ldpex5 203-391-LdPEX14 interaction on the secondary structure was examined by the method of Greenfield (Greenfield, 2004). The far-UV CD difference spectrum generated by subtracting the calculated spectra generated from the unmixed ldpex5 203-391 and LdPEX14 spectra from the CD spectrum obtained for the LdPEX14:ldpex5 203-391 (4:1) complex revealed a maxima at ∼215 nm consistent with an increase in the random coil content of the complex (Figure 3.6 panel D, inset). Interestingly, ldpex5 203-391 did not show a prominent circular dichroism signal suggesting that this fragment has a relatively flexible conformation (Figure 3.6 panel D).

3.4.5 Analysis of the LdPEX14 and LdPEX5-LdPEX14 Complex

Sedimentation velocity analytical ultracentrifugation analysis of the LdPEX14 and LdPEX5-LdPEX14 macromolecular complexes by the method of van Holde-Weischet (Van Holde and Weischet, 1978), which correlates the sedimentation coefficient of a protein species with its abundances at the moving boundary, gave rise to curves with a positive deflection (Figure 3.7). This relationship is diagnostic of a protein forming heterogeneous oligomeric structures (Van Holde and Weischet, 1978). As demonstrated previously (Figure 3.3 panel D), LdPEX14 formed disperse structures with sedimentation coefficients ranging from 17 to 52 *S*. Under similar conditions, the LdPEX5-LdPEX14 complexes also exhibited a heterogeneous behavior, but the complexes had a more compact

architecture with sedimentation coefficients ranging from ∼6 to 19 S. These results imply that binding LdPEX5 induced a conformational change in LdPEX14 leading to the formation of a more ordered LdPEX5-LdPEX14 hetero-oligomeric complex (Figure 3.7) and support the ITC finding that docking of LdPEX5 to LdPEX14 induced a striking conformational change.

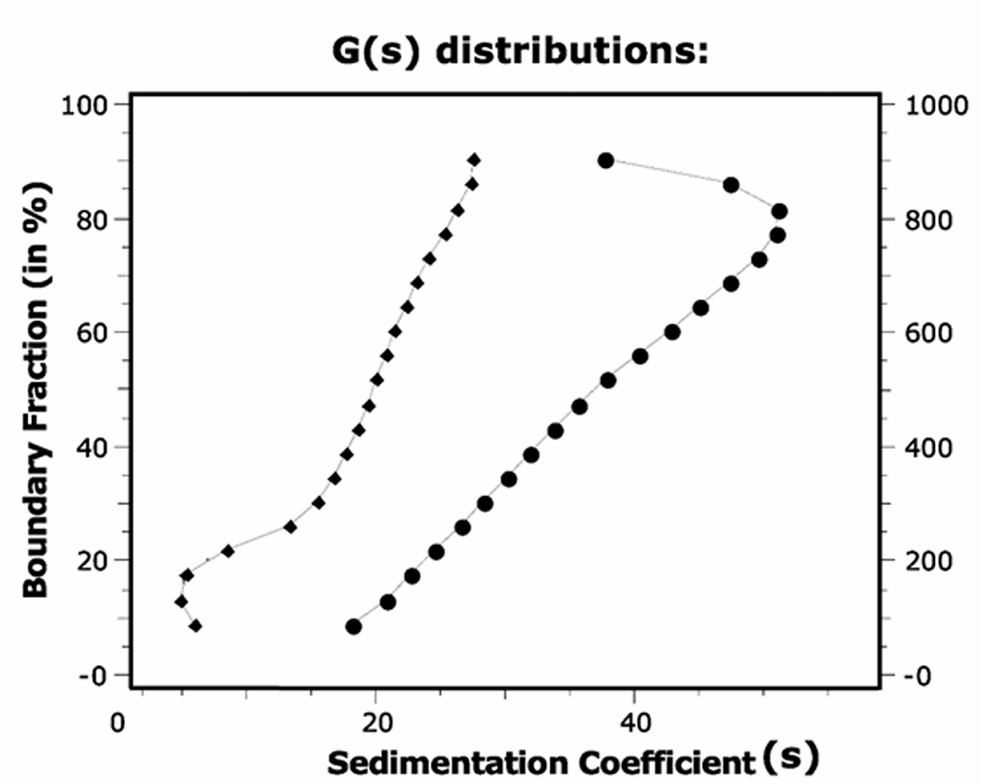

FIGURE 3.7: **Analytical ultracentrifugation analysis of LdPEX14.** Recombinant LdPEX14 (circles) or the LdPEX5:LdPEX14 complex (diamonds) were analyzed on a Beckman Optima XL-I analytical ultracentrifuge using an An-55 AI aluminum rotor. LdPEX14 was analyzed using a rotor speed of 26,000 rpm and the complex LdPEX5/LdPEX14 at a speed of 30,000 rpm. UV scans were collected at 280 nm and analyzed by the van Holde-Weischet method (Van Holde and Weischet, 1978).

3.4.6 Limited Proteolysis

The LdPEX14 conformational changes were next examined using trypsin limited proteolysis. Treatment of recombinant LdPEX14 alone with trypsin resulted in cleavage of this protein to an N-terminal ∼40-kDa fragment that was more resistant to further proteolysis even after a prolonged incubation (3 h at 20°C) (Figure 3.8 panel A and 3.8 panel B). Protease-resistant fragments have been reported for mammalian and *S. cerevisiae* PEX14; however, these fragments were associated with insertion of PEX14 into the peroxisomal membrane (Albertini *et al.*, 1997; Oliveira *et al.*, 2002; Will *et al.*, 1999). Western blot analysis of the digests with Ni^{2+}-NTA-conjugated horseradish peroxidase (Ni^{2+}-NTA-HRP or anti-LdPEX14 antisera (Madrid and Jardim, 2005)) established that this ∼40-kDa proteolytic product corresponds to an N-terminal fragment (Figure 3.8 panel B and 3.8 panel C) exhibiting an electrophoretic mobility similar to ldpex14 1-254 (Figure 3.3 panel C). Smaller N-terminal fragments ranging from ∼19 to 40 kDa were also observed within 15 min of digestion (Figure 3.8 panel C).

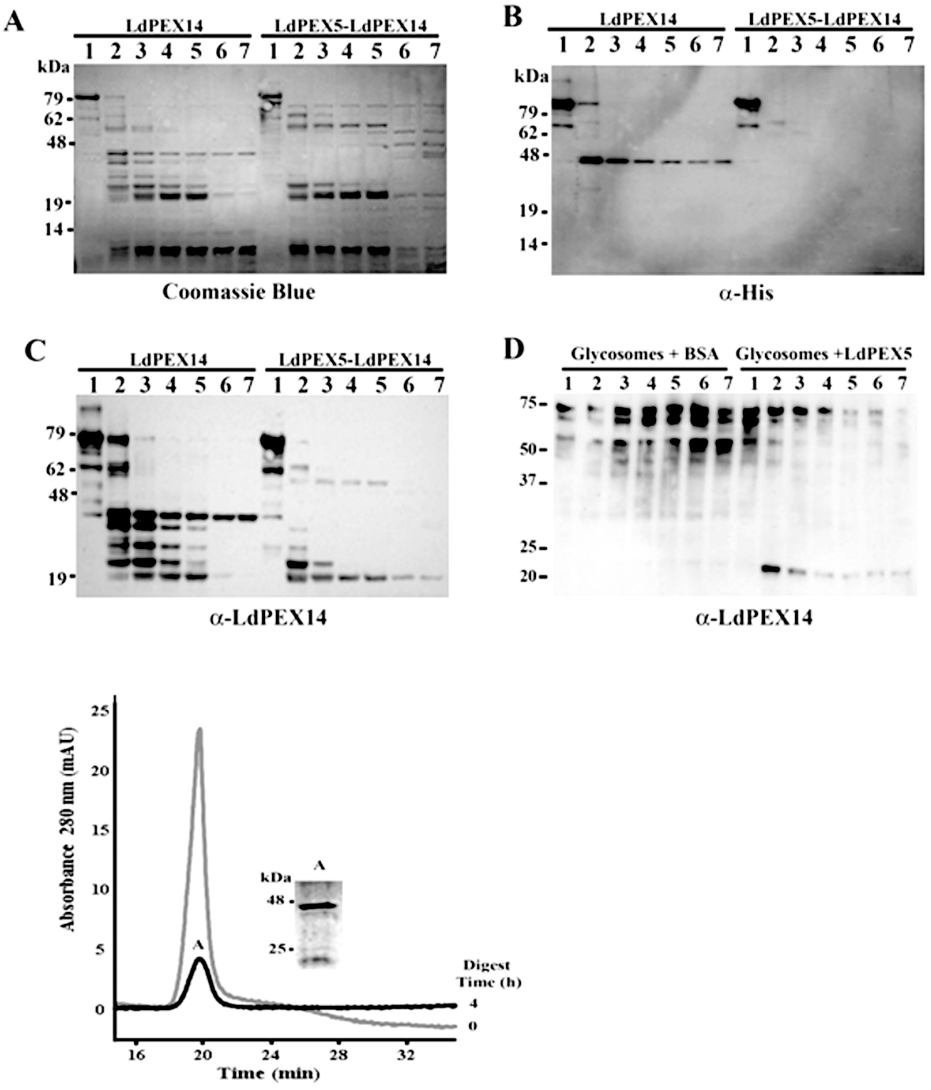
A
LdPEX14
LdPEX5-LdPEX14
1 2 3 4 5 6 7 1 2 3 4 5 6 7
kDa
79
62
48
19
14
Coomassie Blue
B
LdPEX14
LdPEX5-LdPEX14
1 2 3 4 5 6 7 1 2 3 4 5 6 7
kDa
79
62
48
19
14
α-His
C
LdPEX14
LdPEX5-LdPEX14
1 2 3 4 5 6 7 1 2 3 4 5 6 7
79
62
48
19
α-LdPEX14
D
Glycosomes + BSA
Glycosomes +LdPEX5
1 2 3 4 5 6 7 1 2 3 4 5 6 7
75
50
37
25
20
α-LdPEX14
Absorbance 280 nm (mAU)
25
20
15
10
5
0
A
kDa
A
48
25
Digest
Time (h)
4
0
16
20
24
28
32
Time (min)

Figure 3.8

FIGURE 3.8: **LdPEX5 induced conformational changes in the LdPEX14 complexes monitored by limited proteolysis.** Purified LdPEX14 or LdPEX14-LdPEX5 complex were incubated with trypsin (1:300 mole ratio of trypsin:substrate) at 20°C and aliquots were removed at time 0 (lane 1), 2 min (lane 2), 5 min (lane 3), 10 min (lane 4), 15 min (lane 5), 60 min (lane 6), and 180 min (lane 7) after the addition of trypsin. Proteolysis was terminated by adding a large excess of a protease inhibitor cocktail and samples were resolved on a 10% SDS-PAGE. (A) Protein were transferred PVDF membrane stained with Coomassie blue dye and then, (B) probed with Ni^{2+}-NTA conjugated horseradish peroxidase (Ni^{2+}-NTA-HRP) (1:2,000) in PBS. Membranes were stripped with 1% SDS, 5 mM EDTA to remove the Ni^{2+}-NTA-HRP and (C) probed with anti-LdPEX14 antibodies. (D) To evaluate the conformational changes induced LdPEX5, purified glycosomes were incubates with the control protein BSA or recombinant LdPEX5 in the presence of an excess of LdXPRT and treated with trypsin as indicated above. Native LdPEX14 digest produces were analyzed by Western blot. (E) Chromatographic analysis of the LdPEX14 tryptic digest. LdPEX14 was digested for 4 h (LdPEX14:trypsin 200:1 molar ratio) and aliquot was injected onto a Superdex 200 size exclusion column equilibrated with 50 mM phosphate pH 7.5, 150 mM NaCl, 5 mM β-mercaptoethanol to assess the quaternary structure of trypsin resistant LdPEX14 core. The grey trace is a 10 μl injection of intact LdPEX14 and the black trace is a 10 μl injection of the reaction mixture after a 4 h digest. Peak A for the black trace was collected and the protein composition of this peak examined by Coomassie blue dye stained SDS-PAGE.

Formation of the LdPEX5-LdPEX14 (1:4 molar ratio) dramatically altered the susceptibility of LdPEX14 to proteolytic degradation, as shown by the Coomassie Blue-stained gels (Figure 3.8 panel A). Notably, no reactivity was detected with Ni^{2+}-NTA-HRP, suggesting that the proximal N-terminal region of LdPEX14 became exposed resulting in rapid degradation of the hexahistidine tag. Moreover, Western blots probed with anti-LdPEX14 antibodies confirmed that binding of LdPEX5 caused a conformational change resulting in ∼80% of LdPEX14 degradation to an ∼19-kDa fragment within 2 min. This fragment was further degraded, albeit at a significantly much slower rate (Figure 3.8 panel C). Interestingly, the C-terminus of LdPEX14 was very prone to protease digestion, in the presence and absence

of LdPEX5 suggesting that this region may not be tightly folded, possibly because of the high content of proline residues, which appears to be particular to LdPEX14 (Jardim *et al.*, 2002). The ∼60-kDa protein that accumulates in Figure 3.8 panel A corresponds to a proteolytic fragment of LdPEX5 that because of its abundance weakly reacts with the HRP-conjugated secondary antibody accounting for the 60-kDa immunoreactive species in Figure 3.8 panel C. That this protein fragment was derived from LdPEX5 was confirmed by immunostaining with anti-LdPEX5 antibodies.

To evaluate if LdPEX5 triggered similar conformational changes in LdPEX14 anchored to the glycosomal membrane, purified glycosomes in the presence of an excess of recombinant LdPEX5 or the control protein bovine serum albumin (BSA) together with the PTS1 protein LdXPRT (Jardim *et al.*, 1999) were treated with trypsin. Digest mixtures containing BSA revealed that native LdPEX14 was resistant to proteolytic degradation, and ∼50% of the parent protein remained intact even after a 3-h incubation. Only an ∼55-kDa anti-LdPEX14 immunoreactive product was observed to accumulate (Figure 3.8 panel D). This 55-kDa anti-LdPEX14 immunoreactive fragment is likely because of the partial proteolytic degradation of LdPEX14 that occurred during glycosome purification because only low levels of protease inhibitors were used in these preparations (Figure 3.8 panel D). A comparable sized fragment was also observed with the recombinant LdPEX14 (Figure 3.8 panel C). In the presence of an LdPEX5-LdXPRT complex, however, >70 % of the native LdPEX14 was degraded to an ∼19-kDa fragment within 15 min (Figure 3.8 panel D). Because the anti-LdPEX14 antisera recognize an epitope between residues 23 and 63 (Madrid and Jardim, 2005), it is clear that

association with LdPEX5 renders the C-terminus of LdPEX14 extremely susceptible to proteolysis. A similar observation has been reported for mammalian PEX14 (Oliveira *et al.*, 2002). Interestingly, the digest of native LdPEX14, anchored to the glycosomal membrane, closely mimicked the limited proteolysis patterns observed with the recombinant LdPEX14.

It is postulated that the ∼40-kDa trypsin-resistant fragment observed with LdPEX14 complex is protected from further degradation by forming a stable oligomeric complex, similar to the structures formed by ldpex14 1 200 or ldpex14 1-254. This contention is supported by the finding that LdPEX14 treated with trypsin retained a core structure that eluted on a Superdex 200 column with a mass >670 kDa (Figure 3.8 panel E). Western blot analysis of this core structure revealed that the ∼45- and ∼19-kDa fragments cross-reacted with anti-LdPEX14 antibodies. It should be stressed that the resistance of the ∼40-kDa fragment to tryptic degradation is attributed to protein folding because LdPEX14 contains 24 lysine and arginine residues, trypsin cleavage sites, within the first 250 residues.

3.5 Discussion

The membrane-associated protein PEX14 is a crucial component required for the import of PTS1 and PTS2 proteins into glycosomes and peroxisomes (Galland *et al.*, 2007; Azevedo and Schliebs, 2006; Rayapuram and Subramani, 2006; Jardim *et al.*, 2002; Choe *et al.*, 2003; Madrid and Jardim, 2005; Dawson *et al.*, 2002; Kleiger *et al.*, 2002). In glycosome biogenesis, the importance of PEX14 has been underscored by genetic experiments demonstrating that knockdown of PEX14 in the kinetoplastid parasite *T. brucei* results in a lethal phenotype (Furuya *et al.*, 2002; Galland *et al.*, 2007; Moyersoen *et al.*, 2003). Although a considerable amount of knowledge regarding the protein complement involved in the assembly of these microbody organelles has been amassed, far less is known about the molecular dynamics associated with the docking of the PEX5 and PEX7 receptors to PEX14 and the subsequent translocation of folded nascent polypeptides across the lipid bilayer membrane.

