Philipps-Universität Marburg

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An experimental strategy towards the proteome analysis of the parasitophorous vacuole in *Plasmodium falciparum*-infected erythrocytes.

Dissertation

zur
Erlangung des Doktorgrades

Der Naturwissenschaften

(Dr.rer. nat)

Julius Nyalwidhe

aus Nairobi

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Table of Contents

Page

List of Figures

List of Tables.

Page

List of Abbreviations

ALD	Aldolase
AP	Alkaline phosphotase
ATP	Adenosine triphopshate
Bip	Immunoglobulin binding protein
BFA	BrefeldinA
D	Dalton
ER	Endoplasmic reticulum
et al	Together with
EXP1	Exported protein 1
EXP2	Exported protein 2
GBP	Glycophorin binding protein
GFP	Green fluorescent protein
Hb	Hemoglobin
HRP	Horse Radish peroxidase
Hsp	Heat shock protein
Hz	Hemozoin
IRBC	Infected erythrocyte
kD	Kilodalton
KAHRP	Knob associated histidine rich protein
MALDI	Matrix assisted laser desorption ionization
MS	Mass spectrometry
Mw	Molecular weight
N	N-terrminus
NBT	Nitro Blue Tetrazolium
P	Pellet fraction
PfEMP1	*Plasmodium falciparum* Erythrocyte Membrane Protein1
PIC	Protease inhibitor cocktail.
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuole membrane
RBC	Red blood cell
RBCM	Red blood cell membrane
rpm	Revolutions per minute
RT	Room temperature

S	Supernatant
SAv	Streptavidin
SERP	Serine rich protein
SLO	Streptolysin O
TOF	Time of flight
TVN	Tubovesicular network
v/v	volume/volume
w/v	weight/volume

1 Introduction.

Malaria, caused by *Plasmodium* spp afflicts an estimated 300 million to 500 million people per annum. *Plasmodium falciparum* is the etiologic agent of the most pernicious form of human malaria and is responsible for 1.5 - 2.7 million deaths yearly, more than 1 million of which occur in children younger than 5 years (WHO, 1997; WHO, 2000). The significance of malaria as a disease is best documented by the fact that the entire issues of *Nature* (October 3, 2002) and of *Science* (October 4, 2002) were devoted to the genomic analyses of the parasite and the Anopheles vector respectively. *Plasmodium* spp are members of the Phylum Apicomplexa which consists of roughly 350 genera and more than 4500 parasitic protozoan parasites (Levine, 1988). Other prominent members of this Phylum are *Toxoplasma gondii, Cyrptosporidium parvum* and *Babesia spp*, which are causative agents of human disease, and *Eimeria, Theileria, Sarcocystis* and *Perkinsus* species, which infect livestock and which are responsible for considerable economic losses (Ruff, 1999; de Graff *et al*.,1999; Dubey, 1999).

P. falciparum exhibits a complex life cycle (Figure 1), going through a sexual phase in the insect vector, the *Anopheles* mosquito, and multiplying asexually within the human host to precipitate its morbid and lethal sequelae. Sporozoites are inoculated into the host's blood stream by the bite of infected female *Anopheles* mosquitoes. The sporozoites invade hepatocytes where they undergo asexual division (exo-erythrocytic schizogony) to release merozoites. The merozoites invade erythrocytes within which they complete the asexual life cycle inside a parasitophorous vacuole, enclosed by the parasitophorous vacuole membrane (PVM*)*. While in the red cell, the parasite undergoes three distinct stages of development: the ring (0-24 h post invasion), the trophozoite (24-36 h post invasion), and the schizont stage (36-48 h post invasion) (Figure 2), During erythrocytic schizogony, mature trophozoites undergo multiple nuclear divisions to form schizonts. This is followed by cytoplasmic division, resulting in mature schizonts (segmenters) that contain between 16 and 32 merozoites. Approximately 48 h after infection, the infected erythrocyte rupture and release merozoites into the blood stream where they infect new cells and repeat the cycle in the erythrocytes. Some merozoites undergo differentiation into sexual stages, immature macrogametocytes or microgametocytes. These mature but do not undergo further development until a feeding mosquito takes them up. In the mosquito gut, microgametocytes undergo rapid DNA replication and cell division to form eight flagellated male gametes. These are released and one fertilizes a macrogametocyte to form a diploid zygote. The zygotes undergo meiosis and each develops into an elongated form called an `ookinete´. The ookinetes penetrate the epithelial lining of the stomach of the mosquito and develop into oocysts,

which undergo sporogony to produce a large number of haploid sporozoites. These migrate to the salivary glands of the mosquito from where they are injected into a human host when the infected female *Anopheles* bites.

The asexual stage of the parasite is responsible for the most severe pathogenesis of the disease. Severe malaria occurs after parasite proliferation inside erythrocytes and consequent binding of infected erythrocytes to the vascular endothelium (cytoadherence) and to noninfected erythrocytes (rosetting). These bindings eventually lead to the accumulation of parasitized cells in the local post-capillary microvasculature and block the blood flow, limiting the local oxygen supply. The most common clinical symptoms of severe malaria are high fever, progressing anemia, multi-organ dysfunction, and unconsciousness, i.e., coma, which is a sign of cerebral malaria and one of the causes of death (Miller *et al*., 1994).

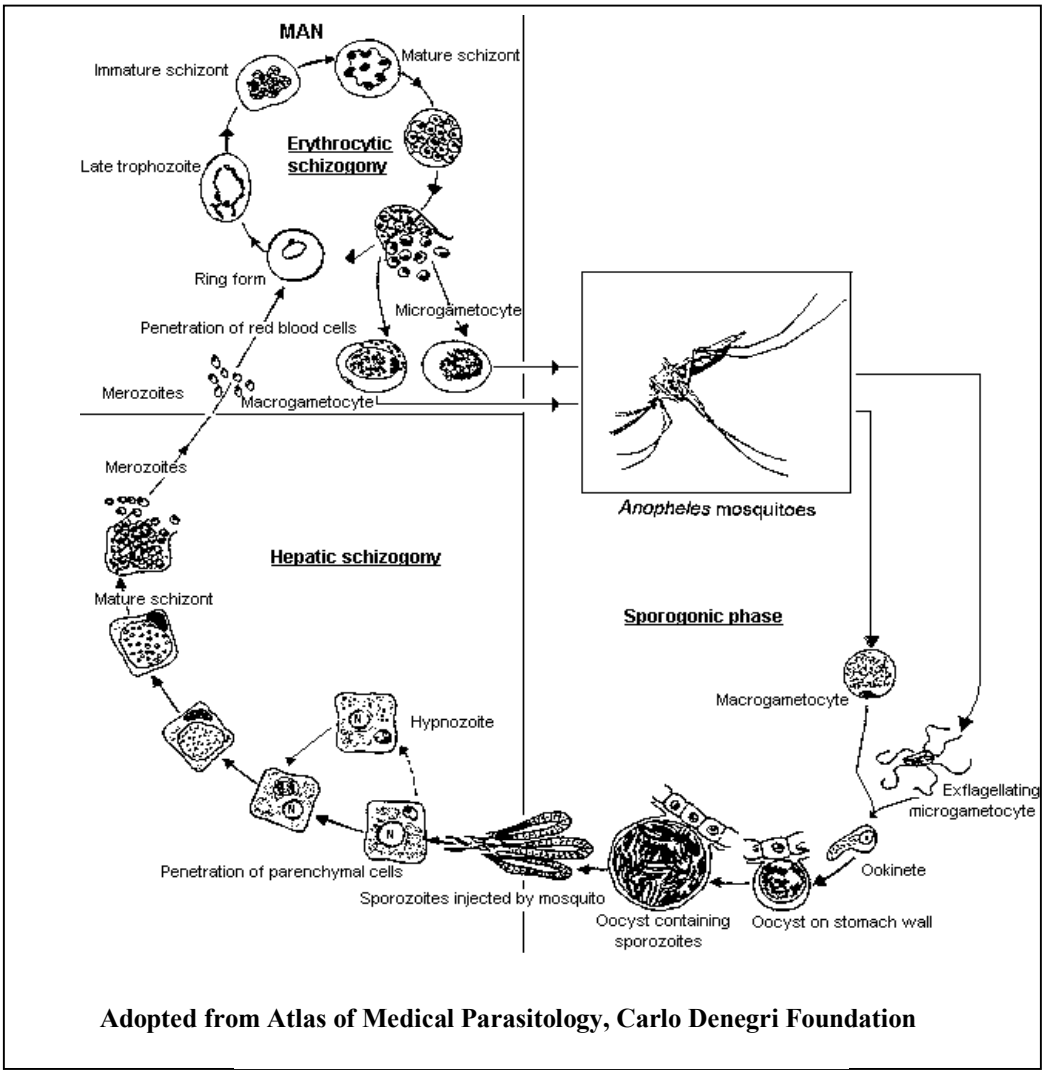


Figure 1. Life cycle of *Plasmodium falciparum*. The infective sporozoites are introduced into the human host by the bite of an infected *Anopholes* mosquito. The sporozoites undergo schizogony in hepatocytes to produce merozoites which are released into the blood stream where they invade erythroyctes. After repeated erythrocytic schizogony steps some merozoites differentiate into sexual gametocytes which mature and are taken up by a feeding mosquito. After sporogony more infectious sporozoites are produced.

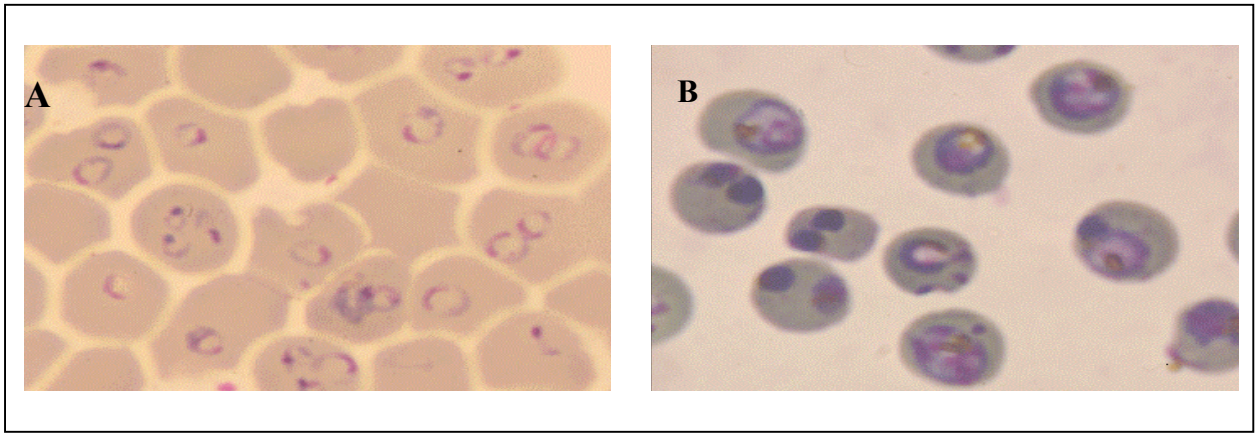


Figure 2. Asexual erythrocytic stages of *Plasmodium falciparum*. Giemsa stain of *P.falciparum* infected erythrocytes from in vitro culture. Panel A shows ring stages of the parasite. Panel B shows a mixture of trophozoites and young schizonts.

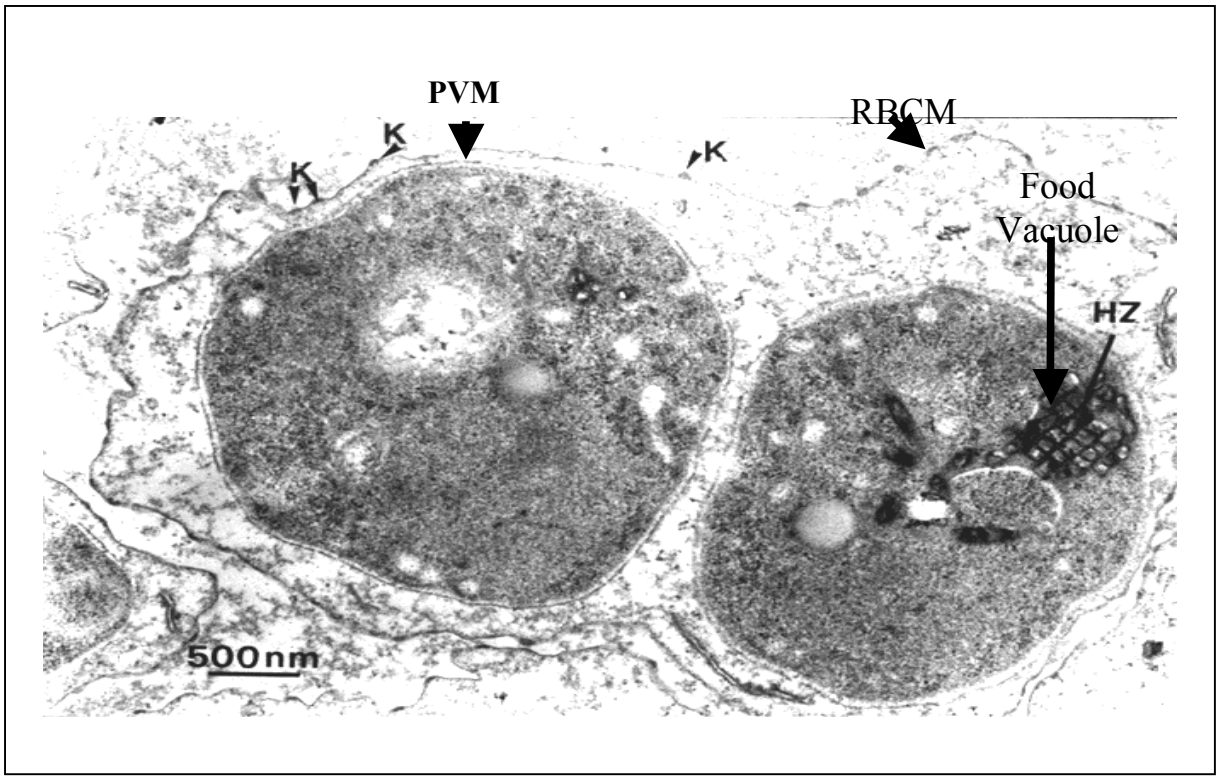


Figure 3. Ultrastructure of *P. falciparum* in permeabilized erythroyctes. The infected erythrocytes were treated with streptolysin O and the host cell cytoplasm extracted before processing them for electronmicroscopy. Electron dense structures `knobs´ are found on the red blood cell membrane (RBCM). The parasite is enclosed within a vacuole surrounded by the parasitophorous vacuole membrane (PVM). The food vacuole is indicated with an arrow and it contains haemozoin crystals (HZ).