In situ cross-linking studies demonstrate that on the glycosomal surface, LdPEX14 forms a homo-oligomeric complex that on sucrose density gradients migrates with a density of ∼800 kDa. Similar PEX14 oligomeric structures that constitute the importomer complex have also been reported in mammals and yeast (Shimizu *et al.*, 1999; Azevedo and Schliebs, 2006; Rayapuram and Subramani, 2006; Shimozawa *et al.*, 2004). Recombinant LdPEX14 expressed in *E. coli* also formed comparable homomeric complex and suggested that oligomerization did not appear to be contingent on accessory proteins or chaperones unique to *Leishmania*. However, it is unclear how these complexes participate in the import of folded proteins across the glycosomal membrane.

Functional domain mapping indicated that the hydrophobic domain, which is predicted to adopt an α-helix configuration (Figure 3.2 panel B), is important for stabilizing the LdPEX14 homomeric complex. In LdPEX14, the hydrophobic domain contains a centrally located GXXXA and SXXS motifs that by molecular modeling are predicted to be on the same face of the α-helix, an architecture that would promote helix-helix packing and oligomerization (Dawson *et al.*, 2002; Kleiger *et al.*, 2002). Indeed, mutagenesis of the analogous GXXXG and AXXXA motifs in the mammalian PEX14 caused disruption of oligomerization (Shimozawa *et al.*, 2004). In contrast to the mammalian PEX14, where deletion of this hydrophobic segment abrogated oligomerization and resulted in cytosolic targeting of the mutant protein, elimination of the LdPEX14 hydrophobic domain alone did not disrupt glycosomal targeting or homo-oligomerization, although ldpex14 Δ149-179 was found to form smaller complexes. The association of ldpex14 Δ149-179 with the glycosome, in contrast to the mammalian PEX14 mutants lacking the analogous hydrophobic domain (Itoh and Fujiki, 2006), was not surprising because recent studies demonstrated that residues 1-23 of LdPEX14 are the crucial elements required for glycosomal membrane attachment. Whether ldpex14 Δ149-179 is biologically functional and capable of mediating protein import into the glycosome is not clear however, because in kinetoplastids PEX14 appears to be essential for parasite viability (Furuya *et al.*, 2002; Moyersoen *et al.*, 2003) and generation of *Leishmania donovani* Δ*ldpex14* mutant cell line required for these studies has been hampered by the absence of RNA interference machinery or tight regulatable expression systems (Robinson and Beverley, 2003). That the hydrophobic domain of LdPEX14 participates in a protein-protein or protein-membrane interaction may be

inferred from the capacity of the ldpex14 1-200 to form large heterodisperse oligomeric structures that are dependent on residues 149-200. It should be noted that by analytical ultracentrifugation, ldpex14 Δ149-179 assembled into structures that were smaller than LdPEX14.

In addition to the hydrophobic region, it is postulated that contacts involving the N-terminal dimerization domain and the coiled-coil motif located in the C-terminal portion of LdPEX14 contribute to stabilization of the homomeric complex. This repeating geometry of intermolecular contacts illustrated in Figure 3.9 would account for the heterodisperse nature of the LdPEX14 complexes observed in sedimentation velocity experiments and also for the oligomerization of the ldpex14 C-terminal truncation mutants (Figure 3.9 panel B and 3.9 panel C).

A pivotal step in the import of PTS1 proteins into the glycosome involves the docking of LdPEX5 to LdPEX14. Thermodynamic studies indicate that this is not a simple bimolecular interaction as four molecules of LdPEX14 bind to one LdPEX5. Complexes isolated from peroxisomes appeared to have comparable PEX5:PEX14 (1:5) binding stoichiometries (Gouveia *et al.*, 2000; Schliebs *et al.*, 1999). Because the N-terminus of LdPEX14 forms dimers, it is unlikely that LdPEX14 monomers bind to LdPEX5 in a sequential fashion, but rather we argue that the initial association occurs at a LdPEX14 dimer that subsequently recruits a second LdPEX14 dimer to complete the interaction. This contention is supported by the glutaraldehyde cross-linking experiments showing 1:2 (∼80 kDa) and 1:4 (∼100 kDa) ldpex5 203-391:ldpex14 1-120 species (Figure 3.5 panel C). However, the possibility that two LdPEX14 dimers associate to form a complete LdPEX5-binding site cannot be discounted. In contrast

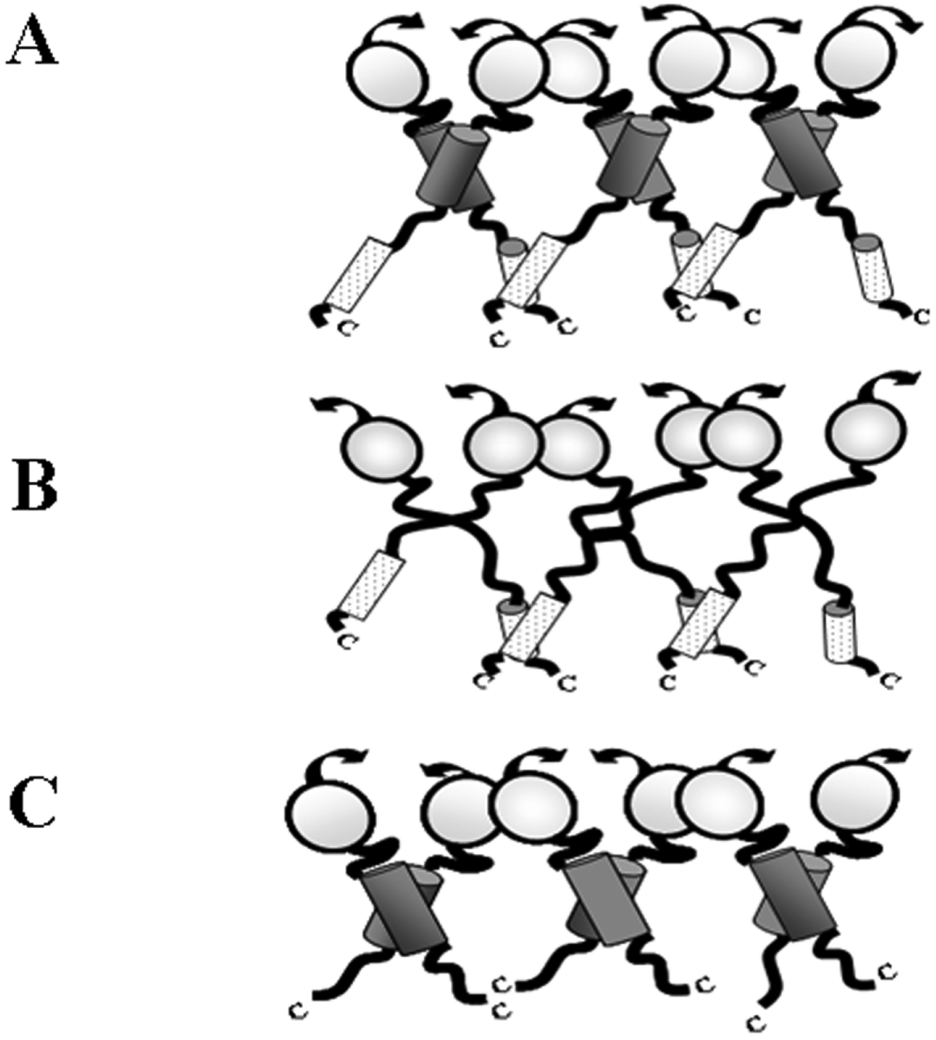

FIGURE 3.9: **LdPEX14 interacting domains.** The diagram illustrates the general architecture of (A) His_6/S-LdPEX14, (B) His_6/S-ldpex14 Δ149-179, and (C) His_6/S-ldpex14 1-200 and the potential interacting domains that mediate homo-oligomerization of this protein. The black arrow represents the N-terminus and spans residues 1-23, a region that has been demonstrated to be important for the anchoring of LdPEX14 to the glycosome surface. The spheres represent residues 23-75, a region that contains the LdPEX5 binding domain and a dimerization domain. The grey cylinders denote the hydrophobic domain that forms an inter helix interaction that is stabilizes LdPEX14 oligomerization. The stippled cylinders correspond to the coiled-coil motif that is known to form protein-protein interactions.

to PEX5-PEX14 complexes previously characterized from rat livers that had an apparent molecular mass of 250 kDa (Gouveia *et al.*, 2000), the LdPEX5:LdPEX14 structures appear to be substantially larger with an apparent mass of >670 kDa.

Several lines of evidence suggest that LdPEX5 triggers marked conformational changes in LdPEX14. First, analytical ultracentrifugation revealed that binding of LdPEX5 caused a rearrangement of the LdPEX14 leading to the formation of more compact complexes. Second, limited proteolysis experiments intimate that binding of LdPEX5 or LdPEX5-PTS1 dramatically impacted the quaternary and tertiary structures of both the soluble recombinant LdPEX14 and native LdPEX14 anchored to the glycosome, a consequence that rendered these proteins highly susceptible to proteolytic degradation. Third, intrinsic fluorescence measurement using the single tryptophan residue, located immediately adjacent to the hydrophobic domain of LdPEX14, confirmed that association with LdPEX5 caused a conformational perturbation that shifted this tryptophan from a nonpolar buried environment to a more polar and solvent-exposed environment. It should be emphasized that in the absence of an LdPEX5-PTS1 complex, LdPEX14 is attached to the glycosomal membrane via an N-terminal domain that requires the first 23 amino acids. Given these structural changes, it is tempting to suggest that docking of the PTS1 receptor leads to the exposure of the hydrophobic domain, which would interact and possibly insert into the glycosomal membrane to form a potential pore-like structure that would facilitate the import of PTS1 proteins into the glycosomal lumen. This speculation is supported by the finding that the hydrophobic domain of mammalian PEX14 is

required for membrane insertion (Itoh and Fujiki, 2006). Moreover, the proposed architecture of LdPEX14 would be consistent with this protein being directly involved in the formation of a potential structure that is analogous to a transient pore that has been advanced for peroxisomal protein translocation (Albertini *et al.*, 1997; Erdmann and Schliebs, 2005; Rayapuram and Subramani, 2006; Reguenga *et al.*, 2001).

3.6 Chapter-Related Acknowledgements

LdAPRT antisera was generously provided by Dr. Buddy Ullman of the Oregon Health Sciences University. We thank Dr. Joanne Turnbull of Concordia University for providing access to the spectropolarimeter for the circular dichroism experiments.

3.7 Connecting statement

Here we have shown that PEX14 from *Leishmania donovani* forms large oligomeric structures that remain soluble in solution despite the hypothesized *in vivo* membrane-bound localization of the protein. Such large oligomeric structures of varying size could be explained by the necessity to facilitate the transport of natively folded proteins of different molecular weight across the membrane of the glycosome.

We also observed that the binding process to LdPEX5 involved significant conformational changes which included the solvation of an hydrophobic domain, as well as the formation of a more compact quaternary structure. Such observations led to the hypothesis that the hydrophobic domain of LdPEX14 could be implicated in both glycosomal membrane attachment and pore formation.

In the following chapter, these hypotheses will be verified using various biochemical and biophysical techniques. This will shed light further on the involvement of LdPEX14 in the formation of a translocation pore

Chapter 4

The Hydrophobic Region of the *Leishmania donovani* Peroxin 14 is Required for Glycosome Membrane Anchoring and Mediating Membrane Insertion of the *Leishmania donovani* Peroxin 5-PTS1 Trafficking Complex

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4.1 Abstract

Leishmania parasites use a specialized trafficking and import system to target proteins to the microbody organelle known as a glycosome. Import of proteins containing a PTS1 signal sequence into the glycosome matrix require the receptor peroxin 5 (LdPEX5) to be properly targeted to glycosomes via LdPEX5-peroxin 14 (LdPEX14) docking complex. Immunogold labeling confirmed that native LdPEX14 is localized on the cytosolic face of the glycosomal membrane where it forms an oligomeric rosette-like structure. Flotation centrifugation studies demonstrated that recombinant LdPEX14 spontaneously binds through hydrophobic contacts to large unilamellar vesicles (LUV) containing phosphatidylethanolamine (55%), phosphatidylcholine (27%), phosphatidylglycerol (16%), and phosphatidylinositol (2%), a composition mimicking the *L. donovani* glycosomal membrane. However, no LUV binding was detected with the mutant protein ldpex14 Δ149-179, indicating that the amphipathic region spanning residues 149-179 was critical for anchoring LdPEX14 to the glycosomal membrane. Robust LUV binding was observed with protein ldpex14 (120-200). Intrinsic fluorescence and dye leakage assay revealed that this association involved insertion of a segment containing Trp152 into the hydrophobic core of the lipid bilayer which was accompanied by disruption and release of carboxyflourescein from LUVs and is consistent with this protein forming a pore-like structure in the membrane. Flotation experiments showed that in contrast to other eukaryotic PEX5 proteins, LdPEX5 exhibits no membrane binding activity; however loading LUVs with LdPEX14 facilitated the recruitment LdPEX5 or LdPEX5-PTS1 complex. More importantly the docking of LdPEX5 to LdPEX14 induces

structural changes that lead to the insertion of LdPEX5 into the LUV lipid bilayer. These data suggest that LdPEX5 and LdPEX14 function cooperatively in assembling a transient pore in the glycosomal membrane required for import of protein into the matrix.

4.2 Introduction

The human parasites *Leishmania* and *Trypanosoma*, are etiologic agents that cause a spectrum of often fatal diseases known as leishmaniasis, Chagas' disease, and African sleeping sickness. These protozoan parasites contain a unique microbody organelle called a glycosome that is distantly related to peroxisomes of yeast, fungi, plants, and mammalian cells (Opperdoes *et al.*, 1977b; Parsons, 2004; Michels *et al.*, 2005, 2006). Glycosomes compartmentalize the enzymatic machinery required for a number of vital metabolic and biosynthetic pathways that include; glycolysis, pentose phosphate pathway, ether lipid biosynthesis, and purine salvage (Parsons, 2004; Michels *et al.*, 2005). Proper targeting of proteins to the glycosome, in particular glycolytic enzymes, is crucial for parasite viability . Proteins destined for the glycosome matrix typically contain one of two major topogenic signal sequences designated peroxisomal targeting signal 1 (PTS1) which consist of a C-terminal tripeptide with the archetype sequence Ser-Lys-Leu (Gould *et al.*, 1989; Keller *et al.*, 1991; Blattner *et al.*, 1992) or peroxisomal targeting signal 2 (PTS2) which corresponds to an N-terminal proximal nonapeptide with the consensus motif [RK]-[ILV]-X_5-[HQ]-[LA] (PTS2) (Swinkels *et al.*, 1991; Blattner *et al.*, 1995) that are bound by the cytosolic receptor proteins peroxin 5 (LdPEX5) and peroxin 7 (LPEX7), respectively with nanomolar affinity. These receptors also form bimolecular LdPEX5-LPEX7 complexes with stabilities that are modulated by the binding of cargo proteins (Pilar *et al.*, 2008; Strasser *et al.*, 2012). In kinetoplastid both receptors are necessary for glycosome biogenesis as depletion of PEX5 or PEX7 by RNAi leads mistargeting of both PTS1 and PTS2 proteins (Galland *et al.*, 2007) and is consistent with the recent

observations showing that in addition to forming tight LdPEX5-LPEX7 interaction, the cargo loaded LdPEX5 and LPEX7 pre-assemble into a heteromeric complex in the *Leishmania* cytosolic compartment and that loss of either receptor impairs the trafficking or import of matrix proteins into the glycosome (Pilar *et al.*, 2008; Strasser *et al.*, 2012). This contrasts with findings obtained in mammalian and yeast Δpex5 or Δpex7 mutant cell lines where loss of a functional PEX5 or PEX7 receptor results in the mislocalization of only PTS1 or PTS2 proteins to the cytosol, respectively (Grunau *et al.*, 2009; Mukai and Fujiki, 2006; Matsumura *et al.*, 2000). Proteins lacking either of these topogenic sequences are imported into the glycosomal/peroxisomal matrix by piggy-backing mechanism as part of a folded heteromeric complex (Häusler *et al.*, 1996; Walton, 1996).