1.1 Morphology of *Plasmodium falciparum* and the infected host cell.

1.1.1. The Parasite.

Most of the apicomplexans, including *Plasmodium* spp. are obligate intracellular protozoa that actively invade the host cell in which they propagate and live within the parasitophorous vacuole (PV) formed during invasion (Figure 3). The apicomplexan parasites, as exemplified by *P. falciparum* above, have complex life cycles involving transmission within and between hosts by specialized cell-invasive stages (generically termed zoites). These show directional gliding motility, relative to their anterior-posterior axis and they characteristically possess three sets of secretory compartments, the apical organelles at their leading (apical) end (Aikawa, 1971; Scholtyseck and Mehlhorn 1970). Two of these, the rhoptries and micronemes, secrete their contents apically, whilst the third, the dense granules, release their contents elsewhere on the zoite's surface, and are not necessarily apical in position depending on stage and species (Sam-Yellowe, 1996; Carruthers and Sibley 1997; Perkins 1992; Lingelbach and Joiner 1998; Preiser *et al*., 2000). The apical organelles secrete substances that enable zoites to adhere selectively to and invade host cells and once within, to cause further modifications and eventually escape.

The rhoptries are the largest apical organelles, they are club shaped (hence their name from the Greek letter rho). The rhoptries contain densely packed granular material and their number varies with stage of life cycle and species. *Plasmodium falciparum* merozoites and sporozoites possess two rhoptries, the ookinetes appear to have none at all (Aikawa and Seed 1980). *Theileria parva* merozoites have up to six (Shaw and Tilney 1992) and *Toxoplasma gondii* has eight or more per tachyzoite while Cryptosporidium sporozoites are reported as having only one (Tetley et al., 1998). The size and shape of the rhoptries also vary within the different genera. In *Babesia* and *Theileria* spp they are relatively small (Kawai *et al*., 2000; Shaw and Tilney 1992). In contrast *Plasmodium* sporozoites and zoites of *T. gondii* have very elongated rhoptries and there is a correlation between the size of the zoites and their sizes, where large zoites have large rhoptries, their size and number probably being important in the invasion process and creation of the optimally sized vacuole within which they eventually reside. The merozoite rhoptries of *P. falciparum* and *P. knowlesi*, as in zoites of *T. gondii* (and probably all rhoptries) have two distinct regions: a basal bulbous portion and a narrow apical duct differing in detailed structure and protein composition (Bannister *et al*., 1986; Crewther *et al*., 1990; Sam-Yellowe *et al*.,1995; Bannister *et al*., 2000). Rhoptries change their appearance during invasion after they discharge their contents, most noticeably they collapse and various membranous vesicles and tubules becoming apparent (Bannister *et al*., 1986).

Micronemes resemble rhoptries in being membrane-bounded and in having dense granular contents. However, they are smaller than rhoptries, flask-like in shape and usually occur in larger numbers. In *P. falciparum* merozoites they are small and few (Langreth *et al*.,1978) and they seem to be absent in *T. parva* merozoites (Shaw and Tilney 1992). In *Plasmodium* sporozoites and zoites of *Toxoplasma* and *Eimeria* they are numerous and can secrete large amounts of protein onto the zoite's surface possibly assisting in the gliding movement (Scholtyseck and Mehlhorn 1970; Aikawa and Seed 1980; Aikawa and Sterlin 1974). The micronemal proteins are involved in host cell recognition, binding and, possibly motility. Recent biochemical studies have shown that the penetration of host cells by *Toxoplasma gondii* is an active process involving the parasites own actomyosin system and it is distinct from phagocytosis (Herm-Götz *et al*. , 2002). Proteins secreted by micronemes share extensive domains of homology between members of Apicomplexa, supporting the hypothesis of a common molecular mechanism for host recognition, attachment and invasion (Tomley and Soldati, 2001)

Dense granules are membrane-bounded vesicles with densely packed granular contents found in apicomplexans. They vary in number, size and shape with stage and species. In *P. knowlesi* and *P. falciparum* merozoites they are spheres, about 80nm in diameter (Bannister and Mitchell 1989; Bannister *et al*., 1975; Torii *et al*., 1989). In *Plasmodium* spp the contents of the dense granules are released after merozoites enter the erythrocyte and they play a role in the extension of the PVM (Aikawa *et al*.,1990; Culvenor *et al*., 1991; Bannister and Mitchell 1989; Torii *et al*., 1989). The same sequence of events has been described in other apicomplexans including *Toxoplasma* (Leriche and Dubremetz, 1990) and *Sarcocystis* (Entzeroth, 1985).

More recently an additional compartment, the plastid has been identified in the Apicomplexan parasites including *P. falciparum* (Wilson *et al*.,1996; Mc Fadden *et al*.,1996). This organelle contains a self-replicating circular genome comparable to that of chloroplast in plants. All plastids including the apicomplexan plastid (apicoplast) arose by endosymbiosis of a cyanobacterial-like prokaryotic cell. Engulfment of this cell, referred to as primary endosymbiosis generated a plastid characterized by two membranes, such as those found in red and green algae, and plants. Following this primary endosymbiosis, plastids were presumably transferred laterally into several eukaryotic lineages that normally lacked plastids, by a process known as secondary endosymbiosis. In secondary endosymbiosis, plastid-containing primary endosymbionts, were themselves engulfed and retained by other eukaryotes (Mc Fadden *et al*., 1996; McFadden *et al*.,1997; McFadden and Waller, 1997). The ultimate function of the relict plastid is thought to be anabolic synthesis of some component(s) possibly a fatty acid or lipid (Waller *et al*., 1998). Since the pathways are bacterial in nature they are sensitive to antibacterial compounds. It has been

demonstrated that ciprofloxacin kills *T. gondii* by blocking apicoplast-DNA replication (Fichera and Roos, 1997), is ciprofloxacin also moderately active against *C. parvum* (Woods *et al*., 1998) thus proving that the plastid is unique compartment and an effective drug target which may offer new avenues in the development of parasiticidal drugs.

1.1.2. The infected erythrocyte.

The invasion of erythrocytes by merozoites occurs very rapidly (Dvorack *et al*., 1975) and to date, the molecular mechanisms involved in the process are not well understood. Morphological studies at the light and electron microscope levels have revealed that invasion is a sequential, multi-step process (Dvorack *et al*., 1975; Bannister and Dluzewski, 1990; Aikawa *et al*., 1978; Miller *et al*., 1979). Upon contact with an erythrocyte, the merozoite attaches and orients its anterior end towards the erythrocyte. A region of tight association (Aikawa *et al*., 1978), develops between the membranes of the two cells. Compartments at the anterior end of the merozoite, the rhoptries, discharge their contents onto the erythrocyte surface, which begins to indent at the point of contact. The junction transforms from a localized patch to an orifice, through which the merozoite penetrates into a progressively deepening, membrane bound vacuole. The membrane surrounding the fully internalized parasite is known as the parasitophorous vacuole membrane (PVM). The asexual stage of development of the parasite is completed within the parasitophorous vacuole (PV).

The differentiated erythrocyte appears to be incapable of receptor-mediated endocytosis (Haberman *et al*., 1967; Schekman and Singer, 1976; Zweig and Singer, 1979), and the formation of the PVM is not yet fully understood. It has been reported that erythrocyte proteins (McLaren *et al*., 1977; Aikawa *et al*., 1987; Atkinson *et al*., 1987; Dluzewski *et al*., 1989) and lipids (Dluzewski *et al*., 1992) are excluded from the PVM, leading to the suggestion that the PVM is formed from proteins and lipids stored in the rhoptries and secreted into the erythrocyte membrane during invasion (Bannister and Dluzewski, 1990; Dluzewski *et al*., 1992; Joiner, 1991). Using fluorescent probes and low-light-level video fluorescence microscopy, Ward *et al*., 1993 investigated PVM formation and found that while erythrocyte membrane proteins are indeed excluded from the PVM, the lipids forming the PVM are indistinguishable from those of the host cell membrane. The observation by Pouvelle *et al*., 1994 also indicate that the PVM lipids are to a great extent derived from the erythrocyte plasma membrane.

As the parasite grows and replicates within the vacuole, changes occur in the infected RBC cytoplasm. These changes have been related variably to either inward or outward trafficking between parasite, RBCs and the extracellular medium (Aikawa *et al*.,1986). Various studies using

either electronmicroscopy (EM), confocal microscopy or immunofluorescences have attempted to visualize the structures involved with somewhat conflicting results. Structures unique to *P. falciparum*- infected erythrocytes, including Maurer's clefts and membranous whorls that are somewhat reminiscent of Golgi compartments have been detected by EM (Aikawa, 1977; Langreth *et al*.,1978). Lipidfluorescence assays have revealed a network of tubovesicular membranes (called the tubovesicular network, TVN) extending from the PVM membrane to the RBC membrane (Elmendorf and Haldar, 1993; Behari and Haldar,1994; Elmendorf and Haldar, 1994). The enlargement of the membrane most likely are due to metabolic processes by the parasite since mammalian erythrocytes do not synthesize lipids or proteins *de novo*.

The tubular elements projecting from the PVM to the red cell have been imaged by advanced digitized fluorescence microscopy (Haldar *et al*.,1989; Grellier *et al*., 1991; Elmendorf and Haldar, 1994) and these studies suggest that the PVM and TVN are all interconnected compartments with other intraerythrocytic structures such as the Maurers clefts. However there is an alternative view that the PVM is distinct and that other intra-erythrocytic compartments such as Maurers clefts form different entities. Experiments by Waller *et al.,* 2000; Wickham *et al.,*2001 using chimeric proteins fused to the green fluorescent protein (GFP), show that the distribution of the protein that is exported show a restricted localization within the vacuole. If the two compartments, the PVM and the TVN were continuous the GFP should also distribute into the TVN. Since this does not happen it seems that the two structures are not interconnected. The trafficking of PfEMP-1 through the Maurer's clefts suggests that they are not connected to the PV (Wickham *et al*.,2001).

The most prominent ultra structural change of the surface of infected erythrocytes is the appearance of electron-dense protrusions with a diameter of approximately 100µm generated by the parasite, called knobs. (Luse and Miller, 1971). These structures are only found on the infected erythrocyte surface and are thought to be composed of several polypeptides (one of which is PfEMP1) synthesized and transported from the intracellular parasite. Knobs have been confirmed to be to the sites where infected erythrocytes bind to other cell surfaces, including the surface of normal erythrocytes (e.g., in a rosette). Knobs were also determined to the sites of attachment of infected erythrocytes to brain endothelium of patients who died of severe malaria (Aikawa, 1988; Aley *et al*.,1984).

1.2 Physiological alterations of the infected erythrocyte.

In the IRBC the PVM forms a barrier between the host cell cytosol and the parasite surface. It prevents the host cell proteins from having access to the vacuolar space. However the parasite

enclosed in the parasitophorous vacuole still relies on nutrients from extracellular sources, principally from human serum. The nutrients include some amino acids, fatty acids and other important molecules such as pantothenic acid and purine bases. Unlike mammalian cells which are capable of synthesizing purine nucleotides *de novo*, all protozoan parasites studied to date (Berens *et al*., 1995) including *Plasmodium* spp. (Gero and Sullivan, 1990) are incapable of making the purine ring and the purines must be salvaged from the host. These nutrients generally must be transported across the erythrocyte membrane (RBCM), the parasitophorous vacuolar membrane (PVM), and the parasite plasma membrane (PPM). These nutrient permeation pathways may involve a variety of complex and novel pathways including transporters, channels, and the tubovesicular membranes (TVM) (Gero and Kirk 1994; Haldar 1994; Upston and Gero 1995; Lauer et al., 1997; Ginsburg and Kirk 1998; Kirk *et al*., 1999; Kirk, 2001).