The import of newly synthesized matrix proteins is dependent on four major steps that are orchestrated by a several heteromeric complexes located in the cytosolic compartment and glycosome/peroxisome membrane surface. These events include (i) sorting/trafficking of nascent matrix proteins by the receptors PEX5 and PEX7 (ii) docking of the PEX5 and PEX7 loaded receptor complexes to docking/translocation machinery or importomer complex situated and exposed to the cytosolic face of the glycosome/peroxisome membrane (iii) translocation of the cargo proteins across the organelle membrane, and finally (iv) recycling of the PEX5 and PEX7 receptors back into the cytosol. Studies in mammals, yeast, fungi, plants and kinetoplastid parasites have suggest multimeric ∼800 kDa importomer complex which minimally contains the membrane proteins PEX14 and PEX13 provides the initial docking site and a point of convergence for the recruitment of the cargo loaded PEX5 and PEX7

receptors to the glycosome/peroxisome surface (Miyata and Fujiki, 2005). Movement of PTS1 and PTS2 proteins across the glycosome/peroxisome membrane requires recruitment of the RING subcomplex composed of the subunits PEX2, PEX10, and PEX12 and has been implicated in multiple functions which include; the translocation of matrix cargo proteins, ubiquitin ligases that mono or polyubiquitinated PEX5 modifications that direct the recycling of this receptor back into the cytosol or to proteasome for degradation, respectively, and peroxisome formation (Okumoto *et al.*, 2000; Guerra-Giraldez *et al.*, 2002; Platta *et al.*, 2004; Kragt *et al.*, 2005; Krazy and Michels, 2006; Platta *et al.*, 2009; Prestele *et al.*, 2010). Translocation of cargo proteins into the organellar lumen appears to proceed via an energy independent mechanism; while the recycling of the unloaded PEX5 and PEX7 receptors back into the cytosol is a ubiquitin and ATP dependent step that requires the soluble AAA+ protein family members PEX1 and PEX6 (Erdmann and Schliebs, 2005; Tamura *et al.*, 2006; Platta *et al.*, 2007; Fujiki *et al.*, 2008; Miyata *et al.*, 2012; Grimm *et al.*, 2012).

Although considerable advances have been made in identifying the machinery required for the trafficking and import of proteins into the glycosome/peroxisome, little is known about the mechanisms that mediate translocation of large protein complexes across the organelle membrane without compromising the permeability barrier of these microbodies. The amassing evidence supports the notion that the docking of the cargo loaded PEX5 receptor to PEX14 on the importomer complex is sufficient to induce structural changes that drive insertion of the PTS1 receptor into the lipid bilayer, where it associates with PEX14, and aids in formation of a tightly

gated channel through which cargo proteins are postulated to pass (Gouveia *et al.*, 2003; Erdmann and Schliebs, 2005; Kerssen *et al.*, 2006; Meinecke *et al.*, 2010). This model however, does not take into account PEX7 and its interactions with PEX5 and PEX14 for the delivery of PTS2 proteins into the glycosome/peroxisome lumen. Questions arising from the transient pore model regarding organelle biogenesis include; (i) how PEX5, which has no apparent transmembrane domain, inserts into and is stabilized within the membrane, (ii) what are the mechanics that facilitate the reversible penetration of the PEX5-PEX14 complex into the lipid bilayer to form a pore, (iii) how is the diameter of the translocation pore altered to accommodate the import of diverse array heteromeric cargo protein into the glycosome/peroxisome lumen without the leakage of small molecular metabolites into the cytosol, (iv) does PEX7, which shuttles through the peroxisome/glycosome lumen (Nair *et al.*, 2004; Pilar *et al.*, 2008), also translocates PTS2 cargo proteins via a transient pore?

To address questions regarding the architecture and function of the PEX5-PEX14 transient pore we have exploited the recombinant *Leishmania* glycosomal biogenesis proteins LdPEX14 and LdPEX5 which can be expressed in *E. coli*.

In kinetoplastid parasites, PEX14 (Furuya *et al.*, 2002; Jardim *et al.*, 2002; Moyersoen *et al.*, 2003; Cyr *et al.*, 2008) is key component required for import of PTS1 and PTS2 protein into the glycosome (Moyersoen *et al.*, 2003; Kessler and Parsons, 2005; Haanstra *et al.*, 2008). This protein has been demonstrated to form tight interactions with LdPEX5 and LPEX7. In mammals, *Hansenula polymorpha*, *Pichia pastoris* and *Trypanosoma brucei*, PEX14 behaves as an integral membrane protein , whereas the

Leishmania (Jardim *et al.*, 2002) and *Saccharomyces cerevisiae* (Albertini *et al.*, 1997; Brocard *et al.*, 1997; Niederhoff *et al.*, 2005) PEX14 proteins exhibit features more characteristic of a peripheral membrane protein that is anchored to the cytosolic surface of the glycosome/peroxisome. In addition to PEX14, the docking/translocation platform in mammals, yeast and *T. brucei* also contains PEX13, an intrinsic membrane protein that associates with PEX14, PEX5, and PEX7 and is thought to function in the recycling of the unloaded PTS1 and PTS2 receptors back into the cytosolic compartment (Verplaetse *et al.*, 2009; Brennand *et al.*, 2012; Girzalsky *et al.*, 1999; Elgersma *et al.*, 1996; Gould *et al.*, 1996). This role remains to be characterized in *Leishmania*.

Recent investigations revealed that *Leishmania donovani* PEX14 (LdPEX14) forms large homo-oligomeric structures in solution via the interaction of three conserved domains including the *L. donovani* PEX5 (LdPEX5) binding domain, a predicted coiled-coil domain and a hydrophobic amino acids-rich region (Cyr *et al.*, 2008). This feature was also observed in other species (Brocard *et al.*, 1997; Itoh and Fujiki, 2006; Oliveira *et al.*, 2002). Data using recombinant *L. donovani* proteins illustrated that significant conformational changes occurred upon binding to LdPEX5 in the vicinity of the hydrophobic region of the protein where this domain moved to an environment more exposed to the polar environment, thus hypothesizing that such conformational change would reflect a penetration of the hydrophobic region deeper in the lipid bilayer, in the presence of such structure. Moreover, isothermal titration calorimetry studies of the LdPEX14-LdPEX5 interaction demonstrated that upon binding to LdPEX5, the solvation of a hydrophobic region from LdPEX14

was occurring in solution. This led to the hypothesis that the LdPEX14 receptor would be potentially involved in the formation of a pore for the translocation of PTS1 and PTS2 proteins (Cyr *et al.*, 2008).

Here we demonstrate that the ubiquitous hydrophobic domain present in PEX14 proteins is critical for not only facilitating the spontaneous binding of LdPEX14 to large unilamellar vesicle (LUV) that mimic the *L. donovani* glycosomal membrane phospholipid composition but also for mediating pore formation in these model membranes. Moreover, we show that LdPEX5 also associate LUVs, however this event is dependent on membrane bound LdPEX14 to promote the docking of the PTS1 receptor but to trigger structural changes induce insertion of LdPEX5 into the lipid bilayer. These studies provide a molecular basis for understanding how the binding of LdPEX5 to LdPEX14 causes the LdPEX14 hydrophobic domain to insert into the glycosomal membrane and form transient transmembrane pore through which matrix proteins traverse.

4.3 Material and Methods

4.3.1 Materials

All restriction endonucleases and DNA-modifying enzymes were purchased from Invitrogen or New England Biolabs. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was purchased from GE Healthcare. HRP-conjugated goat anti-mouse and donkey anti-guinea pig was purchased from Sigma-Aldrich. Synthetic phospholipids and cholesterol (Chl) were supplied by Avanti Polar Lipids. All other reagents were of the highest quality commercially available.

4.3.2 Electron microscopy

L. donovani promastigotes (4 x 10^8) in the logarithmic growth phase were harvested, washed three times with 10 ml of PBS (50 mM phosphate pH 7.6, 150 mM NaCl) and the cell pellet was gently resuspended in 4% paraformaldehyde in 250 mM Hepes pH 7.4 and incubated for 1 h at 20°C. Fixed cells were centrifuged at 2,500 rpm for 15 min at 4°C and then gently resuspended in 8% paraformaldehyde in 250 M Hepes pH 7.4 and stored in this solution at 4°C until sectioning. Fixed parasite pellets were infiltrated, frozen and sectioned as previously described. The sections were immunolabelled with anti-LdPEX14 or anti-LPEX7 antibodies (1:250 in PBS/1% fish gelatin), then with goat anti-rabbit IgG or donkey anti-guinea pig IgG antibodies, and bound antibodies detected using 10 nm protein A-gold particles before examination with a Philips CM120 Electron Microscope under 80 kV.

4.3.3 Negative Stain Electron Microscopy

Five μl of sample containing purified LdPEX14 at 0.5 mg/mL was applied to negatively glow-discharged carbon-coated EM copper grids and incubated for 1 min. The protein sample was blotted; 5 μl of 2% (w/v) uranyl acetate was added for 1 min and removed. Micrographs for image analysis were collected at 1.5 μm defocus on a FEI Tecnai 12.

4.3.4 Protein expression

The *L. donovani* PEX14 coding sequence and an internal deletion mutant lacking the amino acids 149-179, ldpex14 Δ149-179, were amplified by PCR from previously generated constructions and cloned into the NdeI/XhoI sites of the pET30b(+) expression vector (Novagen) to generate C-terminal hexahistidine-tagged pET30-LdPEX14-His_6 and pET30-ldpex14 Δ149-179-His_6, respectively. An LdPEX14 fragment encompassing amino acids 120 - 200 was amplified by PCR and cloned into the NdeI/XhoI sites of pET30b(+) to generate pET30b-ldpex14(120-200)-His_6 expression construct.

LdPEX14/ldpex14 proteins were expressed in *E. coli* strain ER2566 transformed with the expression constructs pET30-LdPEX14-His_6, pET30-ldpex14(Δ149-179)-His_6 or pET30b-ldpex14(120-200)-His_6 with 0.5 mM isopropylthiogalactoside for 4 h at 20°C. Bacterial cell pellets (1.0 l culture) were resuspended in 20 ml of 40 mM Tris-HCl pH 8.0 and lysed with three passes through a French Press. NaCl was added to the lysate to a final concentration of 500 mM, and then clarified by centrifugation. The supernatant was applied to 1.0 ml Ni^{2+}-NTA resin (Qiagen) column

equilibrated with 40 mM Tris-HCl pH 8.0 containing 500 mM NaCl (TBS500) and the column was sequentially washed with 100 ml of TBS500, 100 ml of 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 TBS500 containing 500 mM imidazole).

For purification of ldpex14 (120-200), the bacterial cell pellet from 1.0 l culture, was resuspended in 20 ml TBS500 passed three times through a French Press and the lysate was centrifuged at 12,000 rpm for 20 min at 4°C. The pellet containing ldpex14 (120-200) inclusion bodies was resuspended in 20 ml TBS500 containing 8.0 M urea and then clarified by centrifugation. The clarified lysate was loaded onto a 1.0 ml Ni^{2+}-NTA resin (Qiagen) column then washed with 100 ml of TBS500 containing 8.0 M urea to remove unbound proteins. Ldpex14 (120-200) was eluted with TBS containing 500 mM imidazole, 4.0 M urea, concentrated to 6.0 mg/ml using Amicon Ultra filter 3K NMWL unit (Millipore), and dialyzed against TBS containing 4.0 M urea and stored as aliquots at -80°C. Removal of urea caused high concentration of ldpex14 (120-200) to precipitate.

LdPEX5 and the *L. donovani* PTS1 protein hypoxanthine-guanine phosphoribosyltransferase (LdHGPRT) were purified as previously described (Jardim *et al.*, 2000; Allen *et al.*, 1995b). Purified proteins were concentrated and buffer exchanged for 40 mM Tris-HCl pH 8.0 150 mM NaCl (TBS150) using an Amicon Ultra filter 3K or 10K NMWL unit (Millipore) and the protein concentration determined 280 nm using the calculated extinction coefficient for each protein as previously described . Proteins were then stored at -80°C.

4.3.5 Glycosome isolation

Leishmania donovani promastigotes (∼5 x 10^{10} cells) grown in DME-*L* media containing 10% FBS were harvested and wash twice in 10 ml of cold PBS and twice in 20 ml of hypotonic buffer (HB) (2 mM EGTA, 2 mM DTT) prior to lysis by three passes through a French pressure cell (Thermo Scientific) at 200 psi on medium range. Lysates were made isotonic by adding 0.25 volumes of 100 mM Hepes-NaOH pH 7.4, 400 mM sucrose, 1.6 mM ATP, 1.6 mM EGTA and 3.2 mM DTT). Cellular debris was removed by centrifugation at 5,000 x *g* 10 min, 4°C and the supernatant centrifuged at 45,000 x *g* for 45 min and a crude organellar was recovered in the pellet. The organelle pellet was resuspended in 1 ml of 25 mM Hepes-NaOH, pH 7.4, applied to the top of a 20-70% sucrose gradient in 25 mM Hepes-NaOH pH 7.4 (w/v), and the organelles were resolved by centrifugation at 55,000 x *g* for 16 h at 4°C in a Beckman-Coulter SW28 rotor. The gradient was fractionated (1.5 ml fractions) and analyzed by Western blot. Fractions enriched for LdPEX14 and hexokinase were pooled, diluted with two volumes of PBS and the organelles were recovered in the pellet by centrifugation at 45,000 x *g* for 1 h in a Beckman-Coulter SW41 rotor for 1 h at 4°C. The organelle pellet was resuspended in 1.5 ml TBS150 and applied to a linear 20-30% Optiprep gradient in TBS150 and the organelle were resolved by at 125,000 x *g* for 1 h at 4°C on a Beckman-Coulter SW41 rotor. The gradients were fractionated (0.5 ml) and analyzed for the presence of LdPEX14 (glycosome), hexokinase (glycosome), cytochrome oxidase IV (mitochondria), and Bip (endoplasmic reticulum) by Western blot using all primary antibodies at a dilution of 1:10,000. Fractions enriched for glycosomes were pooled and used for phospholipid analysis.

4.3.6 Lipid composition of the glycosomal membrane

Phospholipids were extracted from purified glycosomes using the Folch protocol (Folch *et al.*, 1957). Phospholipids were separated by two-dimensional thin layer chromatography (2D-TLC) using the previously described method. Briefly, glycosomal phospholipids were resolved on Silica-G TLC plates (EMD Chemicals) in the first dimension using chloroform:methanol:ammonia:water (90:74:12:8) and in the second dimension using chloroform:methanol:acetone:acetic acid:water (40:15:15:12:8). Phospholipids were visualized by charring with 2 M sulfuric acid and spots quantified by densitometry. A mixture containing the standards, dioleylphosphatidic acid (DOPA), dioleylphosphatidylcholine (DOPC), dioleylphosphatidylethanolamine (DOPE), dioleylphosphatidylglycerol (DOPG), dioleylphosphatidylinositol (DOPI), dioleylphosphatidylserine (DOPS), and bovine brain sphingomyelin, and bovine phosphatidylinositol (PI) were resolved by 2D-TLC. Phospholipid spots were scraped from digested with perchloric acid for 1 h at 150°C and the inorganic phosphate quantified by the Bartlett method (Bartlett, 1959).

4.3.7 Mass spectrometry analysis

Samples were suspended in chloroform/methanol (1:2 v/v), and an aliquot of total lipid extract was analyzed with a Micromass LCT mass spectrometer equipped with nanoelectrospray source. Samples were loaded into thin-wall nanoflow capillary tips (Waters) and analyzed by ES-MS in both positive and negative ion modes using a capillary voltage of 0.9 kV and cone voltages of 50 V. Analysis by ESI-MS-MS was conducted on a ABSciex

4000 QTrap (linear ion trap). Samples were loaded into thin-wall nanoflow capillary tips (Presearch) and analysed with capillary voltages between1.0-1.5kV for both negative and positive ion modes, Tandem mass spectra (MS-MS) was used with nitrogen as collision gas and various collision offset energies to obtain various precursor and neutral loss scans both in positive and negative ion mode and MS-MS daughter ions scans were conducted to confirm identification.

Protein arrays were analyzed in a ProteinChip biology system reader (PCS-4000) from Biorad Laboratories. Spots on a NP20 chip array were pre-activated by applying 5 μl of dH_2O and left to air-dry. Five microliters of sample (0.5 μg/μL protein) were applied per spot, air-dried, and washed with the addition of 5μl of dH_2O. The array was air-dried and two applications of 1μl each of sinapinic acid (SPA) solution (12.5 mg/ml in 0.5% trifluoroacetic acid, 50% acetonitrile) were added to the spots. The spots were read at high-energy (HE) laser intensity (4500nj). Data was collected up to m/z of 200,000. All spectra were subjected to mass calibration based on the settings used to collect the data, using external calibration standards (hirudin BHVK, 6964 Da; bovine cytochromec, 12,230.9 Da; equine myoglobin, 16,951 Da; bovine RBC carbonic anhydrase, 29,023 Da; *S. cerevisiae* enolase, 46,671 Da; bovine albumin, 66,433 Da; and IgG, 147,300 Da).