The pathways by which these different molecules gain access to the erythrocyte and eventually to the parasite are still unknown but there are three models that have been suggested to try to explain the phenomenon. According to the direct access model, suggested by Pouvelle *et al*., 1991, the PVM fuses with the plasma membrane and allows free access of molecules, including proteins, via a so called `parasitophorous duct´. Further studies by Goodyer *et al*., 1997 also support this model to be involved in the transport of macromolecules in infected erythrocytes. An alternative model suggests that nutrients gain access to the erythrocyte cytosol owing to an increased permeability of the erythrocyte plasma membrane (Ginsburg, 1994), however proteins that could be responsible for the changes in the plasma membrane's permeability are yet to be identified. Once the nutrients are in the erythrocyte cytosol, access to the parasite surface would occur through non-selective pores postulated to be present in the PVM and allowing the free exchange of molecules up to 1300 Da (Desai and Rosenberg, 1997). However these two models of transport into the PV are essentially incompatible: If a direct connection ("duct") existed together with the pores in the PVM, then the host cell would leak low molecular weight solutes into the extracellular space. A third model by Lauer *et al*., (1997), postulates that extensions of the PVM come into close proximity with the erythrocyte plasma membrane, fusion does not occur but the contact site acts as a molecular sieve which allows the selective uptake of small molecules but excludes macromolecules. To date there is no conclusive experimental data confirming the basis of nutrient acquisition between the external medium, through the PVM and ultimately to the parasite. But there is evidence that infected erythrocytes show increased permeability for a number of small molecular weight substances with preference for anionic molecules as compared to no infected erythrocytes (Ginsburg, 1994; Penny *et al*., 1998). The access of these molecules into

infected erythrocyte is non-saturable and it can be inhibited by various anion selective inhibitors such as furosemide among others (Upston and Gero 1995; Kirk *et al*., 1994).

Apart from nutrient acquisition from the host cytosol the PVM may have other functions including protecting the parasite from a potentially unfavorable environment. Initially it has been proposed that the possession of the PVM would avoid the presentation of parasite proteins by major histocompatibility complex (MHC) class I molecules. However there are now reports that show lysis of *T. gondii*-infected professional antigen presenting cells by parasite-specific CD8+ T-cells of both human and murine origin (review Lüder and Seeber, 2001). The implication is that parasite antigens are able to pass through the PVM and that the classical pathway of MHC class I presentation operates in the parasite. However, there is no conclusive evidence to explain how the mechanism is modulated. Nonetheless it would mean that protection of the obligate intracellular parasite by the PVM might not be complete in *Toxoplasma gondii*. The most obvious difference between *T. gondii* and *Plasmodium* spp is that the latter infect non-nucleated cells and the presentation of antigens in a MHC I context may probably not apply in the erythroyctic stages but possibly to the liver stages.

However, in *P. falciparum*, as the parasite develops within the erythrocyte, several parasite encoded proteins which mediate the most severe form of pathogenicity of the disease become associated with the erythrocyte cytoskeleton or are inserted into the host cell membrane. The proteins are exported across the parasite plasma membrane and the parasitophorous vacuole membrane to achieve extensive modifications of both the cytoplasm and the plasma membrane of the host erythrocyte (Lingelbach 1993; Foley and Tilley 1995). As discussed in the previous section, in mature stages of the intra-erythrocytic cycle, the membrane of the erythrocyte becomes distorted with knobby protrusions that are involved in cytoadherence of these cells to the vascular endothelium (Kilejian, 1979). These adhesive structures are formed by deposition of parasite proteins below the erythrocyte membrane and insertion of parasite proteins into the erythrocyte bilayer. The major components of these "knobs" are a peripheral membrane protein the knob associated histidine-rich protein (KAHRP), which forms the major structural element of the knob (Kilejian *et al*.,1986; Pologe *et al*., 1987; Triglia *et al*., 1987), an integral membrane protein, the *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1), which is inserted into the erythrocyte membrane and which acts as the ligand for binding to endothelial cell receptors (Baruch *et al*., 1995; Smith *et al*.,1995 and Su *et al*.,1995) and *P. falciparum* erythrocyte membrane protein-3 (PfEMP3) (Pasloske *et al*., 1993). KAHRP is essential for the formation of the knobs that deform the erythrocyte surface, and deletion of the corresponding gene results in the loss of knobs. (Crabb *et al*.,1997a). This does not completely prevent the expression of PfEMP1 on the erythrocyte

surface, but it reduces cytoadherence of infected cells under continuous flow (Crabb *et al*., 1997b). The function of PfEMP3 is unknown although truncation of the protein has been shown to affect the trafficking of PfEMP1 to the plasma membrane of the infected erythrocyte leading to a reduction in the density of PfEMP1 on the erythrocyte surface and also cytoadherence (Waterkeyn *et al*., 2000). PfEMP1 is an antigenically-variant protein family and there are 50-150 var genes (Su et al 1995). This polypeptide of high molecular mass (200-400kDa) is trypsin sensitive, insoluble in nonionic detergents and is readily labeled by conventional surface iodination techniques. PfEMP1 is purported to be composed of several extracellular Duffy binding-like domains (DBL 1 to 5), with one or two cystein-rich interdomain regions (CIDRs) distributed in between the DBLs, a transmembrane (TM) region, and an intracellular acidic segment (ATS) (Smith *et al*.,1995 and Su *et al*.,1995). The most conserved regions are located in both the N-and C- terminal domains. DBL-1α together with CIDR1α are thought to form a semi conserved head structure in which at least six regions in DBL-1α and several cysteine residues in CIDR1α are almost 100% conserved (Su et al 1995). The common structures harbored in different PfEMP1 molecules indicate that they mediate similar binding activities, yet their multiple and polymorphic nature also suggest an effective role in immune evasion. When using different receptors, CD36, TSP and ICAM-1, only PfEMP1 could be precipitated from SDS soluble fraction of infected erythrocytes, suggesting that PfEMP1 is indeed the adhesive ligand involved in binding to those receptors (Baruch *et al*., 1997).

These observations demonstrate a specific distribution of parasite proteins within the infected erythrocyte. Therefore even though the PV and PVM form a barrier between the parasite and the erythrocyte cytoplasm, parasite-encoded proteins have been shown to translocate through these compartments to sites outside the parasite.

1.3 Secretory pathways in *Plasmodium falciparum*.

In a typical eukaryotic cell there are several thousands of different proteins. For the proper functioning of the cell these proteins must be incorporated into different compartments. Protein trafficking is the process by which proteins reach their destinations either within a cell or outside cells. The proteins that are synthesized on cytosolic ribosomes are released into the cytosol; some remain there while others are specifically incorporated into organelles such as the mitochondrion, the nucleus and peroxisomes. However a large class of proteins is synthesized on ribosomes bound to the rough endoplasmic reticulum and they enter the secretory pathway. This class includes both integral membrane proteins that become inserted in the ER membrane and proteins that become soluble in the lumen of the ER. These proteins are subsequently sorted to their different destinations. Protein sorting involves `retention signals`, which result in the retention of a

polypeptide within a compartment and `sorting signals` which facilitate forward sorting. The classical protein secretory pathway in eukaryotic cells involves the transport of proteins between intracellular organelles by the budding and fusion of small vesicles and it requires a very complex machinery.

While the basic principle applies to protein trafficking within the parasite. Trafficking within the infected erythrocyte poses a number of conceptual problems. Other than its plasma membrane the parasite is enclosed by the PVM and the host cell plasma membrane. The proteins that are exported out of the infected erythrocyte have to transverse the three membranes. The parasite must have novel mechanisms of achieving this since the host cell is devoid of organelles.

Other than the adherence antigens that are exported to the surface of the infected erythrocytes, a number of parasite encoded proteins are exported to different destinations including (i) the cytoplasmic face of the erythrocyte membrane (such as KAHRP, Wickham et al 2001), (ii) the Maurer's clefts (Hui and Siddiqui, 1998; Etzion and Perkins, 1989; Stanley *et al*., 1989; Li *et al*., 1991; Albano *et al*., 1999; Blisnick *et al*., 2000; (iii) the parasitophorous vacuole, the serine rich protein (SERP) Knapp *et al*., 1989; ABRA, Weber *et al*., 1988); (iv) the host cytoplasm, where the histidine rich protein (HRP) is localized (Howard *et al*., 1986).The glycophorin binding protein (GBP) is also localized in the cytoplasm (Kochan *et al*., 1986) and it was been demonstrated that the protein is released into the parasitophorous vacuole before it is translocated across the PVM into the host cell cytosol (Ansorge *et al*.,1996). These results suggest that in addition to GBP and KAHRP (Wickham *et al*., 2001), it is possible that other proteins destined for the erythrocyte cytosol, are first released into the vacuolar space before they are translocated across the PVM. (v) EXP1 and EXP2 are targeted to the PVM as integral and peripheral membrane proteins, respectively (Simmons *et al*., 1987; Günther et al., 1991; Johnson *et al*., 1994). Other parasite proteins such as the ATP-binding cassette protein (PfGCN20) and a fatty acid acyl coenzyme A synthetase-1 (PfFACS-1) are delivered to less-defined membranous structures in the host cytosol (Bozdech *et al*., 1998; Matesanz et al., 1999). The distribution of the parasite proteins in infected erythrocytes is shown in figure 4.

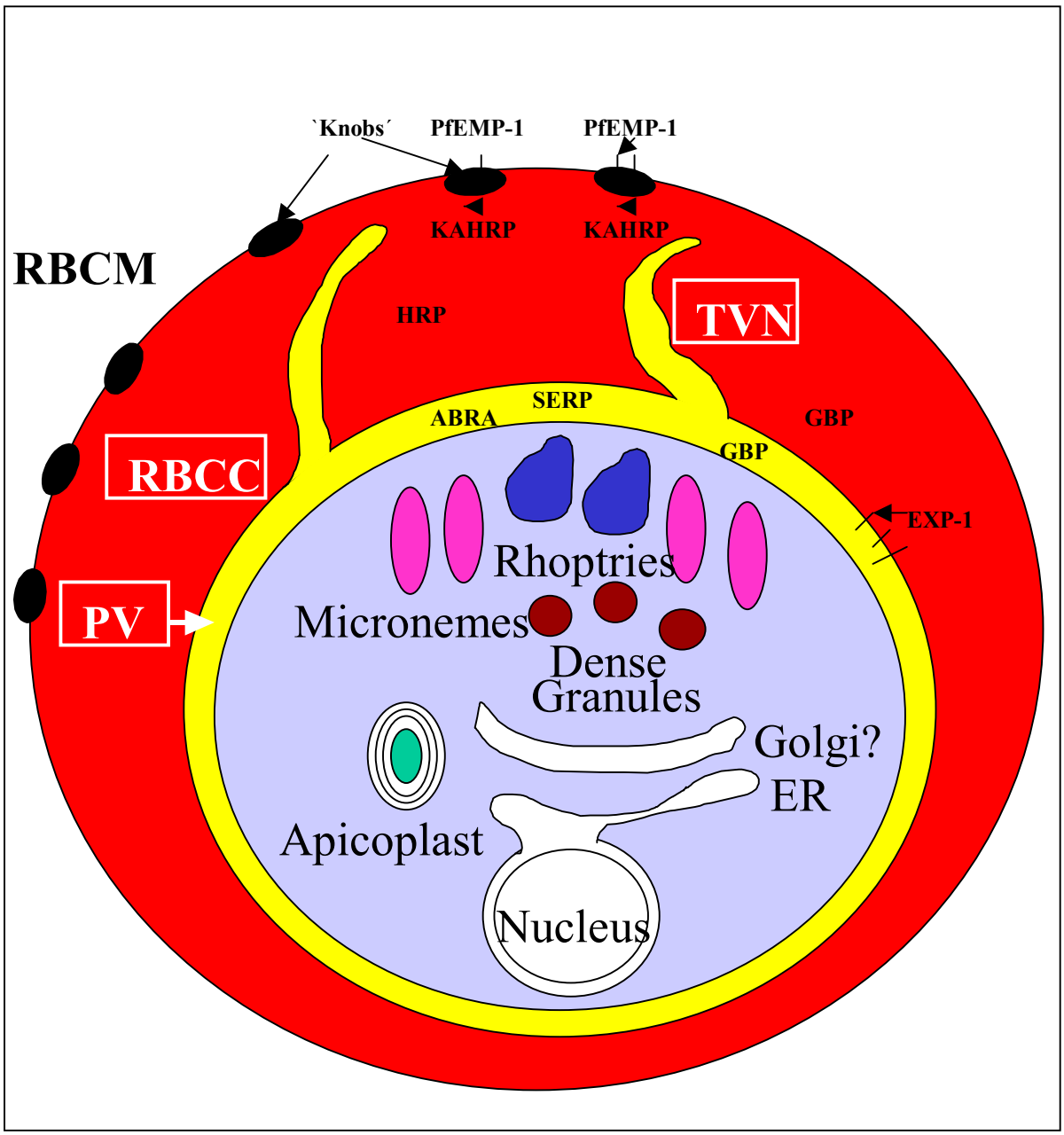


Figure 4. Destination of secreted parasite proteins in *Plasmodium falciparum* infected erythrocytes. The proteins are targeted to secretory organelles within the parasite including the rhoptries, micronemes, dense granules and the apicoplast. The proteins are also exported out of the parasite to the PV, PVM, TVN, erythrocyte cytosol (RBCC) and the erythrocyte plasma membrane (RBCM).

In eukaryotes the main mechanism to export proteins is to allow their recruitment into the secretory pathway. The first step requires a polypeptide signal that targets the protein to the ER membrane. The signal targets nascent or complete proteins from the cytosol to translocation sites in the membrane of the ER. After membrane insertion, signal sequences can be cleaved from the

precursor protein by a membrane bound signal peptidase. The characteristic feature of signal sequences is a hydrophobic core (h-) region comprising, for cleavable signal sequences, six to fifteen amino acid residues. Analysis of signal sequences mutants showed that this h-region is the most essential part required for targeting and membrane insertion (von Heine, 1985). The h-region is flanked on its C-terminal side by a polar (c-) region which often contains helix-breaking proline and glycine residues as well as small uncharged residues in positions –3 and –1 that determine the site of signal peptide cleavage (von Heine, 1990). On its N-terminal side, the h region is flanked by a rather polar n-region, which usually has a net positive charge. Comparative analysis of a large number of signal sequences reveals substantial variability in their overall length, ranging from 15 to more than 50 amino acid residues (von Heine 1986). The n-region contributes most to the variation in overall length. Signal sequences can be located within a protein or at its C-terminal end (Kutay *et al*.,1995). Additional targeting signals are used to divert proteins to other compartments (e.g. to the lysosome or to a regulated secretory compartment, (Keller and Simons 1997).