4.3.8 Fatty acid composition of the glycosome phospholipids

For fatty acid analysis, glycosomal phospholipids were dissolved in hexane containing the internal standard 1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine (C17-PE) (Avanti Polar Lipids) and sodium methoxide was added and the methylation reaction incubated for 5 min at 20°C. Samples (1 μl) were injected onto a Varian CP-3800 gas chromatograph system equipped with a flame ionization detector, and a CP-Sil 88 capillary column (100 m x 0.25 mm, 0.20 m film thickness, Varian). The injector and detector temperature were maintained at 260°C with helium as the carrier gas while the oven temperature increased from 60°C to 230°C with a run time of 75 min. Methyl esters were identified by comparing retention times the elution of a fatty acid methyl ester (FAME) standard mixture (Nu-Chek Prep, MN, USA). Quantification of the FAMEs from C10 to C22:6 were calculated relative to the amount of the internal standard C17PE recovered using Galaxie software (Varian).

4.3.9 Liposome preparation

Individual phospholipids or mixtures of DOPE:DOPC (2:1), DOPC:DOPG; (1:1), DOPE:DOPC (1:1), and DOPE:DOPC:DOPG:PI:cholesterol (53:24:15:4:4), the latter of which resembles the composition of *L. donovani* glycosomal membrane, were dissolved in chloroform and a thin film prepared by evaporation under a nitrogen stream. Residual chloroform was removed under vacuum for 16 h. Multilamellar vesicles were prepared by re-suspending the lipid film in PBS at a concentration of 5 mg/ml.

The suspension was then extruded through a 0.2 μm polycarbonate membrane (Millipore) 20 times to generate large unilamellar vesicles (LUV) with a diameter of 200 nm, which is comparable to the diameter of glycosomes. Alternatively, rehydrated lipid films were sonicated with a probe sonicator to generate small unilamellar vesicles (SUV) for circular dichroism experiments.

4.3.10 LUV leakage assay

Thin films of the phospholipid mixture were re-suspended in PBS containing 200 mM 5(6)-carboxyfluorescein (CF) (SigmaAldrich) and then extruded through a 0.2 μm polycarbonate membrane. CF loaded LUVs were purified by gel filtration on Sephadex G-50 column (1 x 20 cm) equilibrated in PBS. ldpex14 (120-200) was titrated into CF loaded LUVs and dye release was monitored at an excitation wavelength of 492 nm and an emission wavelength of 515 nm. The total CF content of the LUVs was determined by addition of Triton X-100 to a final concentration of 0.1% v/v.

4.3.11 Sucrose density flotation centrifugation

LUVs were incubated with proteins (molar ratio of 500:1 phospholipid:protein) in 300 μl of PBS for 40 min at 23°C, mixed with 1.2 ml of 66% sucrose in PBS (w/v), transferred to a 5.2 ml ultracentrifuge tube and overlaid with 3.0 ml of 40% sucrose in PBS, and 1.0 ml of PBS. Samples were centrifuged at 75,000 x g for 16 h at 4°C in a Beckman-Coulter SW55 rotor. The gradient was fractionated (0.65 ml/fraction) and the proteins precipitated with trichloroacetic acid (15%) prior to Western blot

analysis. It should be noted that Western blots of the flotation experiments show a notable amount of LdPEX14 degradation. This is likely due to the predicted native disordered structure of LdPEX14 which renders this protein highly susceptible to proteolytic degradation .

4.3.12 Alkaline carbonate extraction

LUVs loaded with the peroxin proteins LdPEX5, LdPEX14, or ldpex14 (120-200) were isolated by flotation and then treated sequentially with 500 mM NaCl, 100 mM Na_2CO_3 pH 11.5, or 100 mM Na_2CO_3 pH 11.5 containing 4.0 M urea at 0°C. Following each treatment samples were separated into a supernatant and pellet fraction by centrifugation at 100,000 x *g* for 30 min at 4°C in a TLA100.3 rotor on a Beckman-Coulter table top ultracentrifuge. Proteins in the pellet and supernatant fractions were precipitated with 15% TCA and then analyzed by Western blot.

4.3.13 Limited proteolysis

Protein complexes in solution or bound to LUVs were resuspended in 400 μl of PBS containing 1.0 mM $CaCl_2$ and 2.5 mM DTT then treated with the arginine specific protease clostripain (Worthington Biochemical Corp), at a substrate:protease ratio of 50:1. The reaction was incubated at 0°C and 75 μl aliquots were removed at 0, 2, 5 30, and 60 min time points. Digest mixtures were treated with 1.0 ml of ice-cold acetone to precipitated proteins for Western blot analysis. Western blot were quantified by densitometry and quantified using ImageJ software (Abràmoff *et al.*, 2004).

4.3.14 ldpex14 (120-200) oligomerization

Purified ldpex14 (120-200) was dialyzed against dH_2O and the precipitate protein was resuspended in 4.0 M urea in TBS at a concentration of 5 mg/ml and 5.0 μg of protein was resuspended in 20 μl of SDS-PAGE sample buffer and then resolved on 12% Bis-Tris PAGE using a MES-Tris (pH 7.25) running buffer with and without 0.5% SDS, or 0.02% Coomassie Blue G250 dye (Hachmann and Amshey 2005; Swamy, Siegers et al. 2006). Alternatively, ldpex14 (120-200) was incubated and loaded on to DOPE:DOPC:DOPG:PI:Chl LUVs and the liposomes were solubilized with 0.5% of either CHAPS, octylglucoside, taurodeoxycholate, or SDS prior to electrophoresis on Bis-Tris PAGE. Protein bands were visualized by silver stain.

4.3.15 Fluorescence spectroscopy

Intrinsic fluorescence measurements were performed on a Varian Cary Eclipse spectrofluorometer at 25°C using an excitation wavelength of 290 nm to record emission spectra from 305-400 nm at a scan rate of 120 nm/min with slit widths of 5 nm. A 5 μM solution of ldpex14 (120-200) in 40 mM Tris pH 8.0, 500 mM NaCl, was titrated with LUV to a final concentration of 1.0 mM phospholipids. Solvent accessibility of tryptophan residues was assessed by using acrylamide as the fluorescence quenching reagent. ldpex14 (120-200) (5.0 μM in PBS) was titrated with acrylamide in the presence or absence of LUVs containing a total of 1.0 mM phospholipids.

4.3.16 Circular dichroism (CD) analysis

A stock solution of ldpex14 (120-200) (0.5 mM), in 4 M urea, 10 mM sodium phosphate, 10 mM NaCl pH 7.5, was titrated with SDS and CD spectra were recorded from 205 nm to 260 nm. Spectra were recorded at 23°C on a JASCO 810 instrument using a 1 mm cuvette at a scan rate of 100 nm/min and a bandwidth of 1 nm. Five spectra were collected and averaged.

4.3.17 Bioinformatics

A hydrophathy plot for LdPEX14 was generated using Kyte and Doolittle algorithm (Kyte and Doolittle, 1982). The I-TASSER *ab initio* modeling server (Zhang *et al.*, 2008; Roy *et al.*, 2010; Wu *et al.*, 2007) was used to generate a three dimensional model of ldpex14 (120-200). The disordered probability of ldpex14 (120-200) was predicted using the DisEMBL algorithm (Linding *et al.*, 2003). The capacity of ldpex14 (120-200) to form amphipathic helices was determined using the Heliquest program (Gautier *et al.*, 2008).

4.4 Results

4.4.1 Native LdPEX14 in glycosomes

To further validate the subcellular location and orientation of LdPEX14 on the glycosome membrane we performed an immunoelectron microscopy (IEM) analysis on sections prepared from whole *L. donovani* promastigotes. IEM sections stained with anti-LdPEX14, a polyclonal sera that recognizes an epitope(s) between residues 23-63 (Madrid and Jardim, 2005), showed that ∼70% of all glycosomes were stained and that 82% of the 10 nm gold particles were associated with the glycosomal membrane, while 7% appeared to localize to the glycosome matrix, and 11% showed nonspecific staining (Figure 4.1, panels A and B). In ∼30% of the stained glycosomes, the gold particles associated to the membrane formed rosettes with a gold particle-to-particle a diameter of ∼30-40 nm (Figure 4.1, panel B), which is consistent with previous reports showing that LdPEX14 formed an oligomeric structure on the glycosome surface. This ring-like configuration detected by IEM analysis suggests that LdPEX14 in the absence of cargo loaded LdPEX5 or LdPEX7 is capable of forming a pre-pore structure without compromising the permeability barrier of the glycosomal membrane.

Recombinant and native LdPEX14 extracted from glycosomes has been shown to form complexes of ∼600-900 kDa complex (Cyr *et al.*, 2008). To evaluate the topological structure of these complexes, the recombinant LdPEX14 was examined by negative stained transmission electron microscopy. This analysis revealed that the most common

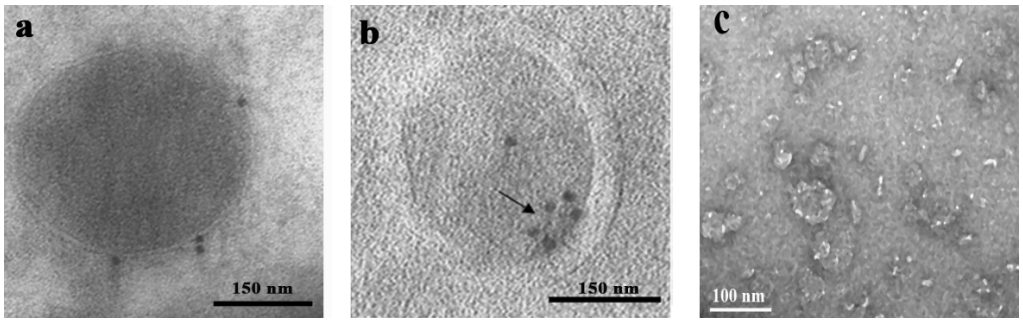

FIGURE 4.1: **Immunoelectron microscopy analysis of LdPEX14.** *L. donovani* promastigotes were fixed in paraformaldehyde, frozen and sectioned. Sections were then immunolabelled with rabbit anti-LdPEX14 sequentially followed by goat anti-rabbit IgG, or donkey anti-guinea pig IgG and 10 nm protein A-gold particles. Specimens were visualized under 80 kV using a Philips CM120 Electron Microscope. Panels A and B show representative images of the distribution of LdPEX14 on the glycosomal membrane.

structure detected in the recombinant protein preparations was a concave bowel-like geometry (Figure 4.1, panel C).

4.4.2 Phospholipid composition of the Leishmania glycosome membrane

To examine the interaction of LdPEX14 with the glycosome membrane, it was necessary to determine the phospholipid composition of this organellar membrane. Glycosomes isolated by sequential ultracentrifugation fraction on a sucrose density gradient followed by an Optiprep density gradient yielded a preparation that was ∼60-fold enriched when anti-LdPEX14 antibodies were used to monitor the purification (Figure 4.2, panel A). Western blot analysis using antibodies specific to the protein markers cytochrome oxidase IV and BiP showed that the enriched glycosome

preparation contained only minor traces of mitochondrial and endoplasmic reticulum, respectively.

Analysis of the glycosome phospholipid composition by 2D-TLC showed four major spots with a relative abundance of 50%, 30%, 15%, and 5% (Figure 4.2, panel B). However, assignment of these spots using a standard mixture of DOPC, DOPE, DOPS, DOPA, DOPG, and sphingomyelin was not possible due to a poor correlation with the Rf values of the standards. As an alternative strategy to identify the glycosome membrane, MALDI-TOF mass spectrometry run in the positive and negative ion modes was used. These analyses also showed the presence of four major classes of phospholipids; phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylinositol (PI) with a relative abundance of 55 ± 10%, 27 ± 5%, 16 ± 5%, and 2 ± 2% respectively (Table 4.1, Figure 4.2, panel C). In *Leishmania* and *T. cruzi* the glycosomal membrane had a 2:1 preponderance PE:PC, a composition distinctive from that of *T. brucei*, rat, yeast, and *Pichia* glycosomal/peroxisomal membranes where PC was the dominant lipid (Table 4.1). Notable levels of the anionic phospholipids phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidaylserine (PS), or cardiolipin (CL) were detected in *T. brucei*, *T. cruzi*, rat, yeast, and *Pichia* glycosomes/peroxisomes. In contrast, the *Leishmania* glycosomal membrane contained high levels of PG as the major anionic phospholipid (Table 4.1). No detectable levels of cardiolipin were found in the *Leishmania* glycosomes.

Gas chromatography analysis of the *Leishmania* glycosomal phospholipids revealed that C18 fatty acids where the predominant species accounting for ∼60% of the total fatty acids; with the unsaturated fatty acid C18:2

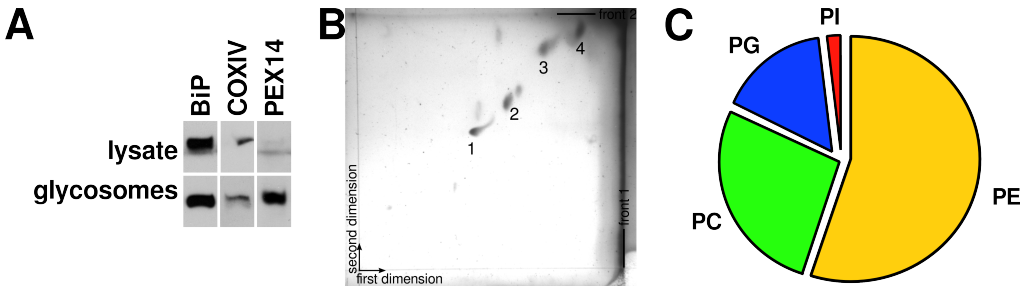

FIGURE 4.2: **Isolation and separation of phospholipid species from *L. donovani* glycosomal membrane.** (A) Western blots showing LdPEX14 enrichment following glycosome purification. (B) 2D-TLC separation of phospholipids extracted from purified glycosomes of *L. donovani*. Phospholipids were resolved with chloroform:methanol:ammonia:water (90:74:12:8) in the first dimension and with chloroform:methanol:acetone:acetic acid:water (40:15:15:12:8) in the second dimension. Four major spots were observed following development by charring with sulfuric acid. (C) Pie chart representing the relative distribution of phospholipid head groups identified by mass spectrometry.

Species	PC	PE	PG	PS	PI	PA	CL	SM	Reference
P. pastoris	54	28	-	5	6	3	4	-	(Wriessnegger *et al.*, 2007)
R. norvegicus	61	30	-	3	5	-	-	-	(Hardeman *et al.*, 1990)
S. cerevisiae	48	23	-	4	16	2	7	-	(Zinser *et al.*, 1991)
T. brucei	61	13	-	7	19	-	-	-	TK Smith, personal comm.
T. cruzi	14	61	-	23	-	-	-	2	(Quiñones *et al.*, 2004)
L. donovani	27	55	16	-	2	-	-	-	our study

TABLE 4.1: **Phospholipid composition of the glycosomal/peroxisomal membranes.** PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; CL, cardiolipin; SM, sphingomyelin.

being more that C18:1, C18:0, or C18:3 (Table 4.2). *Leishmania* glycosomal membranes are also enriched for very long chain unsaturated C22:5 fatty acids (14% of total fatty acids); which in whole *L. donovani* promastigotes is a minor species . A notable amount of the shorter C16 fatty acid was also detected in the glycosomal membrane (Table 4.2).

4.4.3 LdPEX14 - membrane interaction

Biochemical and IEM studies supported the hypothesis that LdPEX14 was anchored to the cytosolic face of the glycosome as a peripheral membrane protein. To assess if recombinant LdPEX14 could spontaneously partition and bind lipid bilayers, flotation experiments were performed using

Fatty acid	% of total
C10:0	2.1
C12:0	1.7
C14:0	2.5
C16:0	6.1
C18:0	12.1
C18:1	23.4
C18:2	16.9
C18:3 (n-6)	5.9
C18:3 (n-3)	0.9
C20:0	1.5
C20:2	0.9
C22:0	0.5
C22:2	1.2
C22:4	3.8
C22:5	13.6
C24:0	2.8

TABLE 4.2: Fatty acid composition of the *L. donovani* glycosomal membrane.