Analyses of protein sequences of *P. falciparum* proteins that are exported out of the parasite have shown some unusual characteristics. The ER located protein, PfERC, has a classical hydrophobic N-terminal signal sequence (La Greca *et al*.,1997). Similarly, the S-antigen and SERP that are exported to the parasitophorous vacuole have N-terminal hydrophobic signals (Knapp *et al*., 1989b). Proteins destined for the PPM, such as the merozoite surface protein 1 (MSP1) (Holder *et al*., 1985), or EXP-1 which is present in the PVM (Kara et al.,1990; Günther et al., 1991) also have classical N-terminal signal sequences.

In eukaryotes, after translocation of the precursor protein, the N-terminal stretch of hydrophobic amino acids is cleaved by a signal peptidase within the ER lumen. This cleavage site is often N-terminal to an acidic or basic residue and often has small amino acids (glycine or alanine) at positions –3 and –1 (von Heijne,1986) A similar system appears to operate in Plasmodium and the secretory signal of Exp-1 has been shown to be cleaved by the malaria parasite at a site adjacent to aspartate 23 (Kara *et al*., 1990). EXP-2 is cleaved at a site N-terminal to aspartate 25 (Fischer et al., 1998).

However, a number of parasite proteins that are directed across the PPM and PVM to the host cell cytosol do not have classical N-terminal signals. GBP and KAHRP and RESA have a stretch of hydrophobic amino acids starting 20-50 amino acids from the N-terminus (Favaloro *et al*., 1986; Kilejian *et al*., 1986; Nolte *et al*., 1991). This internal hydrophobic signal does not appear to be recognized by the translocation machinery of higher eukrayotes as it has been shown that neither KAHRP nor protein 41-2 are translocated across the ER membrane in cell-free

systems using mammalian microsomes (Hinterberg *et al*.,1994). Another group of proteins are exported into the erythrocyte cytosol but they do not have an N-terminal signal sequence, i.e. no hydrophobic patch within the first 100 amino acids, these include PfGCN20 (Bozdech *et al*., 1996), *falciparum* erythrocyte serine threonine kinase (FEST) and Pfsar1p (Albano et al 1999). The pathway of export of these proteins is unknown. PfEMP1 which is exported and inserted into the erythrocyte membrane does not have an obvious N-terminal signal sequence (Taylor *et al*., 1987; Baruch *et al*., 1995).

The study of protein trafficking to organelles within the parasite especially the apicoplast, has also provided interesting results. The apicoplast is a secondary endosymbiont and has attracted attention due to its evolutionary origin and also as a novel drug target since the organelle is unique to the parasites and is absent in the hosts (McFadden and Roos, 1999). As with a primary endosymbiont, many proteins targeted to the apicoplast are nuclear encoded and need signals for their proper delivery to the organelle. The first direct evidence that an ER type signal sequence results in protein release at the parasite plasma membrane came from studies on protein transport to the apicoplast (Waller *et al*., 2000). Since the parasite is a secondary endosymbiont, transport to the apicoplast is a two step process mediated by a bipartite signal that comprises an ER type secretory signal followed by a plastid targeting signal (SS-PTS). It has been demonstrated that a bipartite signal is necessary in plastid targeting in secondary endosymbiont (Sulli and Schwartzbach, 1995; Wastl and Maier, 2000). This signal was shown to be both necessary and sufficient for transport of apicoplast proteins in *T. gondii* and *P. falciparum* (Waller *et al*., 1998; 2000). In *P. falciparum* the ER signal alone targeted the peptide into the PV.

In mammalian cells, once a soluble protein has entered the secretory pathway, the failure to retain it in a compartment or target it to an endosomal or lysosomal destination, results in its release into extra cellular medium (Pfeffer and Rothman, 1987). This is termed the default secretion. Components of this `classical´ vesicle mediated secretory pathway for the export of proteins are present within the cytoplasm of the malaria parasite and appear to be involved in the transport of proteins to the parasite plasma membrane. Homologues of a number of trafficking components have been found including the ER molecular chaperone, Pfgrp (Kumar *et al*., 1991), the *P. falciparum* ER calcium-binding protein (PfERC) which is a reticulocalbin homologue (La Greca *et al*., 1997), a homologue of the KDEL- binding protein, PfERD2 (Elmendorf and Haldar 1993) and homologues of trafficking associated GTPases, PfRab6 (Alves de Castro *et al*., 1996; Van Wye *et al*., 1996) and PfRab11 (Langsley and Chakrabarti, 1992). Despite evidence for a classical secretory pathway, *P. falciparum* lacks a stacked cisternal Golgi (Van Wye *et al*., 1996), its Golgi apparatus shows unusual dynamics (Elmendorf and Haldar, 1994) and there is no

conclusive evidence for N-glycosylation (Dieckmann-Schuppert *et al*., 1993). This makes it difficult to detect secretory transport by following post-translational protein modifications or movement through the Golgi. However recently, a single gene coding for the enzyme N-acetylglucosamine-1-phosphate transferase, the first enyzme in the N-linked glycosylation pathway has been shown to present in the recently published *P. falciparum* genome (Gardner *et al*.. 2002).

In eukaryotic cells, brefeldin A (BFA) inhibits the secretion of polypeptides of proteins by redistributing the *cis*-Golgi components in the ER (Lippincott-Schwartz *et al*.,1989). In mammalian cells, secretion of proteins can be arrested at the cis-Golgi level by incubating the cells at 15° C (Saraste and Kuismanen 1984). The best evidence for the existence of a classical secretory pathway is the inhibition of protein secretion in infected erythrocytes with BFA and at low temperature (Benting *et al*.,1994).). In the ring stages, brefeldin A can be added for extended periods of time, which arrests parasite development in the ring stage (Benting *et al*.,1994). In later stages treatment of parasites with BFA for more than 2 hours is lethal. The drug BFA disrupts the Golgi and proteins are redistributed to the ER, possibly by the retrograde transport mechanism (Lippincott-Schwartz et al., 1990). The accumulated evidence confirms that the export parasite proteins involve a classical secretory pathway and the effects of Brefeldin A are rapidly and fully reversible upon removal of the drug. However there are reports of some parasite proteins being secreted despite the presence of BFA (Elmendorf *et al*., 1992).

At a molecular level, the drug interferes with the exchange of GDP for GTP on ADP-ribosylation factors (Donaldson *et al*., 1992; Helms and Rothman, 1992 Randazzo *et al*., 1993). Recently the target of BFA, namely plasmodial ARF-GEF was detected in a BFA resistant parasite line obtained after selective pressure. The mutant protein has a single methionine-isoleucine substitution in the sec 7 domain, a conserved structure of guanine nucleotide exchange factors (ARF-GEF). The mutation confers resistance to the drug (Baumgartner *et al*., 2001).

The observation that many paraiste proteins which are transported beyond the PVM do not conatin hydrophobic N-terminal signal sequences has led to the suggestion that these proteins are secreted via an laternative secretory pathway (Wiser *et al*., 1997). However this hypothesis has been challenged by others (Mattei *et al*., 1997), and the mechanism of translocation of proteins to destinations beyond the confines of the parasite is still under investigation.

1.4 The parasitophorous vacuole- a transit compartment?

The final step in the secretory pathway is the fusion of transport vesicles with the plasma membrane and the release of soluble proteins into the extracellular space or, in the case of

membrane proteins insertion into the plasma membrane. Some parasite proteins such as the serine rich protein (SERP), are released into the vacuolar space, whereas other proteins, such as GBP are transported beyond the vacuolar membrane into the erythrocyte cytosol. Initially two models for protein transport had been proposed to explain protein transport across the parasite plasma membrane and the parasitophorous vacuole membrane (Howard *et al.*,1987; Haldar, 1996).

In the single-step model, proteins destined for the parasite plasma membrane are targeted to specific sites, where the parasite plasma membrane fuses with the PVM. The proteins are then translocated across both membranes in single step. This model would suggest the presence of two set of secretory vesicles in the parasite, those with a vacuolar destination and others destined for locations beyond the PVM. To date there is no experimental evidence for the existence of transport vesicles that differ with respect to the final destination of their cargoes.

In the two-step model, proteins are secreted into the vacuolar space in a first step and proteins destined for the erythrocytic cytosol are subsequently translocated across the PVM in a second step. Since secretory proteins are already in a folded conformation, the second step of translocation across the PVM would not occur spontaneously. Therefore a prediction would be that proteins destined for the erythrocytic cytosol have no complex conformation or that, alternatively, chaperone-like molecules in the vacuolar space assist in the unfolding of such polypeptides prior to translocation (Lingelbach, 1997). In contrast to the single step model, proteins destined for the vacuolar space and erythrocyte cytosol can be transported within the same vesicle, and sorting would occur in the vacuole (Lingelbach, 1997).

In contrast to the above, an alternative model proposes that the sorting of proteins that are normally exported beyond the parasite (to the parasitophorous vacuolar membrane, the host cell cytoplasm and the host cell plasma membrane) takes place at the level of diversion into a specialized secretory organelle (Wiser *et al*., 1997). The authors showed that proteins exported to the red cell accumulate in a specialized region near the periphery of the parasite cytoplasm in *Plasmodium berghei* and *Plasmodium chaubadi* in the presence of brefeldin A. They proposed that this compartment might represent a secondary ER, and that proteins targeted to destinations within the parasite or the parasite plasma membrane traffic through the classic ER-Golgi pathway, while proteins destined for export are targeted to and translocated across the membrane of the secondary ER from which they are transferred across the parasite membrane (Wiser *et al*., 1997; Wiser *et al*., 1999). The conclusion was based on the observation that this site appeared to be distinct from the perinuclear location in which a other exported parasite proteins accumulate upon BFA treatment (Benting et al.,1994;Wiser *et al*., 1999), leading to the conclusion that two independent secretory pathways operate in parallel in the parasite.

BFA is known to induce multiple morphological changes in mammalian cells (Lippincott-Schwartz *et al*., 1990). These include the redistribution of Golgi components into the ER (Lippincott-Schwartz *et al*., 1990; Wood and Brown, 1992) and the formation of ´BFA bodies´, derived from ER membranes (Orci *et al*., 1993). BFA also induces the fusion of the *trans*-Golgi network and early endosomes; this fused compartment remains functional, capable of recycling and delivering material (Lippincott-Schwartz *et al*., 1990; Wood and Brown, 1992). Therefore, BFA can induce different morphological changes in distinct subcellular compartments depending on cell type (Pelham, 1991). And in the absence of sufficient data to support the presence of the secondary ER, as proposed by Wiser *et al*., (1997), the BFA-induced peripheral localization of exported proteins may reflect altered membrane organization induced by the drug, rather than a specialized organelle.

Using streptolysin O permeabilized *P. falciparum* infected erythrocytes Ansorge *et al*.,1996, provided the first evidence that translocation of GBP across the parasite plasma membrane and the parasitophorous vacuole membrane is a two step process. In the first step, the protein is secreted into the vacuole. The second step translocates the protein across the PVM and one reason for the ATP-requiremnt may be an ATP-dependent putative translocator located within the PVM (Baumeister et al., 1999). In contrast the serine rich protein (SERP) is secreted into and retained within the vacuolar space (Knapp *et al*., 1989b; Ragge *et al*.,1990). The two-step translocation mechanism has since been confirmed using foreign reporter proteins (Wickham *et al*., 2001; Burghaus and Lingelbach 2001).

Therefore a mechanism appears to exist which discriminates between proteins that are destined to reside within the PV and those that are targeted to specific locations outside the vacuole. Therefore the PV is a compartment where protein sorting occurs. There are resident vacuolar proteins such as SERP whereas other proteins such as GBP and KAHRP transit through the vacuole across the PVM to the erythrocyte cytosol. In the case of GBP the process has been shown to be dependent on the availability of ATP (Ansorge *et al*., 1996). If translocation of proteins across the PVM occurs via a default pathway, a mechanism that retains vacuolar resident proteins would have to be postulated, involving a retention signal. Alternatively, translocation across the PVM could be mediated by a specific signal contained within an amino acid sequence motif in the proteins that are translocated across the PVM. In the case of GBP there is no evidence for the cleavage of the putative signal sequence during or after its translocation across the PVM.

Experiments using transfected parasites expressing chimeric proteins comprising the N-terminal of a number of secreted *Plasmodium falciparum* proteins fused to reporter proteins have been used to try to characterize the secretion pathway in the infected erythrocytes. The

translocation of a hybrid protein comprising the N-terminal sequence of KAHRP fused to the green fluorescent protein GFP has been studied. The translocation is postulated to occur in two steps, the first step being co-translocation into the lumen of the ER, followed by cleavage of the signal sequence at the predicted amino acid sequence, then trafficking to the parasitophorous vacuole. Outward transport from the PV presumably requires recognition of the appropriate sequence element(s) within the histidine region of KAHRP (Wickham *et al*., 2001). Further studies showed that the N-terminal sequence of KAHRP, which is considered to be a non-classical N-terminal signal sequence allows for protein release at the PPM (Wickham *et al*., 2001). The data further shows that in the presence of exon I + exon II there is transport across the PV, while exon I results in the release of the protein into the PV. Therefore it is possible that the sequences in exon II act in conjunction with exon I to facilitate the translocation. However it has not been demonstrated that exon II alone does confer translocational activity (Wickham *et al*., 2001). Exon II is histidine rich but as the sequences of many exported proteins are not histidine rich, it is unlikely that these sequences provide the signal for the export to the erythrocyte. The authors conclude that proteins destined for a location within the erythrocyte require additional sequences for translocation across the PVM. However in the case of KAHRP this can only be confirmed by fusing the Exon II sequence to a reporter protein and analyzing the subsequent localization. In contrast another study proposes that no specific protein sequences are required for translocation across the vacuolar membrane (Burghaus and Lingelbach, 2001). Using transfected parasites expressing a chimeric protein comprising the N-terminal of the *Plasmodium falciparum* exported protein-1, (which has a classical signal sequence) fused to luciferase, the reporter protein was exported into the PV as well as to the erythrocyte cytosol (Burghaus and Lingelbach, 2001). Despite these different observations it is clear that protein sorting occurs within the vacuole and the mechanisms that determine retention and forward sorting still need to be addressed.

Recent work in *P. falciparum* suggests that import into the apicoplast is stage regulated and that apicoplast-targeted proteins can be are released into the PV during the early stages of the development of the parasite. And more intriguing the plastid-targeting signal can signal retrograde transport from the PV to the apicoplast (Cheresh *et al*., 2000). This suggests that the PV is an important compartment for sorting proteins that are destined for export to the host erythrocyte and also for the endocytic uptake of some proteins back into the parasite. All these studies confirm that the parasitophorous vacuole is an important transit and sorting compartment in the secretory pathway in *Plasmodium falciparum.*

In the recently published genome sequence of *P. falciparum* the authors tried to identify additional proteins that may have a role in protein translocation and secretion (Gardner *et al*.,

2002). The *P. falciparum* database was searched with *S. cerevisae* proteins with Gene Ontology assignments for proteins involved in protein export. The authors identified potential homologues of important components of the signal recognition particle, the translocon, the signal peptidase complex and many components that allow vesicle assembly, docking and fusion, such as COPI and COPII, clathrin, adaptin, v- and t-SNARE and GTP binding proteins. The presence of Sec62 and Sec63 orthologues raises the possibility of post-translational translocation of proteins as found in *S. cerevisae* (Gardner et al., 2002). However as discussed earlier, the unusual features of the secretory pathway of the parasite, where some proteins which lack signal peptide sequences are targeted beyond the PVM still remains to be solved. With the expanded list of protein-transport-associated genes identified in the *P. falciparum* genome new approaches should be developed to further elucidate the intra-and extracellular compartments of the parasites protein transport system.

1.5. Additional functions of the parasitophorous vacuole.

It is clear that the vacuolar space contains specific parasite proteins which are not found in other locations in infected erythrocytes and thus it represents a compartment which is clearly distinct from the erythrocyte cytosol and from the parasite cytosol respectively (Lingelbach, 1997). The PV represents a compartment that acts as an interface between the parasite and the host cell. Apart from the proteins with chaperonic activity that may be involved in protein sorting, it is likely that other proteins with different functions that are essential for development and survival of the parasite within the infected erythrocyte are also found within the vacuole. The proteins found within this compartment can be postulated to be involved in nutrient acquisition among other functions. The intracellular parasites acquire nutrients from either the host cell cytosol, the extracellular medium or from both and the proposed mechanisms of acquisition are discussed in 1.2.

A defining feature of merozoite release at the end of the asexual stage of development is its susceptibility to protease inhibitors (Lyon and Haynes 1986). To better characterize the events of merozoite escape at the end of erythrocytic schizogony, purified *P. falciparum* schizonts were cultured in the presence of a csyteine protease inhibitor L-transexpoxy-succinyl-leucylamido-(4-guanidino)butane (designated E64). The result was the accumulation of the merozoites within a single membrane supposedly derived from the PVM (Salmon *et al*., 2001). Importantly, structures identical in appearance can be detected in untreated cultures at low frequency. These studies propose that exit is a two-step process, where merozoites enclosed within the PVM first exit from the from host erythrocyte and then rapidly escape from the PVM by a proteolysis-dependent mechanism (Salmon *et al*., 2001). Therefore the vacuole is most likely to contain proteins with

proteolytic activities, that are involved in the release of the merozoites at the end of the asexual intraerythrocytic stage of development. Indeed, P126 (SERA) also known as SERP has been shown to be a major component of the parasitophorous vacuole and is proteolytically processed upon release of merozoites into the culture medium (Delpace *et al*.,1988).

As described in 1.1.2 above the process of erythrocyte invasion involves several steps that are mediated by proteins on the surface of the merozoite. Merozoite surface proteins are important since they mediate the initial recognition and attachment to the erythrocytes, secondly the proteins are vaccine candidates because they are exposed in the plasma and therefore are potential targets for antibodies that prevent merozoite invasion or lead to opsonisation and parasite killing. The best characterized merozoite surface protein is MSP-1 which is synthesized during schizogony as a large precursor protein of approximately 195 kDa (Holder and Freeman,1984). The protein is anchored by glycosylphosphatidylinositol to the parasite plasma membrane (Gerold *et al*., 1996). The precursor protein is proteolytically processed into smaller fragments in a two-step procedure characterized by primary and secondary processing events within the vacuolar space (Holder *et al*., 1992). Primary proteolytic processing occurs at the end of schizogony. It produces four major polypeptide fragments of approximately 83, 28-30, 38 and 42 kDa (denoted MSP-1_{83}, MSP-1_{30}, MSP-1_{38} and MSP-1_{42}) (Holder *et al*.,1987). The 42 kDa C-terminal fragment is further processed to 33 and 19 kDa fragments at the time of merozoite invasion (Blackman *et al*., 1991). This secondary processing is catalyzed by a Ca^{2+}-activated serine protease, which results in the shedding of a soluble MSP-1 complex (Blackman and Holder, 1992). The remaining glycosyl-phosphatidylinositol-anchored 19 kDa (Gerold *et al*., 1996) contains two epidermal growth-like modules (Blackman *et al*., 1991) and is carried into the erythrocyte upon merozoite invasion (Blackman *et al*.,1990). This fragment has been shown to be the target of invasion inhibiting antibodies (Blackman *et al*.,1990). Therefore the vacuolar space components are important in the processes that lead to the release of merozoites at the end of the asexual cycle and in the proteolytic processing of MSP-1, which is a prerequisite for the successful reinvasion of new erythrocytes.

1.6 From genomics to proteomics

The genomic era of biomedical research has provided massive amounts of information on DNA sequences from many species including *Plasmodium falciparum* whose entire genome was recently published (Gardner *et al*., 2002). This wealth of data has been annotated and is continuing to be analyzed by new bioinformatic algorithms and the main biomedical research activities are shifting to the proteome, the protein complement of the genome. Proteomics may be defined

broadly as the study of all proteins (and alternatively spliced varieties) expressed by a genome, including the concomitant isolation, identification, structural determination (with post-translational modifications), interaction with partners (other proteins, lipids, nucleic acids) development time courses, effects on biological responses, and functional properties (Kenneth and Lee 2002). Proteins are involved in virtually all biological processes, including enzyme catalysis, ion transport, immune protection and signal transduction. In practice, a proteomic approach will focus on limited aspects (e.g. identification and interactions) of this broad menu of protein attributes or may focus on a limited proteome from cell fractionation such as the complement related to a cellular organelle (e.g. mitochondrion) or component (e.g. nucleosome) or compartment (e.g. parasitophorous vacuole). In the recent years new and sophisticated methods have been developed for proteome analysis, because it is at the protein level where biological processes including those relevant for disease can be modulated and studied (Broder and Venter 2000; Banks *et al*., 2000; Chambers *et al*., 2000).

Two dimensional polyacrylamide gel electrophoresis (O´Farrell, 1975) is one of the most widely accepted methods for separating and comparing complex mixtures of thousands of proteins found in biological samples. By separating proteins on the basis of two independent variables isoelectric point and molecular mass (pI and M_r), 2-DE is capable of resolving thousands of proteins expressed simultaneously in a particular cell or tissues (Klose and Kobalz 1995; Berkelman and Stenstedt 1998). This makes 2-D PAGE the core method in comparative proteome studies. In particular, the introduction of immobilized pH gradients (IPG's) has made the method robust and reproducible (Bjellqvist *et al*., 1982). The resolution of the target proteins can be further enhanced by selecting the appropriate pH range for IEF, or by adjusting the percentage of polyacrylamide in the 2-D gel (Berkelman and Stenstedt 1998).

Protein identification by peptide mass fingerprinting (PMF) usually involves digestion of gel separated proteins with trypsin, followed by mass measurement of the resulting peptides. Mass spectrometry is the key element of proteome analysis (Reviews by Petersons and Aebersold 1995; Yates, 1998; Quadroni and James 1999; Gevaert and Vandekerckhove 2000 and Roepstorff, 2000). Basically a mass spectrometer could be considered as an instrument with three different parts: (a) the sample loading side;(b) the ion path, and (c) the detection side. The polypeptides can be loaded either by spraying the sample into the mass spectrometer out of a capillary with a micro- or nano-outlet or by desorbing and ionizing proteins from a solid surface with laser shots.

Matrix-assisted laser desorption ionization (MALDI), developed by (Karas and Hillenkamp 1988) is a `soft ionization´ method that is used to generate gas phase protonated molecules including peptides for mass spectroscopy. The trypsin digested protein sample is mixed with a

large excess of matrix material usually a carefully selected weak organic acid, alpha-cyano hydroxycinnamic acid. A submicroliter volume of the mixture is loaded on a metallic surface and the mixture is allowed to dry. The resulting solid is then irradiated by nanosecond laser pulses, from small nitrogen lasers with a wavelength of 337 nm. This is the absorbance range for the matrix surface and for an unknown reason, a proton moves from the weak organic acid to the protein which gets "vaporized" at the same time. The finally volatile and positively charged polypeptides are accelerated to affixed amount of kinetic energy and they enter a flight tube. The smaller ions have a higher velocity and they are recorded on a detector before the larger ones, producing the time of flight (TOF) spectrum which give the masses and abundance of the flying ions.

Peptide mass mapping with MALDI-TOF-MS (Joubert-Caron *et al*., 2000) remains a practical method for rapidly identifying and partially characterizing numerous proteins in proteomic studies. The positive identification of proteins requires the measurement of enough peptide masses to obtain a definitive match with sequence information recorded in protein databases. Bioinformatics plays an essential role in all proteomic work and numerous proteomic virtual tools have been developed and many of them are accessible through the world wide web e.g.(http://www.expasy.ch/tools/;http://www.prospector.ucsf.edu/; www.matrixsciences.com; http://prowl.rockefeller.edu/cgi-bin/ProFound, http://www.ncbi.nlm.nih.gov/). The ultimate goal of proteomics is to complement genomic efforts to unravel biological processes and function. The growing awareness that the PV forms an important interface between parasite and host cell cytosol highlights the significance the vacuolar proteome. The recent development of technical and bioinformatic tools should greatly facilitate the identification of vacuolar proteins. A major obstacle is the small volume of the PV, which constitutes only a 1:10,000 of the infected erythrocyte. Therefore the first and main objective of this project is the development of an experimental strategy that allows the selective identification of soluble vacuolar proteins, avoiding contamination with soluble proteins, either from the host erythrocyte or from the parasite cell.

1.7 Biotin labeling of proteins

Biotinylation is a highly sensitive method for labeling and analyzing proteins and the method has been used to characterize cell wall proteins of *Candida albicans* (Casanova *et al*., 1992) *Borrellia burgdorfi* (Luft et al.,1989) and *Helicobacter pylori* (Sabarth et al., 2002). Biotinylation is one of the most commonly used means of labeling proteins for easy detection, immobilization, and purification. The utility of biotin as a tag is a result of its interaction with egg white protein avidin and its bacterial analogue streptavidin. The two proteins share a similar

high affinity ($K_a \sim 10^{15} M^{-1}$) for the vitamin biotin, this is several orders of magnitude greater than the constants for antigen –antibody or agglutinin- sugar complexes (Green,1990) and the avidin-biotin system has gained a great importance over the years both as a means of studying bio-recognition phenomenon and as a tool for general application in many biotechnological applications. This powerful approach has facilitated the localization, identification and assay of an almost unlimited number of biological molecules (Wilchek and Bayer 1988; Wilchek and Bayer 1990). Other than the high affinity constant, two other properties of this specific, ligand-protein interaction make it convenient for its applications. The biotin can be attached to target macromolecules by a varied of reactive derivatives (Liu and Leonard, 1979) frequently without altering the biological activity of the biotinylated moiety and secondly mixed conjugates may be prepared which consists of avidin chemically linked to a wide spectrum of perceptible probes (e.g. Feritin, fluorescein, alkaline phosphatase, horseradish peroxidase, and rhodamine), as well as to lectins or solid matrixes (Bayer and Wilchek, 1974).

Pierce, Rockford IL, USA has developed several biotin derivatives including sulfosuccinimidyl-6-(biotinamido)hexanaote (sulfo-NHS-LC-biotin) and Sulfosuccinimidyl-2-(biotinamido)Ethyl-1-3dipropionate (sulfo NHS-SS-Biotin). These water-soluble biotin analogues react with primary amines in amino acids, mainly the epsilon group of lysine of proteins. They have spacer arms between the biotin and the reactive ester molecules to reduce stearic hindrance when binding several biotinylated molecules to one avidin complex. The sulfo NHS-LC-Biotin has a hydrocarbon spacer arm whereas the sulfo NHS-SS-Biotin has a disulfide bond in the spacer arm. The biotin can be separated from the reactive group when the spacer arm is cleaved using reducing agents. This is ideal for use in reversible biotinylation reactions and in purification procedures where a reducing agent is used to release the labeled molecule from biotin bound to avidin or streptavidin fixed to a solid matrix.