LUVs that mimicked the *L. donovani* glycosomal membrane phospholipid composition. Incubation of LdPEX14 with DOPE:DOPC:DOPG:PI:Chl LUVs showed that ∼50% this protein bound to these model lipid bilayers and was recovered at the top of the sucrose gradient (Figure 4.3, panel A). In the absence of LUVs, no LdPEX14 flotation was observed. Addition of 500 mM NaCl, a salt concentration that is typically used to dissociate proteins that bind to membranes via electrostatic interactions, did not significantly alter the capacity of LdPEX14 to bind DOPE:DOPC:DOPG:PI:Chl LUVs and was diagnostic of the LdPEX14-membrane association being stabilized by hydrophobic contacts (Table 4.1 and Figure 4.3, panel A). Interestingly, incubation of LdPEX14 with LUVs at 0°C or 23°C prior to flotation revealed that ∼ 50-70% of the total LdPEX14 was recruited to the LUV membranes; whereas at 37°C, quantitative binding of LdPEX14 to LUVs (Figure 4.3, panel B) was observed. It is unclear, however, if the increased binding was due to an altered membrane fluidity or conformational changes in the LdPEX14 that enhance membrane binding.

To investigate the nature of the interactions stabilizing the association of LdPEX14 with the lipid bilayer, binding studies were performed in the presence of 500 mM NaCl. Fractionation of LUVs showed that large proportion of LdPEX14 remained associated with the LUVs pellet fraction, confirming previous observations showing that this protein-membrane interaction was not dependent on electrostatic forces (Figure 4.3, panels A and C). Similarly, treatment of loaded LUVs with alkaline carbonate revealed that the preponderance of the LdPEX14 partitioned with the membrane pellet, intimating that LdPEX14 binding was stabilized by hydrophobic contacts with the nonpolar core of the lipid bilayer (Figure

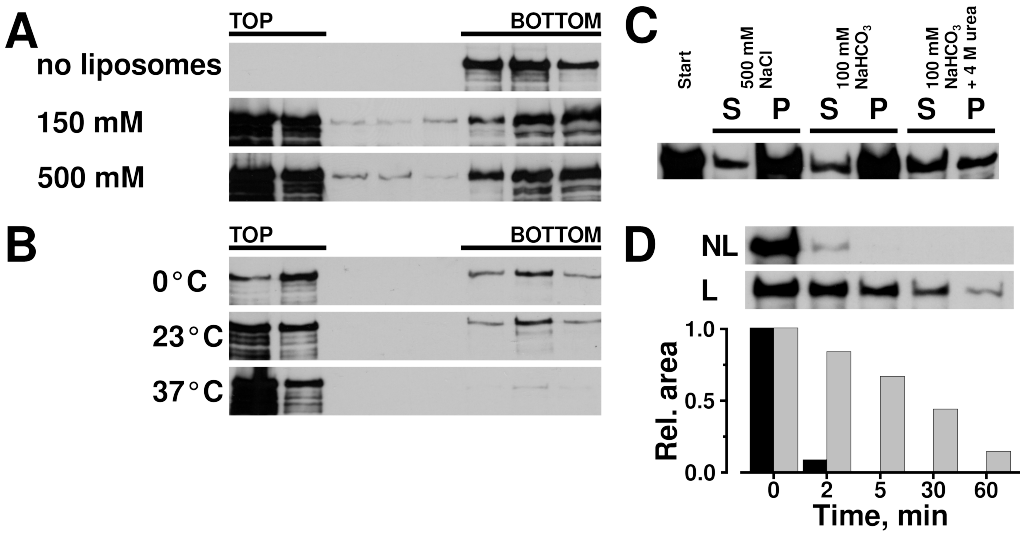

FIGURE 4.3: **Interaction of LdPEX14 with lipid bilayers mimicking the *L. donovani* glycosome.** (A) To assess the requirement of electrostatic interactions for LdPEX14 binding to liposomes mimicking the phospholipid composition of the *L. donovani* glycosomal membrane recombinant LdPEX14 was incubated at 20°C with LUVs in buffers containing 150 or 500 mM NaCl prior to flotation on a sucrose density gradient. (B) To examine the effect of temperature on the LdPEX14 membrane binding, LUVs were incubated with LdPEX14 at 0, 23 or 37°C for 40 min prior to flotation to resolve membrane associated from unbound LdPEX14. Following flotation gradients were fractionated from the top of the gradient and protein precipitated for Western blot. (C) To investigate the forces stabilizing the interaction of LdPEX14-lipid bilayers, LdPEX14 loaded LUVs were sequentially extracted with 500 mM NaCl, 100 mM $NaHCO_3$ and 100 mM $NaHCO_3$ containing 4.0 M urea. Following each extraction step the sample was fractionated into supernatant (S) and pellet (P) prior to Western analysis to assess the partitioning of the LdPEX14 between the two phases. (D) The association of LdPEX14 with the lipid bilayer was examined using the protease clostripain treatment in the absence (NL) or presence (L) of LUVs. Band intensities were quantified using the ImageJ software (Abràmoff *et al.*, 2004).

4.3, panel C). A more stringent extraction of the LUVs pellet with 100 mM alkaline carbonate containing 4.0 M urea resulted in an equal distribution of LdPEX14 into the supernatant and membrane pellet fraction further the conjecture that this interaction was stabilized by nonpolar contacts (Figure 4.3, panel C). These results are consistent with those previously reported for native LdPEX14 bound to the glycosomal membrane (Jardim *et al.*, 2002).

To investigate if membrane binding altered the LdPEX14 structure, a limited proteolytic analysis was performed using the arginine specific endoproteinase clostripain. In the absence of LUVs LdPEX14 exhibited a high susceptibility to protease cleavage and was complete degraded within 5 min (Figure 4.3, panel D), a result that is consistent with the native disordered structure predicted for LdPEX14. In contrast, the binding of LdPEX14 to LUVs dramatically decrease the susceptibility of this peroxin to proteolysis and resulted in ∼50% of the full length LdPEX14 being detected after a 30 min incubation. These data suggest that the recruitment of LdPEX14 to membranes induces structural changes that make this protein more resistant to proteolysis (Figure 4.3, panel D).

4.4.4 The hydrophobic region of LdPEX14 is required for membrane attachment

Hydropathy analysis of the LdPEX14 predict a putative transmembrane domain spanning residues 153-169 (Figure 4.4, panel A), which is situated downstream of the LdPEX5 and LPEX7 binding sites (Figure 4.4, panel B, solid line), and a region that is predicted to adopt an ordered structure

(Figure 4.4, panel B, dashed line). Bioinformatic analysis of residues 120-200 with the HELIQUEST, a program that use the hydrophobic moment to predict amphipathic structures, indicated that residues 152-173 had a high propensity to form an amphipathic helix (Figure 4.4, panel C), structures known to have membrane binding activity. *Ab initio* modeling of residues 140-180 using the I-TASSER algorithm indicate that residues 140-150 favour the formation of a short helix (Figure 4.4, panel D blue) followed by a flexible random coil segment (residues 151-157, green) and long, 18 amino acids, amphipathic helix spanning residues 158-175 that contains a hydrophobic (red) and hydrophilic (yellow) face (Figure 4.4, panel D).

To verify that the LdPEX14 hydrophobic region was required for membrane association an internal deletion mutant lacking residues 149-179 (ldpex14 Δ149-179) was generated and the capacity of ldpex14 Δ149-179 to bind LUVs was assessed by sucrose density flotation. In contrast to LdPEX14, which exhibited robust association with DOPE:DOPC:DOPG:PI:Chl LUVs (Figure 4.6, panel A), no ldpex14 Δ149-179 was detected in liposomes recovered from the top of the gradient supporting our contention that residues 149-179 were essential for membrane binding (Figure 4.6, panel A). To demonstrate that this segment was necessary and sufficient for LdPEX14 to bind the lipid bilayer, a 10 kDa peptide encompassing residues 120-200 (ldpex14 (120-200)) was expressed and assayed for membrane binding activity. Surprisingly, ldpex14 (120-200) bound quantitatively to LUVs (Figure 4.6, panel A).

To assess the capacity of various phospholipids to mediate LdPEX14 binding, vesicles composed of the single phospholipids DOPC, DOPE,

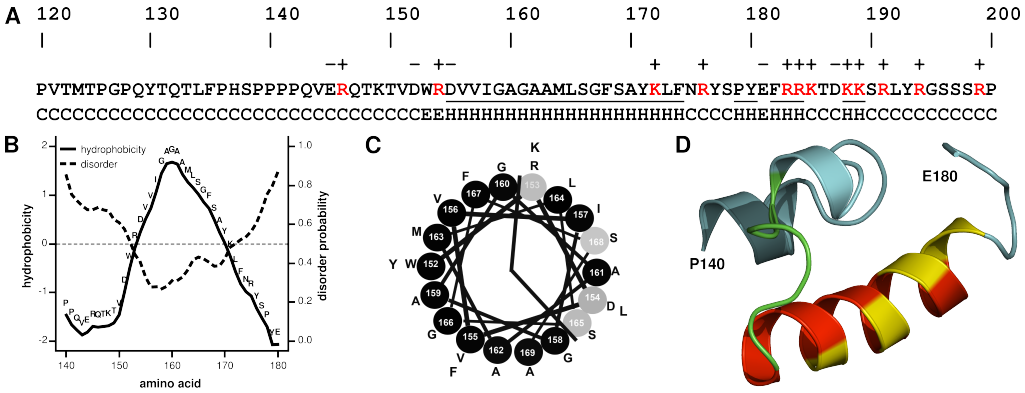

FIGURE 4.4: **Bioinformatic analysis of the LdPEX14 hydrophobic/amphipathic domain.** (A) Amino acid sequence of the predicted hydrophobic region from LdPEX14. Secondary structure prediction was performed using the JUFO program (http://meilerlab.org/index.php/servers) and E denotes extented strands, H α-helix and C random coils. (B) Hydrophobicity (solid line) , Kyte and Doolittle scale (Kyte and Doolittle, 1982) and disorder probability (dashed line, DisEMBL algorithm (Linding *et al.*, 2003)) plots of the predicted hydrophobic region from LdPEX14. (C) Amphipatic character of the helix spanning residues 152-169 was determined using HELIQUEST program (Gautier *et al.*, 2008). (D) I-TASSER in silico model (Zhang, 2008, 2009; Roy *et al.*, 2010) of the 140-180 region from LdPEX14 suggested a tertiary structure containing a short helix (blue) followed by a flexible coil (green) up to residue 157. A long amphipathic helix can be found downstream where red and yellow represents hydrophilic and hydrophobic residues respectively.

DOPA, DOPS, or DOPG or a mixture of DOPE and DOPC were evaluated in flotation experiments. No LdPEX14 flotation was detected in the absence of LUVs or with vesicles composed of only DOPC and DOPE or mixtures of DOPE:DOPC (2:1) (Figure 4.5 panel A). In contrast, liposomes composed of DOPC:DOPG (1:1) or DOPE:DOPG (1:1) or LUVs composed solely of the anionic phospholipids DOPA, DOPG, and DOPS all exhibited robust LdPEX14 binding activity (Figure 4.5 panel A and B), however this interaction was not mediated by electrostatic contacts since binding experiments performed in the presence of 500 mM NaCl had no notable impact on the protein-membrane interaction. In contrast to full length

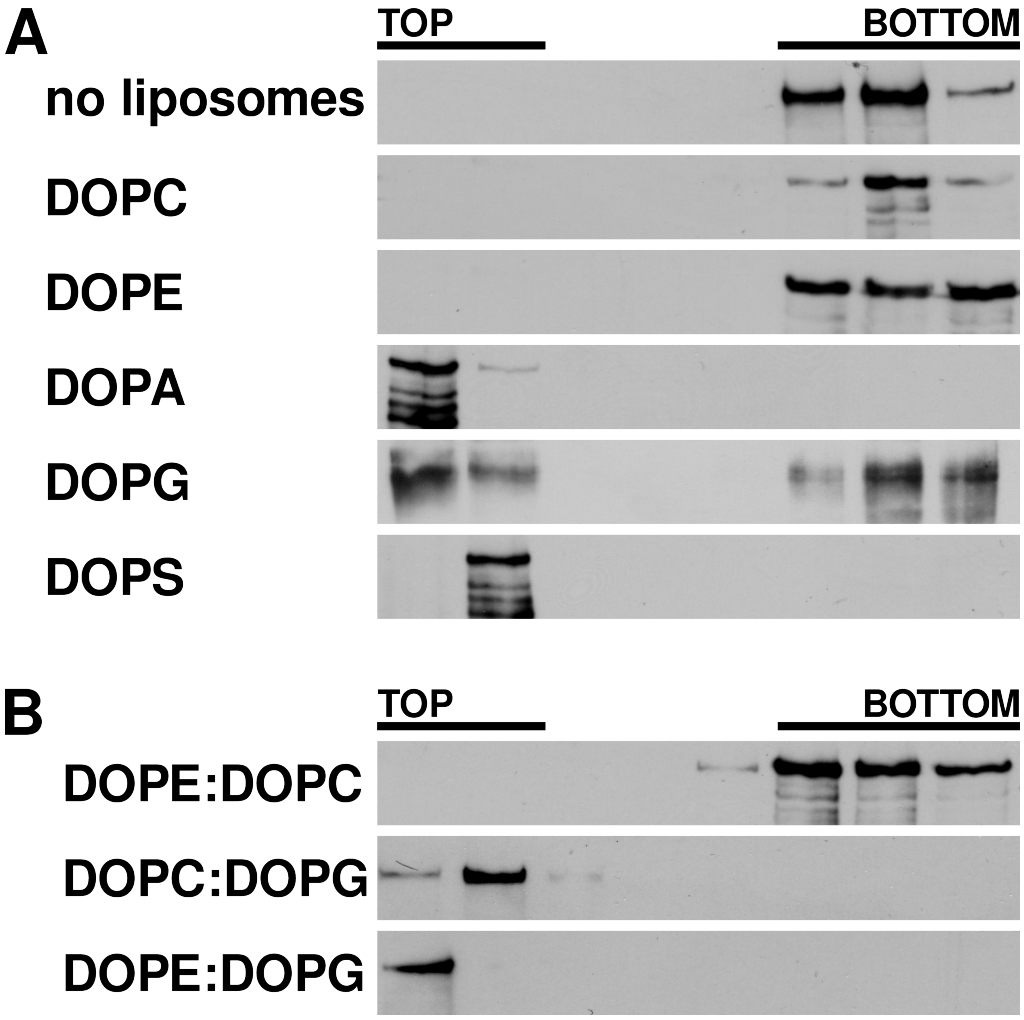

FIGURE 4.5: **LdPEX14 requires anionic phospholipids for membrane binding.** Large unilamellar liposomes prepared from (A) the single phospholipids DOPC, DOPE, DOPA, DOPG or DOPS or (B) mixtures of DOPE:DOPC, DOPC:DOPG or DOPE:DOPG in 2:1 molar ratio, were incubated with 50 μg of LdPEX14 for 40 min prior to submitting the resulting complex to differential density flotation. Following the centrifugation, tubes were fractionated into 8 fractions from top to bottom and assessed for the presence of LdPEX14 by Western blot.

LdPEX14 which required the presence of anionic phospholipids for binding to the lipid bilayer, ldpex14 (120-200) was more promiscuous and bound to both the anionic LUVs, DOPE:DOPC:DOPG:PI:Chl and zwitterionic LUVs DOPE:DOPC (Figure 4.6 panel B). These data suggest that the PG and PI detected in glycosomal membranes are important for mediating insertion of native LdPEX14 into the cytosolic face of the glycosomal membrane and that the requirement for anionic phospholipids appears to be controlled by a region(s) in LdPEX14 other that the membrane binding domain.

Previous studies have reported the presence of cholesterol and ergosterol in glycosomal preparations, however whether these sterols are components of the membrane or products of the sterol biosynthetic pathway housed in the glycosome was unclear. To assess the impact of this sterol on the membrane binding activity of LdPEX14, flotation experiments were performed with LUV prepared with and without cholesterol. Interestingly, no significant difference in LdPEX14 binding was detected for these two different populations of LUVs which suggested that the presence of sterols in the lipid bilayer did not significantly alter the recruitment of ldPEX14 into the lipid bilayer (Figure 4.8).

4.4.5 Insertion of the LdPEX14 hydrophobic region into lipid bilayer

Treatment of ldpex14 (120-200) loaded LUVs with alkaline carbonate showed that this peptide like the full length LdPEX14 remained largely resistant to extraction with only ∼50% of the protein partitioning into

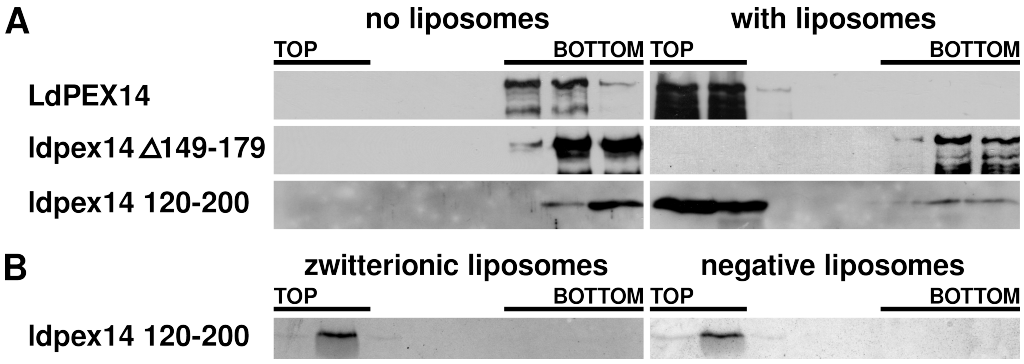

FIGURE 4.6: **The LdPEX14 hydrophobic/amphipathic domain is essential for membrane binding.** (A) Liposomes binding assays by differential density flotation performed on an internal deletion of LdPEX14 (ldpex14 Δ149-179) which removed the predicted hydrophobic region showed that this protein is only found at the bottom of the centrifugation tube where it did not associate with liposomes. In opposition, a fragment compassing the hydrophobic region, ldpex14 120-200, almost exclusively bound to the liposomes. Centrifugation tubes were fractionated into 8 fractions and proteins were detected by Western blot using a rabbit polyclonal anti-LdPEX14 (LdPEX14 and ldpex14 Δ149-179) or a mouse monoclonal anti-His_6 (ldpex14 120-200). (B) ldpex14 120-200 fragment was assayed for binding capability with zwitterionic (DOPE:DOPC, 2:1 molar ratio) or anionic (DOPE:DOPC:DOPG:PI:Chl, 53:24:15:4:4 molar ratio) liposomes. Fractions were analyzed by Western blot using a mouse monoclonal anti-His_6.

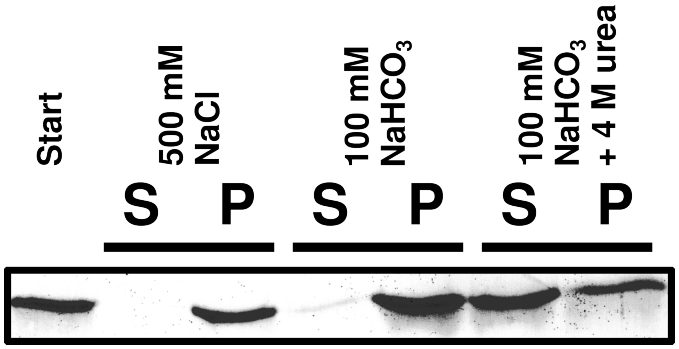

FIGURE 4.7: **Interaction of ldpex14 120-200 with the lipid bilayer.** Floated liposomes loaded with ldpex14 (120-200) were serially extracted with 500 mM NaCl, 100 mM $NaHCO_3$ and 100 mM $NaHCO_3$ in 4M urea and the supernatant (S) and pellet (P) fraction at each step were separated by ultracentrifugation and the partitioning of ldpex14 120-200 between the two fractions assessed by SDS-PAGE and Western blot analysis.

the supernatant fraction when membranes were extracted with alkaline carbonate containing 4.0 M urea, a biophysical feature consistent with the peptide-membrane interaction being stabilized by hydrophobic contacts (Figure 4.7).

The intrinsic fluorescence properties of the single tryptophan residue present in LdPEX14 (Trp152) were used to investigate the interaction of ldpex14 (120-200) with SDS micelles and SUV. Titration of ldpex14 (120-200) with SDS, a detergent that mimics the membrane environment, induced a progressive blue shift in the emission maxima wavelength (λ_{max}) of Trp152 from 350 nm to 325 nm which reached a plateau at 2.0 mM, the critical micelle concentration of SDS (Figure 4.9, panel B, inset). A similar blue shift emission λ_{max} was observed when ldpex14 (120-200) with DOPE:DOPC:DOPG:PI:Chl SUVs with the maximum occurring at phospholipid:protein ratio of 150:1 (Figure 4.9, panel A, inset). This hypsochromic shifts in λ_{max} is diagnostic of tryptophan relocating into a more nonpolar environment such as the hydrophobic core of the lipid bilayer. This contention was validated using acrylamide, a quenching agent that decrease the fluorescence intensity of tryptophan residues that are

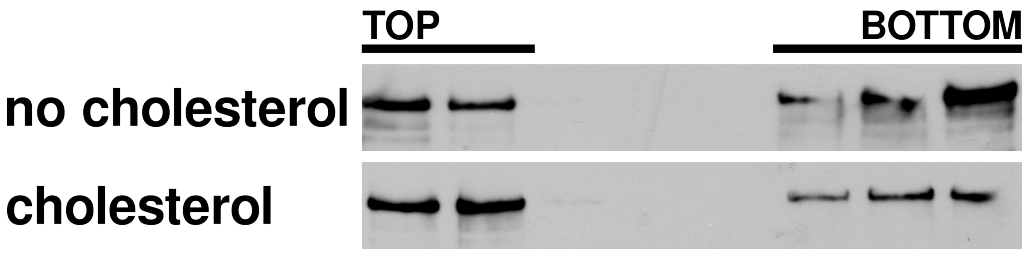

FIGURE 4.8: **Effect of cholesterol on the membrane binding activity of LdPEX14.** Differential density flotation assay of LdPEX14 using liposomes mimicking the *L. donovani* glycosomal membrane were produced in presence, and absence of 5% mol/mol of cholesterol. No difference in the flotation pattern was observed.

exposed to the bulk aqueous solvent. Stern-Volmer plots showed that ldpex14 (120-200) in solution exhibits an upward trending curve that is characteristic of quenching arising from dynamic and static components that have Stern-Volmer quenching constants (K_{sv}) of 9.1 and 0.8 M^{-1}, respectively (Figure 4.9, panel C). In contrast, ldpex14 (120-200) bound to LUVs exhibited a linear response with dynamic K_{sv} quenching constant of 3.2 M^{-1}, a value diagnostic of Trp152 having penetrated into the lipid bilayer and being less accessible to acrylamide quenching (Figure 4.9, panel C). Attempts to monitor the insertion of Trp152 into the lipid bilayer with the full length LdPEX14 was not possible since in the absence of membranes this residue exhibits an emission λ_{max} at 328 nm which is consistent with this tryptophan being sequestered in a hydrophobic environment (Cyr *et al.*, 2008).

Circular dichroism analysis of ldpex14 (120-200) revealed that in solution this peptide adopted predominantly a disordered or random structure (Figure 4.9, panel D, inset, solid line). However, in the presence of SDS micelles, which mimic a membrane environment, the spectra for ldpex14 (120-200) exhibited an increase in the minima at 222 nm, a change in molar ellipticity which reflects an increase in the α-helix content of the protein (Figure 4.9, panel D). CD analysis, together with the fluorescence spectroscopy, suggest that insertion of ldpex14 (120-200) in lipid bilayers is accompanied by a conformational change promoting α-helix formation.

To assess if the insertion of ldpex14 (120-200) into LUVs disrupted the membrane integrity, a carboxyfluorescein (CF) leakage assay was performed. Addition of ldpex14 (120-200) to CF loaded DOPE:DOPC:DOPG:PI:Chl LUVs induced a concentration dependent

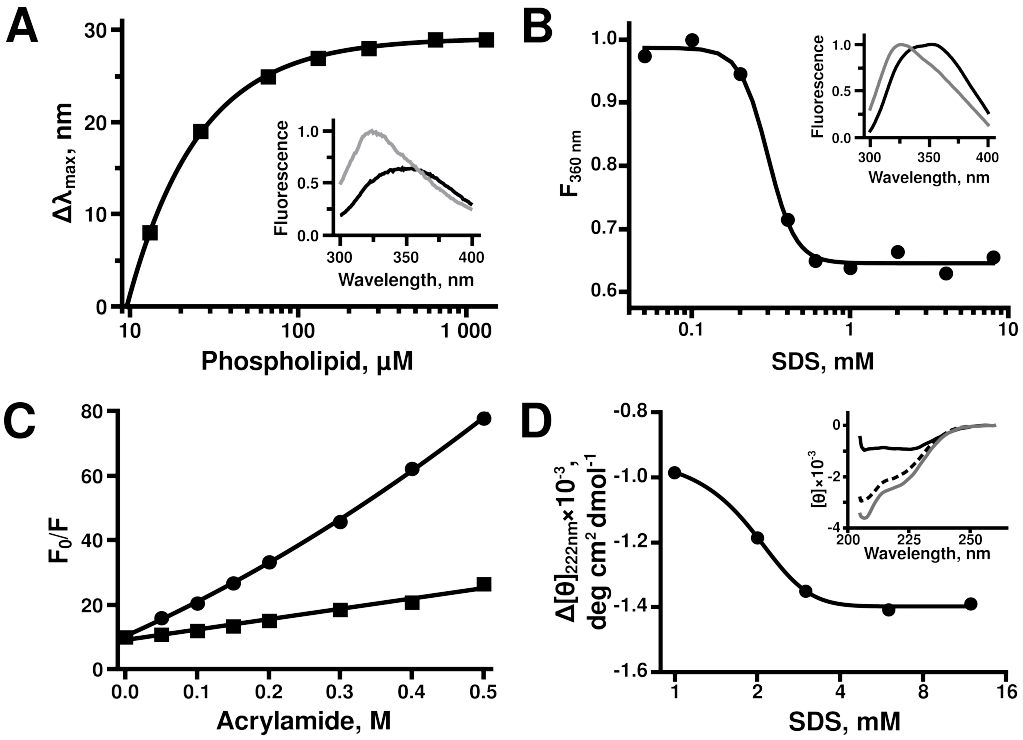

FIGURE 4.9: **Insertion of the ldpex14 (120-200) into lipid bilayer triggers α-helix formation.** (A) Intrinsic fluorescence of Trp152 from ldpex14 120-200 exhibited a hypsochromic shift from 355 nm (inset, black line) to 325 nm (inset, gray line), indicative of a change in the environment of Trp152 towards a more hydrophobic environment. (B) Acrylamide quenching of the Trp152 from ldpex14 120-200 was performed. Strong dynamic and static quenching of tryptophan occurred in the absence of liposomes (circles), whereas presence of liposomes (squares) inhibited such effect drastically. (C) SDS was titrated into a solution of ldpex14 120-200 and circular dichroism in the far UV region was measured. A strong change in molar ellipticity at 222 nm was observed as a function of SDS. As illustrated in the inset, both 1 mM (dashed line) and 16 mM (gray line) exhibited a strong secondary structure change.

leakage of CF with ∼45% dye release of the total dye occurring when 2.0 μM of ldpex14 (120-200) was added to the LUVs (Figure 4.10, panel A, squares). Kinetic analysis showed that at 25°C, insertion of ldpex14 (120-200) into lipid bilayer was rapid and caused maximal CF release within ∼2 s (Figure 4.10, panel B). In contrast, addition of buffer or the soluble glycosomal matrix protein LdHGPRT to CF loaded LUVs showed no appreciable dye release confirming that ldpex14 (120-200) indeed was capable of forming a pore-like structure in LUV membranes. Interestingly, full length LdPEX14 showed only minor ∼12% dye release suggesting that despite binding LUVs, this proteins alone was not sufficient to alter membrane integrity and cause release of CF (Figure 4.10, panel A, diamonds).

Assays performed with CF loaded DOPE:DOPC (2:1) LUVs showed that ldpex14 (120-200) also triggered dye release from zwitterionic vesicles with an efficiency comparable to that of DOPE:DOPC:DOPG:PI:Chl LUVs which suggests that the presence of anionic phospholipids is not essential for insertion of this protein into the lipid bilayer (Figure 4.10, panel C). These results further validate the flotation experiment showing the binding of ldpex14 (120-200) to DOPE:DOPC liposomes (Figure 4.6, panel B).

4.4.6 Oligomerization of ldpex14 (120-200)

Dye leakage assays and CD analysis suggested that following the membrane insertion ldpex14 (120-200) adopts a more ordered structure and potentially assembles to form a pore structure. Analysis of affinity purified ldpex14 (120-200) resuspended in SDS-PAGE sample buffer and resolved on a 12% Bis-Tris polyacrylamide gel using MES running buffer containing

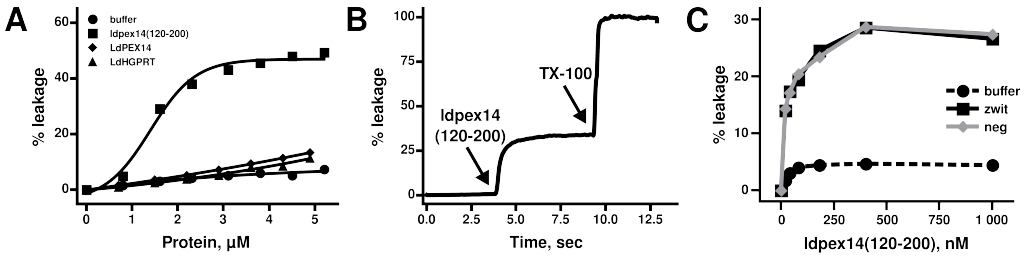

FIGURE 4.10: **Insertion of ldpex14 (120-200) into LUV membranes induces dye leakage.** (A) Proteins were titrated into a solution of 5(6)-carboxyfluorescein-encapsulated liposomes and fluorescence emission at 515nm was monitored (excitation at 492nm). Little increase in fluorescence was observed upon addition of either LdPEX14 (diamonds) or LdHGPRT (triangles). A similiar effect was seen when corresponding volumes of buffer (circles) was added to control for liposomes disruption due to pipetting. On the other hand, addition of ldpex14 120-200 (squares) caused a drastic increase in fluorescence emission, which reached ∼45% of maximum fluorescence observed in presence of detergent. (B) A kinetic experiment was performed where 2 μM of ldpex14 120-200 was spiked into a solution of CF-loaded liposomes at 4 seconds, followed by a complete disruption of the liposomes with the addition of 0.1% v/v Triton X-100 at 9.5 seconds. Emission fluorescence at 515 nm resulting for an excitation at 492 nm was monitored continuously. (C) Similar titration experiment was performed using liposomes composed of the zwitterionic phospholipids DOPE and DOPC in a 2:1 molar ratio (black squares) and compared with a buffer control (black circles).

0.5% SDS showed that this protein migrated as a ladder of bands that increased in size by ∼10-50 kDa in increments consistent with this protein assembling into stable structures corresponding from monomer to tetramer, and possibly higher order structures (Figure 4.11, panel A). To further evaluate the oligomerization capacity of ldpex14 (120-200) in lipid bilayers, DOPE:DOPC:DOPG:PI:Chl LUVs were loaded with this protein then solubilized with 0.5% of the following detergents; sodium dodecylsulfate (SDS), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), n-octyl-b-D-glucoside (OG), or taurodeoxycholate (TDOC) prior to resolution on a Bis-Tris PAGE using a MES-Tris running buffer

containing 0.02% Coomassie blue G250 dye (Swamy, Siegers et al. 2006) or 0.5% SDS. ldpex14 (120-200) extracted from LUVs with CHAPS and OG resolved on blue native gels migrated predominantly as a monomer and trimer. Tetrameric and pentameric species were also observed with these zwitterionic or non-ionic detergents (Figure 4.11, panel B). However, LUVs solubilized with the anionic detergents taurodeoxycholate or SDS migrated as monomer and trimer, although higher molecular weight species that did not enter the resolving gel is apparent. In contrast, ldpex14 (120-200) extracted with the four detergents and resolved using a running buffer containing SDS migrated as monomer and dimeric species (Figure 4.11, panel B)

SELDI-TOF mass spectroscopy analysis of ldpex14 (120-200) co-crystallized with the matrix sinapinic acid showed a dominant species with a mass of 10.4 kDa which corresponded to the monomeric form of ldpex14 (120-200). Surprisingly, high order species of mass 20.8, 31.0, 41.3, 51.6, 61.9, and 72.5 kDa, with diminishing signal intensities, were also detected by SELDI-TOF. These species correspond to ldpex14 (120-200) oligomers with a subunit composition ranging from dimer to heptamer (Figure 4.11, panel C). Collectively, the electrophoretic and mass spectroscopy data support the hypothesis that insertion of ldpex14 (120-200) into the lipid bilayer promotes oligomerization and pore formation.