The reaction of the primary amines with *N*-hydroxysulfosuccinimide (NHS) esters is best at neutral pH values and above. This is due to the fact that the target of the NHS ester is the deprotonated form of the primary amine. The amine reacts with NHS ester by nucleophilic attack forming a stable amide bond. The product of the reaction, *N* –hydroxysulfosuccinimide, is released and the biotin conjugate remains bonded to the protein as shown in figure 5. These derivatives are coupled to a $-SO_4$ group, they are hydrophilic and they cannot permeate lipid bilayers (Pierce, USA) and they have been used in selective labeling to identify surface proteins in different organisms (Luft *et al*., 1989; Manuel *et al*., 1992; Apoga *et al*., 2001;). The biotin labeled proteins can then be detected in western blot analysis by using alkaline phosphatase or horseradish peroxidase conjugated streptavidin. Recently Nyalwidhe *et al*., (2002), showed that

these derivatives do not enter non-infected erythrocytes but they enter trophozoite-infected erythrocytes probably through the novel permeation pathways since biotinylation of the cytoplasmic face peripheral membrane protein, spectrin which closely associates with the host cell cytoskeleton is inhibited by furosemide an inhibitor of the NPPs.

1.8 Permeabilization of *P. falciparum* infected erythrocytes with Streptolysin O

Pore-forming bacterial toxins are increasingly used by cell biologists as tools for controlled permeabilization of cell membranes (Bhakdi *et al*., 1993). The advent of using streptolysin O (SLO) among other bacterial pore forming toxins to permeabilize the erythrocyte membrane without affecting the PVM (Ansorge *et al*., 1996; Ansorge *et al*., 1997; Lingelbach, 1997) has made it possible to sequentially fractionate infected erythrocytes and thus investigate protein transport and the localization of proteins in different compartments, for example, the erythrocyte cytosol, the parasitophorous vacuole and the parasite cytosol. Binding of the toxin occurs via interaction with cholesterol. SLO pores are generated when membrane-bound toxin monomers collide via lateral aggregation to form high-molecular-weight oligomers (Bhakdi et al., 1985). These can be seen in electron microscope as partially or fully circularized ring structures with heterogeneous internal diameters (Bhakdi et al., 1985). The pores formed by these polymers have diameters exceeding 30 nm, which allow free passage of substances thus allowing the introduction of proteases, drugs and many other substances making it possible to study their effect on the physiology of the parasite in a defined system. The toxin does not affect the PVM in infected erythrocytes.

The study of the topology of the PVM has also been achieved with respect to two exported membrane protein exp-1 and exp-2 that are associated with the PVM (Ansorge *et al*., 1996; Ansorge *et al*., 1997) using SLO permeabilization. In addition the study of the mechanisms of translocation of GBP, an abundant parasite protein which is exported across the PVM into the host cell cytosol has also been achieved after the use of streptolysin O,. After the permeabilization process, host cell cytosol is extraction completely and the extra cellular milieu can be reconstituted and the study of the mechanisms involved in the translocation of proteins across the PVM in the selectively permeabilized infected erythrocytes is facilitated (Ansorge *et al*., 1996; Baumeister *et al*., 1999).

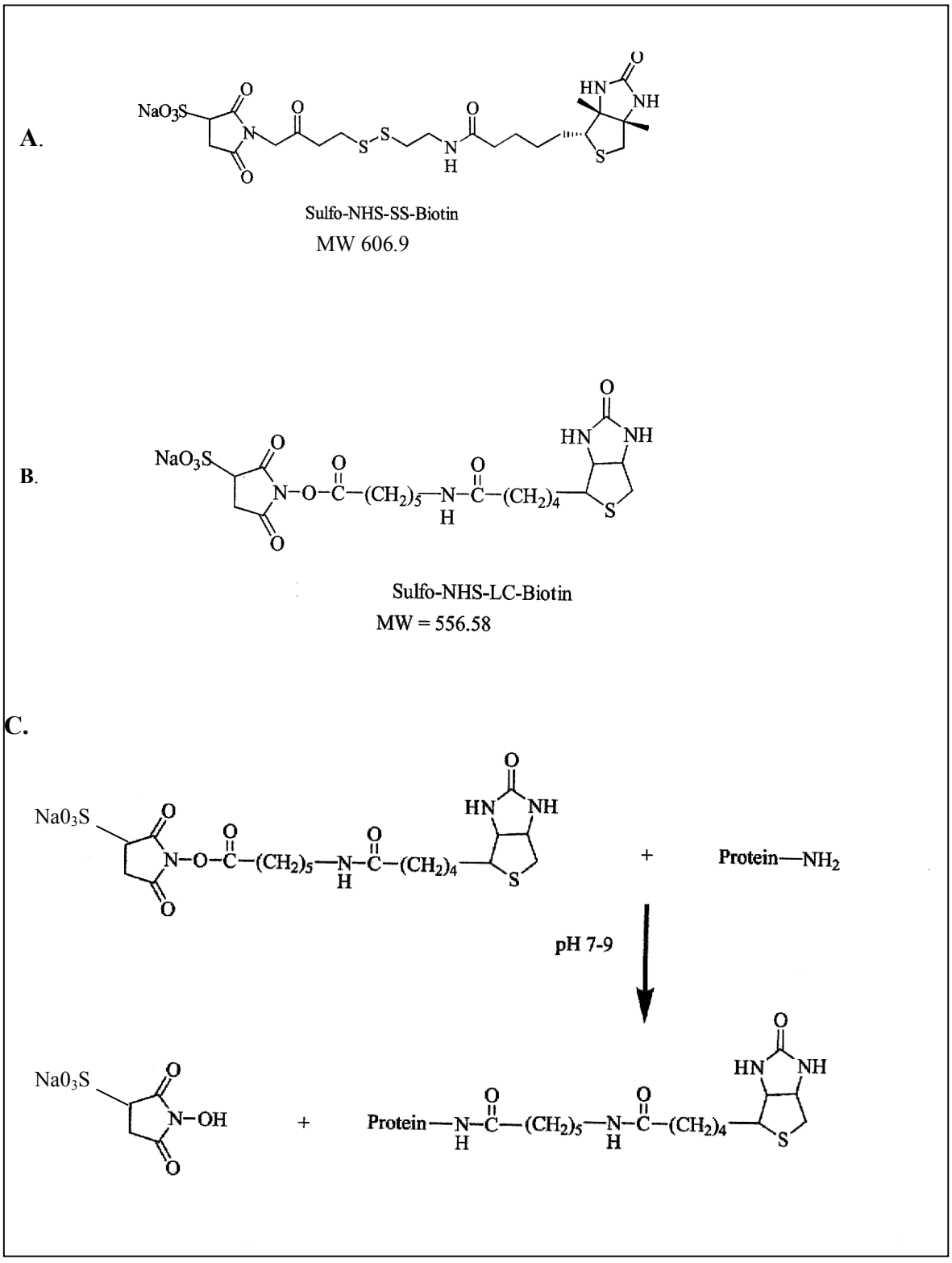


Figure 5. Structures of biotin derivatives and their mechanism of reaction with proteins. The sulfo-NHS-SS-biotin has a linker with a disulphide bond between the biotin molecule and the reactive ester containing group, which can be cleaved by reducing agents. The linker in the sulfo-NHS-LC-biotin is non-cleavable.

1.9 General objectives:

The objectives of this study were

i). To identify soluble *Plasmodium falciparum* proteins in the parasitophorous vacuole.

ii) To characterize the proteins identified under (i).

2. Materials and Methods.

2.1 Materials

2.1.1. Equipment

Blotting Apparatus	Phase	Lübeck
Confocal Microscope	Leica Laser Scanning TCS	
Incubator	Heraeus	Hanau
Film	Cassetes	Rego
Gel Drier	Biotech Fischer	Schwerte
IEF Apparatus Multiphor II	Pharmacia Biotech	Sweden
Labofuge III	Heraeus	Hanau
Lab roller Spiromix	Denley	UK
Magnetic stirrer	Ika Combimag RCH	
MALDI- TOF	Voyager- DE STR	Framingham, MA
Orthoplan Microscope	Leitz	Wetzlar
pH- Meter 766	Calimatic	
Power supply 2130	LKB Biochrom	St.Albans, UK
Spectrophotometer	Beckman	Irvine, USA
Speedvac centrifuge	Uni-Equip	Martinsried
UNO- Thermoblock	Biometra	Göttingen
Vortexer REAX 2000	Heidolph	
Waterbath 2219 Multitemp II	LKB	Bromma

2.1.2 Disposable Materials

Culture flasks	Greiner	Frickenhausen
Eppendorf reaction tubes	Eppendorf	Hamburg
Falcon tubes	Greiner	Frickenhausen
Films	New RX NIF, Fuji	Japan

Glass slides	IDL	Nidderau
IPG IEF strips	Pharmacia Biotech	Sweden
Micro concentrator	Millipore Corp.	Bedford USA
Nitrocellulose membrane	Schleicher & Schuell	Dassel
Pipettes	Gilson	Villiers-le Bel, France
Pipette tips	Greiner	Frickenhausen
Whatman Paper	Schleicher & Schuell	Dassel
Zip Tip gel loading column	Eppendorf	Hamburg

2.1.3 Chemicals and reagents

Acetic acid	Roth	Karlsruhe
Acetone	Roth	Karlsruhe
Acetonitrile	Merck	Darmstadt
Ammonium hydrogen carbonate	Sigma	
Ammonium persulfate	Roth	Karlsruhe
ASB-14	Calbiochem	
BCIP (5-Bromo-4-chlor-3-indolyphosphat)	Biomol	Hamburg
Bovine Serum Albumin Fract.5 BSA	Roth	Karlsruhe
Brilliant blue G-colloidal concentrate	Sigma	
CHAPS	Sigma-Aldrich	
Coomassie Brilliant blue R 250	Roth	Karlsruhe
Cyano-4-hydroxycinnamic acid	Sigma	
DTT	Fluka	
ECL- Reagent	AmershamPharmacia Biotech	Sweden
EDTA	Merck	Darmstadt
Ethanol	Roth	Karlsruhe
Formic acid HPLC grade	Sigma	
Gentamycin	Gibco	Eggenstein
Giemsa solution	Merck	Darmstadt
Glycerin	Roth	Karlsruhe
HEPES	AppliChem	Darmstadt

Hydrochloric acid	Roth	Karlsruhe
Iodoacetamide	Sigma	
IPG buffer	Amersham Pharmacia Biotech.	
Isopropanol	Roth	Karlsruhe
L- [^{35}S] Methionine	ICN	Irvine, California
Magnesium Chloride	Roth	Karlsruhe
Methanoic acid	Roth	Karlsruhe
Methanol	Roth	Karlsruhe
Milk powder	Fluka	
Monomeric avidin agarose beads	Pierce	USA
Nitroblue Tetrazolium Chloride	Biomol	Hamburg
Oligo R3	AppliedBiosytems	
Ponceau S pure	Serva	Heidelberg
Poros R2	AppliedBiosytems	
Potassium Chloride	Roth	Karlsruhe
RPMI 1640 medium	Life Technologies Inc.	
Sequenzyme peptide markers	AppliedBiosystems	
Sodium Azide	Roth	Karlsruhe
Sodium Chloride	Roth	Karlsruhe
Sodium dihydrogenphosphate	Roth	Karlsruhe
Sodium hydrogencarbonate	Roth	Karlsruhe
Sodium hydrogenphosphate	Roth	Karlsruhe
Sodium hydroxide	Roth	Karlsruhe
Streptavidin conjugated agarose	Pierce	USA
Streptolysin O	S. Bhakdi	Universität Mainz
Sulfosuccinimidyl-2-(biotinamido) Ethyl-1-3dipropionate (sulfo NHS-SS-Biotin)	Pierce	USA
Sulfosuccinimidyl -6- (biotinamide) -6-hexanamido hexanoate. Sulfo NHS-LC- Biotin	Pierce	USA
TEMED	Roth	Karlsruhe
Trichloroacetic acid (TCA)	Roth	Karlsruhe

Trifluoroacetic acid (TFA)	Sigma	
Thiourea	Roth	Karlsruhe
Trishydroxymethylaminomethane (Tris)	Roth	Karlsruhe
Trypsin modified sequence grade	Promega	

2.1.4 Solutions and buffers

Acrylamide solution:	30% (w/v) Acrylamide 0.8% (w/v) Bisacrylamide
AP developing buffer:	100 mM Tris-HCL, pH 9.5 100 mM NaCl 5 mM $MgCl_2$
AP developing solution:	66µl NBT stock solution 33µl BCIP stock solution
BCIP stock solution:	5% BCIP in 100% Dimethylformamide
Coomassie destainer:	30% (v/v) Methanol 10% (v/v) Acetic acid Distilled water
Coomassie staining solution:	30% (v/v) Methanol 10% (v/v) Acetic acid 0.25% (w/v) Coomassie Blue R 250 Distilled water
Electrophoresis buffer:	0.124 M Tris 0.96 M Glycin 0.05 % SDS

Gas mixture for *P. falciparum* culture:

5% CO_2

5% 0_2

90% N_2

Giemsa stain: 1% (v/v) Giemsa solution

9% (v/v) Giemsa buffer

Giemsa buffer 10x, pH 6.8: 130 mM Na_2HPO_4

100 mM KH_2PO_4

NBT stock solution: 5% NBT in 70% Dimethylformamide

PBS, pH 7.2: 140 mM NaCl

6.5 mM KCL

2.5 mM Na_2HPO_4

1.5 mM KH_2PO_4

PBS^{++}: PBS containing

0.6 mM $CaCl_2$

1 mM $MgCl_2$

Ponceau stain: 0.2% Ponsceau red

3% Trichloroacetic acid

100ug/ml RNase A

Protease inhibitor cocktail Stock solution:

200 µg/ml of each of the inhibitors antipain, chymostatin, aprotinine, pepstatin, trypsin, leupeptin elastinal and Na-EDTA in PBS.