4.4.7 Assembly of the pre-import complex on LUVs

Flotation experiments confirmed that LdPEX14 formed a tight interaction with LUVs membranes and exhibited biochemical features reminiscent of

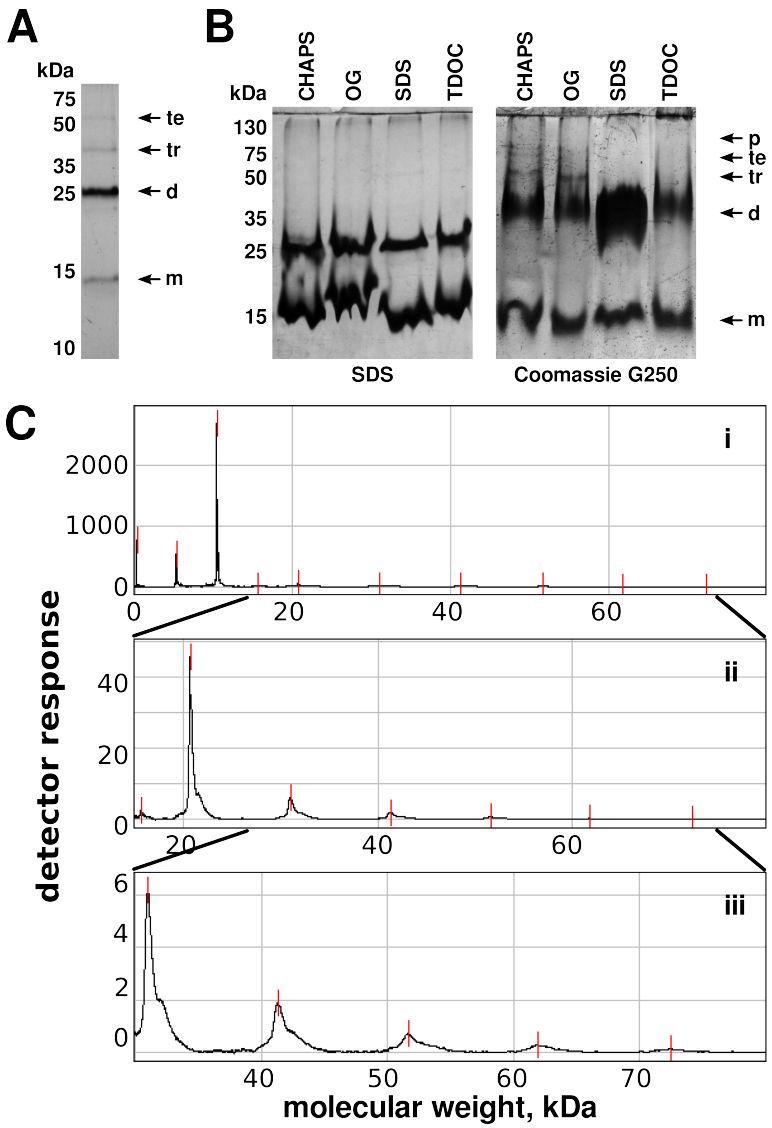

FIGURE 4.11: **Oligomerization of ldpex14 (120-200).** (A) ldpex14 (120-200) migrated as a ladder of bands on a 12% Bis-Tris polyacrylamide gel using MES buffer and 0.5% SDS in the running buffer (m: monomer, d: dimer, tr: trimer, te: tetramer). Proteins were detected by silver staining (B) ldpex14 (120-200) was assembled onto liposomes, then solubilized using various detergents, and finally resolved on a 12% Bis-Tris polyacrylamide gel using MES buffer and either 0.5% SDS or 0.02% Coomassie G250 as carrier. (C) SELDI-TOF analysis of ldpex14 (120-200) showing the presence of oligomers corresponding to multiples of ∼10 kDa, the molecular weight of ldpex14 (120-200) (i: 0-80 kDa, ii: 20-80 kDa and iii: 30-80 kDa).

the association of native LdPEX14 with the glycosomal membrane. We next employed the LUV system to examine the docking of the PTS1-LdPEX5 complex to membrane bound LdPEX14. Incubation of LdPEX5 with DOPE:DOPC:DOPG:PI:Chl LUVs showed that this receptor alone or loaded with the PTS1 cargo protein LdHGPRT exhibited no appreciable binding to liposomes (Figure 4.12, panels A and B), which contrasts with previous reports for the mammalian and *Saccharomyces* PEX5 which found to inserted in model membranes and the peroxisomal membranes. Similarly, no binding of LdHGPRT to LUVs loaded with LdPEX14 was detected (Figure 4.12, panel C); confirming that this PTS1 protein did not associate with membrane anchored LdPEX14. However, addition of LdPEX5 or the LdPEX5-PTS1 trafficking complex to LUVs loaded with LdPEX14 resulted in the recruitment of a major component of LdPEX5 to liposomes (Figure 4.12, panels D and E), an event that was mediated by the LdPEX14-LdPEX5 association. More importantly, the PTS1 protein was also found to associate with the LUVs, suggestion the formation of a intermediate pre-import complex that may form on the glycosome surface. That the association of LdPEX5 with LUVs required the insertion of LdPEX14 into the lipid bilayer was further validated by the finding that no LdPEX5 bound to LUVs when ldpex14Δ149-179, a mutant form of LdPEX14 that lacks membrane binding activity was added to the reaction mixture (Figure 4.12, panel F).

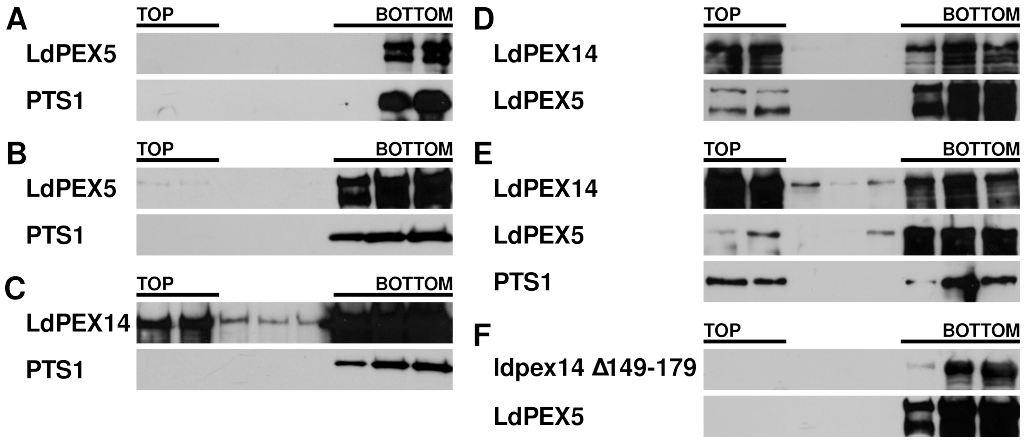

FIGURE 4.12: **LdPEX14 recruitments LdPEX5 and LdHGPRT to the LUV membrane.** Differential density flotation assays were performed by mixing 50g of each protein tested with 400g of a phospholipid mixture mimicking the glycosomal membrane extruded into 200nm liposomes. Combination of proteins were (A) LdPEX5 and LdHGPRT (PTS1 protein), individually assayed, (B) LdPEX5 and LdHGPRT, (C) LdPEX14 and LdHGPRT, (D) LdPEX14 and LdPEX5, (E) LdPEX14, LdPEX5 and LdHGPRT and (F) ldpex14 Δ149-179 and LdPEX5. Following centrifugation, tubes were fractionated into 8 fractions and analyzed by SDS-PAGE and Western blot. Rabbit anti-LdPEX14 (1:10000), rabbit anti-LdPEX5 (1:10000) and rabbit anti-LdHGPRT (1:2000) were used as primary antibodies to detect the presence of their respective proteins.

4.4.8 Docking to LdPEX14 causes membrane insertion of LdPEX5

To further investigate the structure of the PTS1-LdPEX5-LdPEX14 complex bound to the liposome membranes, we performed an alkaline carbonate extraction. Treatment of LUVs loaded with LdPEX14 or LdPEX5-LdPEX14 complexes showed that LdPEX14 remained associated with the membrane pellet when treated with alkaline carbonate and exhibited a 50:50 distribution between the supernatant and pellet when treated with 4 M urea in alkaline carbonate (Figure 4.13, panel A), biophysical characteristics comparable to those of LUVs containing

LdPEX14 alone. A similar examination of the LdPEX5 distribution showed that following treatment of LUVs with 500 mM NaCl or alkaline carbonate the PTS1 receptor remained associated with the membrane fraction and is consistent with this protein on docking to LdPEX14 undergoing a conformational change that promotes its insertion into the lipid bilayer (Figure 4.13, panel B). Subjecting membranes to a more stringent treatment with 4 M urea in alkaline carbonate only resulted in extraction of ∼50% of the protein into the supernatant fraction which is indicative of the LdPEX5 association with the membrane is being stabilized by hydrophobic interactions. Surprisingly, bioinformatic analysis of the LdPEX5 primary sequence failed to reveal any domain with putative membrane active features.

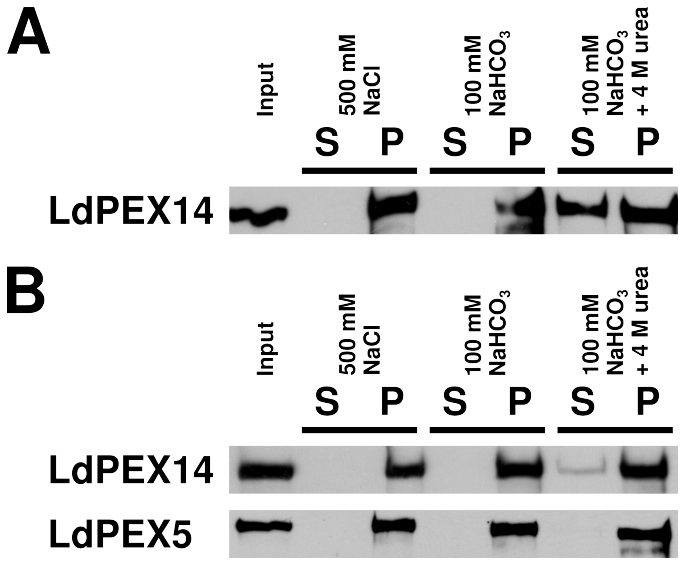

FIGURE 4.13: **LdPEX5 is inserted inside the phospholipid bilayer by LdPEX14 .** Floated liposomes loaded with (A) LdPEX14, or LdPEX14 and LdPEX5 (B) were serially extracted with 500 mM NaCl, 100 mM $NaHCO_3$ and 100 mM $NaHCO_3$ in 4M urea. The supernatant (S) and pellet (P) fraction at each step were separated by ultracentrifugation and the partitioning of LdPEX14 and LdPEX5 between the two fractions assessed by SDS-PAGE and Western blot analysis.

4.5 Discussion

Peroxin 14 has been demonstrated to be a vital component of the docking/translocation machinery that mediates the efficient import of nascent proteins into glycosome/peroxisome (Albertini *et al.*, 1997; Fransen *et al.*, 1998; Johnson *et al.*, 2001; Furuya *et al.*, 2002). Previous reports showing that LdPEX14 was a peripheral membrane protein to the cytosolic face of the glycosome membrane (Jardim *et al.*, 2002; Strasser *et al.*, 2012) were further validated in this study by immunelectron microscopy. The finding that LdPEX14 anchored to the glycosome surface forming rosette patterns is congruent with the suggestion that this protein form an oligomeric structure with a ring or doughnut-like organization that would be reminiscent of a pre-pore structure. Preliminary transmission electron microscopy analysis of negatively stained LdPEX14 complex suggest that this recombinant protein alone assembles into bowel-like structures with a ∼10-40 nm diameter. Yeast PEX5 and PEX14 have also been shown to insert into planar lipid bilayer and generate a dynamically gated pore (Meinecke *et al.*, 2010); however the size of these transient pores were in the order of up to 9nm, which is significantly smaller than those observed for LdPEX14. Moreover, the capacity to tightly regulate the opening and closing of these translocons is particularly crucial in kinetoplastid parasites since leakage of intermediates from the glycosomal matrix is a toxic event that results in parasite death (Furuya *et al.*, 2002; Guerra-Giraldez *et al.*, 2002; Moyersoen *et al.*, 2003; Haanstra *et al.*, 2008).

These data are consistent with our findings that recombinant LdPEX14 spontaneously inserts into large unilamellar vesicles which have a diameter similar to *Leishmania* glycosomes (Figure 4.1 panels A and B, I. Coppens

personal communications) . Although the binding of LdPEX14 and ldpex14 (120-200) was principally stabilized by hydrophobic contacts with the lipid core, the initial insertion of these proteins into membranes was critically dependent on the incorporation of phosphatidylglycerol into the bilayer. This was not surprising since the rat, *Saccharomyces cerevisiae*, *Pichia pastoris*, *Trypanosoma brucei*, and *Trypanosoma cruzi* glycosomes and peroxisomes membranes contain 8-29% of the anionic phospholipids PS, PI, PA, or cardiolipin (CL) (Table 4.2). The *Leishmania* glycosome membrane also contained a notable population of anionic phospholipids with the predominant species being PG (16%), an acidic phospholipid that was absent from the membrane of the microbody organelles of the above organisms. Another unique feature shared by the *Leishmania* and *T. cruzi* glycosomal membranes was the high proportion of phosphatidylethanolamine, a lipid with a small head group that favours a non-bilayer structure, and that like negatively charged lipids, is important for mediating protein insertion and translocation across lipid bilayers, as well as acting as chaperone to aid in folding of membrane proteins (Kusters *et al.*, 1994; Rietveld *et al.*, 1995; van Klompenburg and de Kruijff, 1998). This high content of PG and PE is relatively unusual for eukaryotic cell membranes and is more evocative of the lipid composition found in the inner membrane of *E. coli* (Raetz, 1978). The *Leishmania* glycosomes also have an unusual fatty acid composition and contains an abundance of C:18 and C:22 polyunsaturated fatty acids providing a considerable degree of fluidity to the bilayer which would facilitate pore opening and insertion of proteins into the glycosomal membrane.

The spontaneous insertion of the full LdPEX14, but not the deletion

mutant ldpex14 Δ149-179, into the lipid bilayer mimicking the phospholipid composition of the *L. donovani* glycosomal membrane confirms that the hydrophobic region spanning residues 149-179, a segment that is conserved amongst all PEX14 homlogues (Albertini *et al.*, 1997; Komori *et al.*, 1997; Will *et al.*, 1999; Furuya *et al.*, 2002; Jardim *et al.*, 2002) is essential for membrane binding (Itoh and Fujiki, 2006). Hydrophobic moment analysis of residues 149-179 predicts that this segment favours formation of an amphipathic α-helix, a structural element known to have potent membrane binding activity (Segrest *et al.*, 1990; Cornell and Taneva, 2006).

The capacity of LdPEX14, which in solution assembles into large oligomers (Figure 4.1, panel C and Cyr *et al.* (2008)) and to bind LUVs suggest that this amphipathic helix is surface exposed. Intrinsic fluorescence studies previously demonstrated that this domain on LdPEX14 is highly mobile and becomes more solvent accessible on binding LdPEX5 (Cyr *et al.*, 2008). These observations support the conjecture that docking of the LdPEX5-PTS1 complex to LdPEX14 would promote a tighter interaction or a possible rearrangement of this amphipathic helix in the glycosomal membrane. Limited proteolysis experiments showed that recruitment of LdPEX14 to LUVs alter the structure of LdPEX14, probably by promoting its membrane insertion, and render this protein more resistant to degradation. Likewise, studies with purified glycosomes demonstrated that the native LdPEX14 conformation and its interaction with the glycosomal membrane is dramatically modified, making it more refractory to proteolysis on docking of PTS1-LdPEX5-LPEX7-PTS2 trafficking complex (Strasser *et al.*, 2012). Carboxyfluorescein leakage assays revealed that LdPEX14 did not disrupt the LUV membrane integrity

and is consistent with the amphipathic helix attaching parallel to the membrane, allowing the hydrophobic face of the helix to make contacts with the nonpolar core lipid bilayer. That the lipid-protein interactions stabilizing the association of LdPEX14 with the membrane was limited to the outer leaflet in the absence of the receptor proteins LdPEX5 and LPEX7 may explain why LdPEX14 extraction from the LUV lipid bilayer is facilitated by urea/alkaline carbonate solution but not by an alkaline carbonate solution alone a typical feature of peripheral membrane proteins.

Subcellular fractionation, confocal microscopy (Jardim *et al.*, 2002), and immunoelectron microscopy have demonstrated that LdPEX14 is strictly targeted to the glycosomal membrane and suggests that *Leishmania* employ a selective mechanism to escort newly synthesized LdPEX14 to the glycosome and prevent nonspecific binding to other intracellular membranes. This notion is supported by the finding that deletion of the first 53 amino acids is sufficient to abrogate targeting of these mutant ldpex14 proteins to the glycosome[1]. Bioinformatic analysis of LdPEX14 revealed the presence of a putative LdPEX19 binding motif situated immediately upstream of the LdPEX5 recognition sequence which may be involved in the trafficking of LdPEX14 to the glycosome as previously suggested (Itoh and Fujiki, 2006; Saveria *et al.*, 2007). The possibility that LdPEX14 is targeted to the glycosome by an alternative peroxisomal membrane targeting signal (mPTS) consisting of a cluster of basic residues located adjacent to the hydrophobic region as described in the membrane proteins; PEX3, PEX16, PMP47, and PMP70 (Heiland and Erdmann, 2005) is another favourable possibility since this type of motif is also present in LdPEX14, downstream of the identified hydrophobic region, between residues 183 and 192, and

[1] Banerjee and Jardim, unpublished observation

that we have observed an initial requirement for anionic phospholipids in order to achieve proper attachment to LUVs.

The binding of ldpex14 (120-200) to LUVs, in contrast to the full length LdPEX14, triggered the release of carboxyfluorescein through a pore that was formed by ldpex14 (120-200) inserted into lipid bilayer. The capacity for ldpex14 (120-200) to mediate this event was ascribed to the oligomerization of this protein to form a pore. It is postulated that the amphipathic helix of ldpex14 (120-200) initially binds parallel to the membrane, however the increased flexibility allows this peptide to penetrates through the lipid bilayer and oligomerize to form a pore structure akin to that of barrel-stave model (Shai 1999). Formation of a pore via this mechanism is supported by the fact that residues 151-173 of ldepx14 (120-200) have propensity to fold into a $\sim$30 $\mathring{A}$ long amphipathic helix which is sufficient to span the membrane (Shai, 1999; Hildebrand *et al.*, 2004). In addition this helix contains a central pentameric GXXXA motif that has been implicated in the homo-oligomerization of PEX14 and other transmembrane proteins (Kleiger *et al.*, 2002; Itoh and Fujiki, 2006; Cyr *et al.*, 2008).

In contrast to the yeast and mammalian PEX5, which spontaneously insert into lipid bilayers (Gouveia *et al.*, 2003; Kerssen *et al.*, 2006), no binding of LdPEX5 or PTS1 loaded LdPEX5 to glycosome membrane mimicking LUVs was observed unless these liposomes vesicles were loaded with LdPEX14. This finding is supported by earlier reports showing that only trace amounts of LdPEX5 co-purify with glycosomes (Pilar *et al.*, 2008; Strasser *et al.*, 2012) and suggests that the PTS1 receptor forms only a transient interaction with the glycosome membrane during the assembly of

the pre-importation complex. Interestingly, the recruitment of LdPEX5 to the LUVs indicates that N-terminus of LdPEX14, containing the LdPEX5 binding site (residues 23-63) (Madrid and Jardim, 2005), remains accessible to the bulk solvent.

Our current model assumes that the LdPEX14 macromolecular complex binds to the outer leaflet of the lipid bilayer and ensures that glycosome integrity is retained; which is critical for parasite viability (Furuya *et al.*, 2002; Guerra-Giraldez *et al.*, 2002; Haanstra *et al.*, 2008). On docking of the PTS1-LdPEX5 complex, it is postulated that LdPEX14 undergoes a conformational change that causes the amphipathic helix to penetrate the membrane (Cyr *et al.*, 2008) and form a translocation pore which is supported by the capacity of ldpex14 (120-200) to cause leakage of LUV-encapsulated carboxyfluorescein. The re-orientation of the amphipathic helix is potentially driven by the cluster of basic amino acids (residues 182-193) located immediately downstream of this helix. This basic cluster is reminiscent of the hydrophilic arginine and TAT cell penetrating peptides (Madani *et al.*, 2011) which readily transverse lipid bilayers. A similar mechanism utilizing a basic loop has been proposed for insertion of; PMP47 (Dyer *et al.*, 1996), PEX3 (Fujiki *et al.*, 2006), and PEX16 (Heiland and Erdmann, 2005; Matsuzono and Fujiki, 2006) in the peroxisomal membrane. Dissociation of the empty LdPEX5 receptor from LdPEX14 induces a conformational change in LdPEX14 which would cause the withdrawal of the amphipathic helix to the outer leaflet of the bilayer and effectively closing the import channel. This reversible movement of the amphipathic helix in and out of the membrane would be favoured by the increased fluidity of this membrane due the high content of unsaturated fatty acids. In this

model, the cargo proteins passing through the channel would maintain the glycosomal integrity and prevent leakage of metabolites into the cytosol.

Here we illustrate for the first time that LdPEX14 adopts a 30-40 nm, rosette-like shape at the surface of glycosomes, consistent with the oligomeric nature of LdPEX14. We also demonstrate that LdPEX14 is capable of spontaneously inserting into glycosome mimetic membranes despite the absence of putative protein chaperones (*e.g.* PEX19) and anchors (*e.g.* PEX13) identified as being essential for proper targeting to the peroxisome/glycosome in other systems (Banerjee *et al.*, 2005; Itoh and Fujiki, 2006; Saveria *et al.*, 2007; Neufeld *et al.*, 2009).

We also propose a mechanism by which the amphipathic helix (putative transmembrane domain) of LdPEX14 may function in forming a large gated pore on the glycosomal membrane. Despite proper recruitment of LdPEX5 and PTS1 cargo to the LUVs, no import of the PTS1 cargo proteins into the LUVs was detected indicating that additional membrane components likely corresponding to the *Leishmania* homologues PEX2/PEX10/PEX12 subcomplex (Chang *et al.*, 1999; Okumoto *et al.*, 2000; Otera *et al.*, 2000; Kerssen *et al.*, 2006; Krazy and Michels, 2006; Platta *et al.*, 2007; Saveria *et al.*, 2007); and PEX13 (Girzalsky *et al.*, 1999; Gouveia *et al.*, 2000; Otera *et al.*, 2002; Itoh and Fujiki, 2006; Williams and Distel, 2006; Zhang *et al.*, 2006; Verplaetse *et al.*, 2009), peroxins that in other systems have been shown to interact with PEX5 and PEX14, may be required to for assembly of a functional pore and for protein import step.

4.6 Chapter-Related Acknowledgements

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Chapter 5

Summary and conclusions

5.1 Summary

Neglected tropical diseases (NTDs) are found to be poverty-promoting by impairing child growth, intellectual development and by reducing the productivity of the affected populations. Leishmaniasis remains an important tropical disease that affect millions of people, mainly in developing areas of the world that include Africa, the Middle East, India and Latin America (Alvar *et al.*, 2012).

As of 2001, it is estimated that 59,000 people died annually from leishmaniasis. However, these estimates tend to be largely underestimated due to the lack of available diagnostic tools (Ostyn *et al.*, 2011; Alvar *et al.*, 2012; Vogel, 2012). Moreover, leishmaniasis, like other NTDs, lacks intense pharmaceutical research favourable to the development of effective chemotherapeutic treatments required for the elimination of these infections from endemic areas. These caveats underscore a need for further fundamental research essential to better understand the biology of *Leishmania* sp., the protozoan parasite causing leishmaniasis.

The digenetic parasitic lifestyle involving two different hosts (insect and mammal), has required that *Leishmania* sp. evolves molecular mechanisms that permit this parasite to rapidly adapt to different environments. This includes the compartmentalization of glycolysis and other vital metabolic pathways in the glycosome, an organelle that is evolutionarily related to peroxisomes and glyoxysomes of other eukaryotes.

Glycosomes, aside from being recognized as an attractive chemotherapeutic target for treating kinetoplastid infections, provide a unique system for addressing fundamental biological questions regarding the evolution of

parasitic and nonparasitic kinetoplastid organisms. First, the evolutionary aspect of the glycosome, especially with respect to the parasitic lifestyle of *Leishmania* and other trypanosomatids presents challenges with respect to the understanding of how and why the transport machinery evolved. Second, the transport of folded, often oligomeric, proteins across a membrane bilayer presents a great platform to study pore-forming proteins and the dynamics involved in the formation of such structures.

The efforts highlighted in this thesis tackled mainly this second aspect, via the investigation of the role of *Leishmania donovani* peroxin 14 in the glycosomal import machinery. We have shown that LdPEX14 assembles into large oligomeric structures of varying sizes, having sedimentation coefficients ranging from ∼10 to 70 S in solution. Similarly, as seen by electron microscopy, recombinant LdPEX14 adopted a bowl-shaped quaternary structure of varying diameter, ranging from ∼ 20 to 40 nm. Three distinct domains on LdPEX14 were responsible for assembly and stabilization of this macromolecular complex (see Figure 4.12), which include a LdPEX5 binding region (residues 23-63), a hydrophobic domain reminiscent of a transmembrane domain (residues 153-165) and a leucine zipper (residues 270-321).

Elucidation of the LdPEX5:LdPEX14 binding stoichiometry using various biophysical techniques demonstrated that each unit of LdPEX5 binds four molecules of LdPEX14. This protein-protein interaction resulted in an exothermic heat release (ΔG ∼-36 kJ/mol) which suggests that the LdPEX5-LdPEX14 interaction is stabilized by a combination of hydrogen bonding and hydrophobic interactions. Moreover, LdPEX14 exposed an hydrophobic region to the solvent upon binding to LdPEX5 in solution.

This tertiary structure change is hypothesized to be linked to the formation of a translocation pore across the membrane of the glycosome, and correlates with the formation of a more compact LdPEX14:LdPEX5 quaternary structure which could facilitates its insertion into a lipid bilayer.

Immunoelectron microscopy imagery revealed that LdPEX14 protein complexes were found to be associated with the exterior surface of the glycosomal membrane where they assembled into rosette-like structures of 30-40 nm in diameter. An initial phospholipid analysis of the glycosomal membrane of *Leishmania donovani* showed that fatty acids present in the glycosomal membrane are generally highly unsaturated, which encourages fluidity of the phospholipids packing and facilitates protein insertion and pore formation. The phospholipid composition of the glycosomal membrane also presented a great proportion of both phosphatidylethanolamine and of negatively charged phospholipid headgroups. The relative proportions were the following: 27% phosphatidylcholine, 55% phosphatidylethanolamine, 16% phosphatidylglycerol and 2% phosphatidylinositol.

Recombinant LdPEX14 bound spontaneously to liposomes composed of the phospholipids enumerated above. This membrane-protein interaction was dependent on incorporation of negatively charged phospholipids into the lipid bilayer (phosphatidylglycerol in this case). Furthermore, the hydrophobic region of LdPEX14 was essential for attachment and insertion of this protein into membranes mimicking the glycosomal membrane phospholipid composition. Deletion of this regions spanning residues 149-179 abrogated the binding.

The insertion of the hydrophobic region of LdPEX14 affected the integrity of liposomes mimicking the glycosomal membrane phospholipid

composition by forming a pore. Encapsulated fluorescent dye leaked out of those liposomes following addition of a peptide spanning the hydrophobic region of LdPEX14. LdPEX14 bound to liposomes recruited the LdPEX5-PTS1 protein complex, and upon attachment, LdPEX5 inserted in the lipid bilayer and adopted a conformation reminiscent of integral membrane proteins.

5.2 Conclusion

5.2.1 Model, revisited

In order to illustrate the findings reported in this thesis, the general *Leishmania* glycosomal import mechanism presented in Figure 2.12 has been updated in Figure 5.1.

It remains unclear whether LdPEX14 acts alone or in conjunction with other glycosomal membrane proteins that aid in pore formation and regulation of the pore opening and allow protein complexes to translocate across the bilayer of the organelle. Nonetheless, the role of LdPEX14, and particularly of its hydrophobic region, is undoubtedly critical in the process.

The model elaborated here will facilitate the further decipherment of the various steps implicated in the targeting and translocation of proteins destined to the glycosome of *Leishmania donovani* and other trypanosomatids. Current research efforts are now put in place to link the role of LdPEX7 and PTS2 proteins and their contributions to the translocation process.

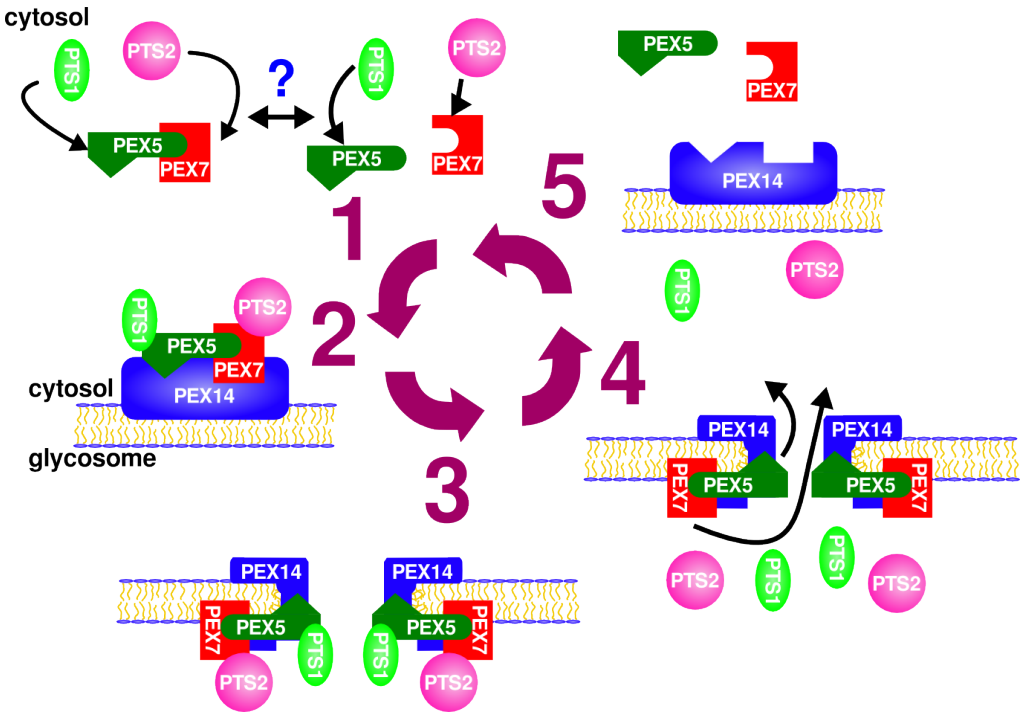

FIGURE 5.1: **Glycosomal translocation model in *Leishmania*.** Recent model for the import of PTS1 and PTS2 proteins in the glycosome of *Leishmania donovani* based on previous data, and improved from Figure 2.12 using the data presented in this thesis. (1) Proteins destined for the glycosome are recruited by their corresponding cytosolic receptor (PTS1-LdPEX5, PTS2-LdPEX7), and are carried to the glycosome where (2) the complex dock on LdPEX14. (3) Conformational changes occur where LdPEX14, LdPEX5 and LdPEX7 insert in the membrane and participate in the formation of a translocation pore. PTS cargoes are brought inside the glycosome and (4) are released in the lumen of the organelle. Following this, LdPEX5 and LdPEX7 are recycled back into the cytosol. (5) The translocation pore is closed and the cytosolic receptors LdPEX5 and LdPEX7 restart their recruiting activity. (Jardim *et al.*, 2000, 2002; Madrid and Jardim, 2005; Pilar *et al.*, 2008; Cyr *et al.*, 2008; Pilar *et al.*, 2012; Strasser *et al.*, 2012).

5.2.2 Putative new drug targets

Knowing that the specificity of LdPEX14 for glycosomal membrane appears to be driven by the presence of phosphatidylglycerol, it could be interesting to expand the research and look for this requirement *in vivo* by altering the phospholipid composition of the glycosomal membrane via interrupting the synthesis phosphatidylglycerol. Such mechanism represents a potential drug target since no phosphatidylglycerol was detected in the glycosomal membrane of other trypanosomatids.

Also, the presence of very long chain fatty acids that are highly unsaturated suggest that elongase and desaturase machineries for very long unsaturated fatty acids are active in trypanosomatids (Lee *et al.*, 2007). Whether their role in glycosome biogenesis is essential remains to be verified. A brief search through the genome of *Leishmania infantum*[1] highlighted the presence of 10 predicted desaturases and 16 predicted elongases. Interestingly, one putative elongase (LinJ.05.1160) appears to contain a PTS1 signal (-AKA), absent in *Trypanosoma* sp. homologues. The localization of this enzyme remains to be verified.

[1] via TriTrypDB web site - http://www.tritrypdb.org

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