Working solution 1:200 dilution

Rehydration buffer:	8M Urea
	2M Thiourea
	4% DTT
	4% CHAPs
	1.6% ASB-15
	2% IPG buffer
	Trace bromophenol blue
RPMI 1640 Medium	31.784 g RPMI 1640
	25 mM HEPES sterile filter
	40 µg/ml Gentamycin
	in 1litre distilled water
RPS- Medium:	867 ml RPMI
	33 ml 8.125% $NaHCO_3$
	100 ml heat denatured human plasma
SDS equilibration buffer:	50 mM Tris-HCl
	6 M Urea
	30% 8 (v/v) Glycerol
	2 % (w/v) SDS
	supplemented with either
	64 mM DTT or 135 mM Iodoacetamide
SDS-PAGE concentrating gel pH 6.8:	
	0.5 M Tris/HCL
	0.4% SDS
SDS sample buffer stock solution:	63 mM Tris/HCl
	0.5 M sucrose
	0.01% Bromophenol blue
	5 mM EDTA
	1% L-methionine

SDS sample Loading buffer:	200 µl 0.5 M DTT
	200 µl 20% SDS
	600 µl SDS sample buffer stock solution
SDS-PAGE separating gel pH 8.8:	
	1.5 M TrisHCl
	0.4% SDS.

Western Blot Buffer:	48 mM TrisHCl
	39 mM Glycine
	0.0375% SDS
	20% Methanol.

2.1.5 Host cells and parasite isolates

Human erythrocytes, blood group A Rh^+ University blood bank Lahnberge Marburg.

Plasmodium falciparum isolate FCBR Philipps University Marburg

2.1.6 Antibodies and working concentrations

Cy3 (red) goat anti rabbit	Sigma, Diesenhofen	Dilution 1: 200
Cy5 goat anti mouse	Sigma, Diesenhofen	Dilution 1: 200
Goat anti rabbit alkaline phosphatase (AP)	DAKO Glostrup	Dilution 1:2000
Goat anti rabbit horse radish peroxidase (HRP)	DAKO Glostrup	Dilution 1:2000
Monoclonal anti biotin peroxidase Conjugate	Sigma, Diesenhofen	Dilution 1:4000
Mouse anti band 3	DAKO Glostrup	Dilution 1:2000
Rabbit anti aldolase	our laboratory	Dilution 1: 500
Rabbit anti Bip	our laboratory	Dilution 1:500
Rabbit anti GBP	our laboratory	Dilution 1:500
Rabbit anti human HSP 90	Santa Cruz Biotech. Inc.	Dilution 1:2000

Rabbit anti PfHsp 70	Dr. Tatu, India	Dilution 1:200
Rabbit anti PfHsp 90	Dr. Tatu, India	Dilution 1:200
Rabbit anti Mouse alkaline Phosphatase (AP)	DAKO Glostrup	Dilution 1:2000
Rabbit anti mouse horseradish Peroxidase (HRP)	DAKO Glostrup	Dilution 1:2000
Rabbit anti SERP	our laboratory	Dilution 1:500
Streptavidin avidin alkaline phosphatase (SAV-AP)	Pharmingen, USA	Dilution 1:10,000
Streptavidin horseradish Peroxidase (HRP)	Pharmingen, USA	Dilution 1:10,000

2.2 Methods

2.2.1 Parasite Cultures

Parasites, *P. falciparum* FCBR were cultured in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% heat in-activated (56°C, 30 min) human plasma and erythrocytes of blood A^+ group (Marburg Blood Bank) using standard procedures (Trager and Jensen, 1976). Trophozoites-infected erythrocytes (iRBC) were enriched to a parasitaemia of > 90% by gel floatation (Pasvol *et al*., 1978). For metabolic labeling of newly synthesized proteins, the enriched iRBC were washed twice in methionine free RPMI 1640 medium and cultured for 2 hours in methionine free medium without the plasma supplement in the presence of 50 µCi/ml L-[^{35}S] methionine.

2.2.2 SLO permeabilization of infected erythrocytes

Metabolically labeled iRBCs were permeabilized with SLO, (kindly provided by S. Bhakdi). Routinely, SLO was titrated as described by Ansorge *et al*., 1996. Briefly, 2 x10^8 iRBC were washed twice using RPMI 1640 medium before resuspension in 200 µl of the same media and incubation with various amounts of SLO at room temperature for 6 minutes. The solution was centrifuged for 5 min at 1300 g and the supernant analyzed spectrophotomerically at 412 nm to determine the amount of hemoglobin released. In parallel the same amount of cells were lysed completely in 200 µl of water by three cycles of freezing and thawing to determine the total hemoglobin content. The amount of SLO that resulted in release of 50% of the hemoglobin is defined as 1 hemolytic unit (Ansorge *et al*., 1997). Subsequent experiments were done using 2 x 10^8 iRBC incubated in 200µl of RPMI containing 3-4 hemolytic units of SLO. The samples were centrifuged at 4000 x g for 5 minutes to extract the erythrocyte cytosol from the permeabilized host cells. This resulted in the release of all the hemoglobin within detectable limits as determined by spectrophotometry. The pellet containing intact parasites, the vacuolar content and membranes was washed twice with 200µl RPMI 1640 medium before biotinylation.

2.2.3 Biotinylation of SLO permeabilized iRBC and extraction of soluble proteins

Permeabilized iRBC were washed with PBS containing 0.6 mM $CaCl_2$ and 1mM $MgCl_2$ pH 7.6 (PBS^{++}). The reaction of primary amines with N-hydroxysulfosuccinimide (NHS) esters is best at neutral pH values and above. This is due to the fact that the target for the NHS ester is the deprotonated form of the primary amine. For the biotin labeling of the total parasite proteins, permeabilized iRBC were routinely resuspended in 800 µl of distilled water containing the protease inhibitors antipain, chymostatin, aprotinin, trypsin-inhibitor, Na-EDTA, pepstatin,

leupepetin and elastatinal each at a concentration of 1 μg ml^{-1}. The lysates were prepared by 3 cycles of freezing and thawing. The soluble proteins obtained after centrifugation at 10,000x g for 15 minutes were used for further analysis. The proteins were biotin labeled in PBS^{++}, pH 7.6 containing 1mg ml^{-1} of Sulfo-NHS-LC biotin or Sulfo-NHS-SS biotin for 30min at 4° C with frequent light shaking. The incubation at 4° C reduces endocytic activity of the parasite and thus prevents the internalization of the biotin derivative by endocytosis. The solution was further centrifuged at 3,000 x g using a microconcentrator with a size exclusion of 5 kDa to remove any free biotin. This step was repeated two more times and the protein solution was stored at 4° C until use.

For the selective biotin labeling of soluble vacuolar proteins, permeabilized cells were incubated with Sulfo-NHS-LC biotin or with Sulfo-NHS-SS biotin as described above. The solution was centrifuged at 1300 x g for 5min at 4°C and the pellet was washed 3 times in PBS^{++} containing 100mM glycine to react with any unreacted biotin derivative that was still present in solution. After a final wash in PBS^{++}, iRBC were lysed and processed as above.

2.2.4 Biotin labeling of Bovine serum albumin (BSA)

To investigate the effect of biotin labeling on the molecular mass and subsequent separation pattern on 1D SDS-PAGE, BSA was labeled with different mole ratios of the Sulfo-NHS-LC-biotin derivative. In one experiment, 10 mg of BSA were dissolved in 1 ml of PBS^{++} before adding 1 mg of biotin derivative to give a mole ratio of 12:1 excess of biotin. In a parallel experiment 2 mg of biotin were dissolved in 1ml of PBS^{++} before adding 0.3 mg of the biotin derivative giving a mole ratio of 1:1 biotin to BSA. The mixtures were incubated at for 30 minutes at 4°C. The unreacted derivative was removed by using micro concentrators as described above and the BSA was subsequently analyzed by SDS-PAGE, western blot and Coomassie staining.

2.2.5 Affinity purification of biotin labeled proteins

Streptavidin and avidin conjugated agarose beads were used in the affinity purification of biotin labeled proteins as follows. A total lysate comprising of non-biotinylated but radiolabeled soluble proteins of the SLO pellet from 2 x 10^8 iRBCs was incubated with washed streptavidin conjugated agarose beads, at 4°C for 2 hours under constant shaking. The beads were sedimented by centrifugation at 10,000 x g for 5min. The supernatant was collected and stored at –20° C until further use. The beads were sequentially washed in buffers (A10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% NP40, 2 mM EDTA; B 10 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.2% NP40, 2 mM EDTA; C 10 mM Tris-HCl pH 7.5 and finally with PBS^{++}. The beads were then washed with 10

mM HEPES buffer pH 7.4 containing 20 mM $MgCl_2$ and 5 mM ATP. The bound proteins were eluted by boiling the beads in SDS-PGE sample buffer before analysis.

In a similar experiment a total lysate comprising soluble proteins of the SLO pellet from 2 x 10^8 iRBCs was incubated with 1 mg/ml sulfo-NSH-LC-biotin at 4° C for 30 min. The unreacted biotin derivative was removed by using micro concentrators as described above and the biotinylated and radiolabeled proteins were incubated with washed streptavidin conjugated agarose beads, at 4°C for 2 hours under constant shaking. The beads were sedimented by centrifugation at 10,000 x g for 5min. The supernatant was collected and stored at –20° C until further use. The beads were processed as described above before analysis by SDS-PAGE.

2.2.6 Immunoprecipitation of the marker proteins

The marker proteins SERP, GBP and aldolase were precipitated from the lysates as follows; soluble proteins from 2 x 10^7 permeabilized iRBCs were dissolved in 400µl of distilled water and 500µl of solubilization buffer containing 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% NP40 and 4 µg/ml PMSF. 5 µl of the corresponding antisera were added and the mixture was incubated at room temperature for 30min after which 30 µl of 50% packed volume of the protein A sepharose beads were added for 1h. The beads were sedimented by centrifugation and the supernatant was collected. The Protein A sepharose beads were washed sequentially with buffers A, B and C and PBS^{++} as outlined above and the precipitated proteins were eluted by boiling the beads in SDS-PAGE sample buffer before analysis. Proteins were precipitated from the supernatant with 10% trichloroacetic acid on ice for 10 minutes. The samples were centrifuged at 10,000 x g at 4°C for 15 minutes, the precipitate was washed using cold acetone and dissolved in SDS-PAGE sample buffer before analysis. The protein A sepharose beads were washed sequentially with buffers A, B and C and PBS^{++} as describe in 2.25 above and the bound proteins were eluted by boiling the beads in SDS-PAGE sample buffer and analysed.

2.2.7 Gel Electrophoresis

For 1D SDS-PAGE protein samples were dissolved in SDS-sample buffer and separated on 7.5%, 10%, 12% or 5%-20% gradient gels under reducing conditions. The protein bands were visualized by Coomassie staining before exposure to X-ray film for the detection of radiolabeled proteins.

For two-dimensional (2-D) PAGE, two different approaches were used. In the first procedure 2 x 10^9 infected RBCs were labeled metabolically with [^{35}S] methionine, permeabilized with SLO and treated with Sulfo NHS-SS-biotin. After cell lysis and processing as described in

2.2.3 above the soluble fraction was incubated with washed streptavidin agarose beads at 4°C overnight under constant shaking. The beads were sedimented by centrifugation at 10,000 x g for 5min. The supernatant was collected and stored at –20° C until further use. The beads were sequentially washed as described in section 2.2.5. Two compounds Tris (carboxyethyl) phosphine Hydrochloride (TCEP·HCl, Sigma-Aldrich) or dithiothreitol, DTT were used to elute the bound biotin labeled proteins from the beads. They act by reducing the sulphide bond between the biotin molecule and the protein, thereby releasing the proteins from the avidin-biotin complex. TCEP·HCl, a tri-aklyphosphine has been shown to be a more effective reducing agent compared to DTT (Han et al., 1994). The beads were incubated with TCEP at a concentration of 1mg/ml at room temperature for 2 hours and in parallel experiments the beads were incubated with 100 mM DTT at room temperature for 2 hours. The solutions were centrifuged at 10,000 x g for 30 minutes and the supernatants obtained were incubated with 3 volumes acetone at –20°C overnight to precipitate the eluted proteins.

The precipitate was dissolved in rehydration medium (8 M urea, 2M thiourea, 4% CHAPS, 4%DTT, 2% ASB and 2% IPG buffer pH 4-7, Pharmacia Biotech, Sweden), vortexed and incubated at room temperature for 1h to solubilize the proteins. Dry linear precast immobilized pH gradient gel strips (IPG, 13cm, pH 4-7) were rehydrated for 24 hours with 250µl rehydration solution containing the dissolved protein.

In the second approach, 2 x 10^9 infected RBCs were labeled metabolically with ^{35}S methionine, permeabilized with SLO and treated with Sulfo NHS-LC-biotin. After cell lysis processing as described above the soluble fraction was precipitated using 3 times volume 10% TCA in acetone containing 20mM DTT at –20° C for 1 hour. The proteins were sedimented by centrifugation and the pellet was washed 3 times with cold acetone containing 20 mM DTT before drying and dissolving in 250 µl of rehydration medium (8 M urea, 2M thiourea, 4% CHAPS, 4%DTT, 2% ASB and 2% IPG buffer pH 4-7, Pharmacia Biotech, Sweden) and rehydrating linear precast immobilized pH gradient gel strips (IPG, 13cm, pH 4-7, Pharmacia Biotech) for 24 hours under low viscosity paraffin oil to prevent evaporation and subsequent crystallization of the urea from the rehydration medium. After the passive rehydration the IPG strips were rinsed with a minimal amount of distilled water to remove any crystals on the surface before isoelectricfocusing (IEF) in a Multiphor II apparatus (Pharmacia Biotech.), for a total of 28,800 volt hours with a maximum of 50 µA per strip.

Table 1. **Rehydration and isoelectricfocussing conditions for 4-7 linear immobilized pH gradient strips.**

Step	Voltage	Duration (h)	Gradient Type
Rehydration	-	24	-
1	300	1	Stepped
2	500	1	Stepped
3	3500	8	Stepped

After focusing the individual IPG strips were consecutively incubated in SDS equilibration solutions A and B (50 mM Tris-HCl, pH 6.8, 8 M urea, 30 % v/v glycerol, 2 % w/v SDS and trace of bromophenol blue) complemented with 20mg ml^{-1} DTT (solution A) or 25 mg ml^{-1} iodoacetamide instead of DTT solution B)), each for 15 min with a distilled water rinse between the incubations. The iodoacetamide alkylates and prevents the re-oxidation of the cysteine residues. The equilibrated strips were transferred to a 10% or 12% slab gel (1mm thickness x 15.5 cm width x 17 cm), sealed with 0.5% (w/v) agarose before separating the proteins in a second dimension. The separated protein spots were either visualized by colloidal Coomassie blue stain or by silver staining or transferred to nitrocellulose membranes for western blot analysis. Parallel gels were dried and radiolabeled spots were visualized by autoradiography. The images of gels were acquired using Photoshop 3.5 software (Adobe Inc.) on a Vistascan flatbed scanner.

2.2.8 Western Blot Analysis

Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes. For detection of biotin labeled proteins, the membranes were blocked using 2% BSA in PBS pH 7.4 for 1 hour at room temperature. Filters were then incubated at room temperature for 20 minutes with streptavidin alkaline phosphatase-conjugate diluted 1:10,000 in 2% BSA in PBS, pH 7.4. The membranes were washed in 10 mM Tris/HCl pH 7.4, 150 mM NaCl for 5 min, then in the same buffer containing 0.05 % Triton-X 100 for another 5 min and finally in the buffer without Triton-X 100. The filters were then incubated for 5 minutes in the alkaline phosphatase detection buffer and the biotin labeled proteins stained with nitro blue tetrazolium and 5-bromo-4 chloro-indolylphosphate following standard procedures. The same membranes were then incubated with specific rabbit antisera at a dilution of 1:500 at 4° C overnight. Specific marker proteins were

detected after incubation with horseradish peroxidase-conjugated anti-rabbit IgG (Dako) for 1h at room temperature and development of the filters using the ECL system (Amersham Biosciences) following standard procedures.

2.2.9 Indirect Immunofluorescence

Permeabilized and biotin treated iRBC were spread on glass slides and air dried. Subsequently, they were fixed in acetone-methanol (1:1) for 10 min at –20 °C. The slides were incubated with the primary antibodies for 2 hours at room temperature. The monoclonal anti-band 3 IgG was diluted 1:20, and the polyclonal rabbit antisera specific for SERP and aldolase, were each diluted 1:10 in PBS, pH 7.2. After 3 washes with PBS, pH 7.2, slides were probed with a mixture of the corresponding secondary antibody (1:100) and Cy3-conjugated streptavidin (1:200) for 30 min at room temperature in the dark. Autofluorescence levels were determined by viewing control samples of either biotin-treated or untreated iRBCs not incubated with Cy3-conjugated streptavidin and secondary antibodies. The cells were mounted in glycerol containing 0.1% of the antifade reagent 1,4-diazobicyclo (2,2,2)-octane. Fluorescence microscopy was performed using a Leica TCS SP2 scanning microscope.

2.2.10 Immunoelectronmicroscopy

Permeabilized and biotin treated iRBCs were fixed overnight at 4° C in 1% gluteraldehyde in 0.1 M phosphate buffer, pH 7.4, then washed with 0.1 M phosphate buffer. The fixed cells were dehydrated by incubating them in successively increasing ethanol concentrations from 30% through 50%, 70%, 80%, 90% and finally 100% allowing 10 min for every step. The cells were then suspended in 1:1 ethanol / Lowicryl resin overnight in open eppendorf tubes to allow the evaporation of the ethanol. The cells were pelleted by centrifugation and the pellet mixed with fresh lowicryl and placed overnight under UV light overnight to allow the polymerization of the resin in a gelatin capsule. Ultra thin sections were cut with a diamond knife and mounted onto copper grids. Sections were blocked with 2% BSA in PBS before incubation with a 1: 800 dilutions of streptavidin conjugated with 10 nm gold particles. The sections were contrasted and examined under electronmicroscope.

2.2.11 Processing of proteins for analysis by MALDI-TOF

Proteins separated by two dimensional gel electrophoresis were fixed for one hour in 7% glacial acetic acid and 40% methanol before staining overnight in colloidal Coomassie blue containing 20% methanol to visualize the spots. The gel was destained in 10% acetic acid, 25% methanol for 60 seconds with shaking, and then rinsed with 25% methanol overnight to remove excess stain.

Protein digestion was performed according to the method of Hellman *et al*., 1995 with minor modifications. Briefly, protein spots were precisely cut out of the gels using an eppendorf blue pipette tip to minimize the collection of unstained gel and placed in micro centrifuge tubes (Eppendorf, Germany) containing 200 µl washing/ destaining buffer (200 mM NH_4HCO_3; 50% CH_3CN). The gel pieces were incubated at 37 °C for 30 min with continuous shaking. The buffer was aspirated and the gel pieces dried under vacuum for 30 minutes. 10 µl of the in gel digestion solution containing 10% CH_3CN, 40 mM NH_4HCO_3 pH 8.1 and 0.02 µg ml^{-1} sequencing grade trypsin was added to the gel and incubated for 45 min at room temperature to allow the gel pieces to soak in the solution. A further 5 µl of the solution was added and the mixture was incubated for 1 hour to ensure that the gel pieces were fully saturated with the digestion solution. The supernatant was then aspirated and the gel pieces incubated at 37 °C for 18 hours for in-gel trypsin digestion. The eppendorf micro centrifuge tubes were inverted to minimize evaporation of the digestion solution. Subsequently 15 µl of diffusion solution, 10% CH_3CN in 1% trifluoroacetic acid were added and the mixture was sonicated at 37 °C for 45 minutes. The supernatant containing the tryptic digests was transferred to a gel loader tip with a 5mm high column of reverse phase material consisting of POROS 50 R2 and Oligo R 3(AppliedBiosystems), 1:1 in 50% acetonitrile to desalt and concentrate the peptides. Before loading, the column was washed sequentially with 10µl methanol then 10µl methanoic acid. After loading the tryptic digests, the column was again washed with 10µl methanoic acid before adding 0.7 µl the elution medium consisting of the MALDI matrix and marker peptides (2 mg α-Cyano-4-hydroxycinnamic acid (Sigma) in 170 µl 70% CH_3CN in 0.1 % trifluoroacteic acid complemented with 0.25 µl of calibration mixtures I and II (AppliedBiosystems) diluted 1:500) and directly loading the eluted peptides directly on to the gold or steel plated MALDI target. The analytes were dried before loading the target to the MALDI apparatus.

Mass spectrometry was performed using a Perseptive Biosytems Voyager DE-STR (Framingham, MA). The instrument employs nitrogen at 337nm as the laser source and it has a 20 kV accelerating voltage. Briefly, the digested peptides co-crystallized with the matrix were irradiated by laser beam, leading to sublimation and ionization of peptides. About 150 ns after the

laser pulse, a strong electric field is switched (delayed extraction), which imparts a fixed kinetic energy to the ions produced by the MALDI process. These ions travel along a flight tube and are turned around in an ion mirror (reflector) to correct for initial energy differences before being directed to the detector. The mass-to-charge ratio is related to the time it takes an ion to reach the detector; the lighter ions arrive first. The ions are detected by a channeltron electron multiplier that gives the mass and the abundance of the flying ions. Peptide mass fingerprint data were acquired in the reflector mode with delayed extraction (150ns) using approximately 100 laser shots and calibrated using internal standards I and II (Applied Biosytems). The spectrum obtained was an average of the spectra obtained from the 100 laser shots. Typically the performance of the mass spectrometer is in the range of a few parts per million in mass accuracy as shown by the masses obtained from the internal standards.

2.1.12 Peptide sequencing by tandem mass spectrometry

The sequences of some peptides were determined by interpreting data resulting from fragmenting the peptides in a tandem mass spectrometer (Bieman and Scoble 1987; Hunt *et al.*, 1987). This technique was used to determine the presence of the biotin label on the peptides. The peptides that showed the presence of biotin in the first mass spectrometer were selected and then dissociated with a higher laser strength 355 nm nitrogen lasers or fragmented in a second collision cell using argon as a laser source. The resulting fragments were separated in the second part of the tandem mass spectrometer, producing the tandem mass spectrum, or MS/MS spectrum. Multiple collisions impart energy onto the peptide until it fragments. The ions are produced due to the fragmentation of the amide bond with charge retention on the N or C terminus, which leads to the formation of b and y ions respectively. Since the peptides were generated by trypsin digestion, and since they contain arginyl or lysyl residues as their C-terminal residues, y ions were the predominant ion series observed.

2.1.13 Protein identification in database searches by peptide mass fingerprinting

A key advance in biological mass spectrometry was the development of algorithms for the identification of proteins by mass spectrometry data matched to a database, originally using a set of peptide masses and now increasingly using fragmentation spectra of the original peptides. With the availability of the complete sequence of an increasing number of organisms including *P. falciparum* (Gardner et al., 2002) it is now possible to easily perform data base correlation with a great degree of automation.

Peak harvesting and processing was done using P.E Biosytems Voyager Data Explorer software version 5.01 (Applied Biosystems). The peak resolution was calculated using the Data Explorer software, the raw data was subjected to baseline correction, before being re-calibrated manually using the internal mass standards (Applied Biosytems) to achieve a more accurate data determination of the peptide masses. The spectra were deisotoped and the resulting peptide masses were used in data base interrogation using the program MS-FIT (prospector.ucsf.edu/ucsfhtml/msfit.htm)http://www.prospector.ucsf.edu/; http://prowl.rockefeller.edu/cgi-bin/profound, www.matrixsciences.com and the recently completed annotated Plasmodium genome data base at *www.Plasmodb.org*; The search used all *P. falciparum* proteins in the NCBI nonredundant data base. The modifications considered were; peptide N-terminal glutamine to pyroglutamine carbamidomethylated cysteines, artifactual oxidations of methionines and acrylamide modified cysteines, protein N-terminus acetylated. The modification by sulfo-NHS-LC-biotin derivative which brings in the following set of atoms $C_{12}H_{26}O_3N_3S$ when biotin labeling occurs at a lysine residue (see fig.2 section1.1.4) was also included as a possible modification. A maximum of one missed cleavage site following trypsin digestion was allowed. The monoisotopic masses were used with a mass tolerance of +/- 50.000 ppm and a minimum number of matched masses set as 4 matches. *Plasmodium falciparum* peptide mass finger print data were considered unambiguous when there was a minimum of 4 peptides and 10% sequence coverage (Mann *et al*., 2001). The M_r and pI values of the analyzed spots were obtained from the Coomassie stained 2-dimensional gels and compared to the those obtained in the searches to further confirm the accuracy of the results of the data base searches.

3. RESULTS

3.1 Experimental rationale

The use of SLO to selectively permeabilize and the subsequent fractionation of infected erythrocytes is a valuable technique in the study of protein trafficking in *P. falciparum* (Ansorge et al., 1996; Ansorge et al., 1997). The permeabilization occurs on the erythrocyte membrane with the production of pores with size exceeding 30 nm.The of the host cell cytosol is extracted after a centrifugation step and during the entire procedure the integrity of the PVM is maintained, thus the contents of the vacuolar space are retained.

On the basis of results obtained from electrophysiological studies it has been previously proposed that proteinaceous pores exist within the PVM of *P. falciparum* infected erythrocytes (Desai *et al*., 1993; Desai and Rosenberg, 1997). These pores are postulated to allow the bi-directional passage of molecules with molecular mass less than 1300 daltons across the PVM. If indeed these pores are present, after SLO treatment of iRBCs and the extraction of the host cell cytosol it should be possible to introduce small molecules, including biotin derivatives to the external milieu of the permeabilized cells, these molecules should access the vacuolar space after passing through the SLO pores on the erythrocyte plasma membrane and the proposed pores in the PVM .

This experimental rationale was used and iRBCs were permeabilized before biotinylation with a non-permeant biotin derivatives. These derivatives have a mass of about 600 daltons and they are highly hydrophilic. If the proposed pores are present in the PVM, the biotin derivatives are small enough and they should pass through these pores to access and react exclusively with the PV resident proteins. The hydrophilicity of the derivatives renders them non-permeant to the hydrophobic parasite plasma membrane. This should prevent the biotin derivatives from entering the parasite thus preventing the biotinylation of the internal parasite proteins.The biotin labeled PV proteins can be isolated and characterized using molecular and biochemical approaches. The experimental procedure is summarized in figure 6.

The conceptual problem with the approach is to ensure the removal of the host cell cytosol and to ensure that biotinylation of internal parasite proteins does not occur.

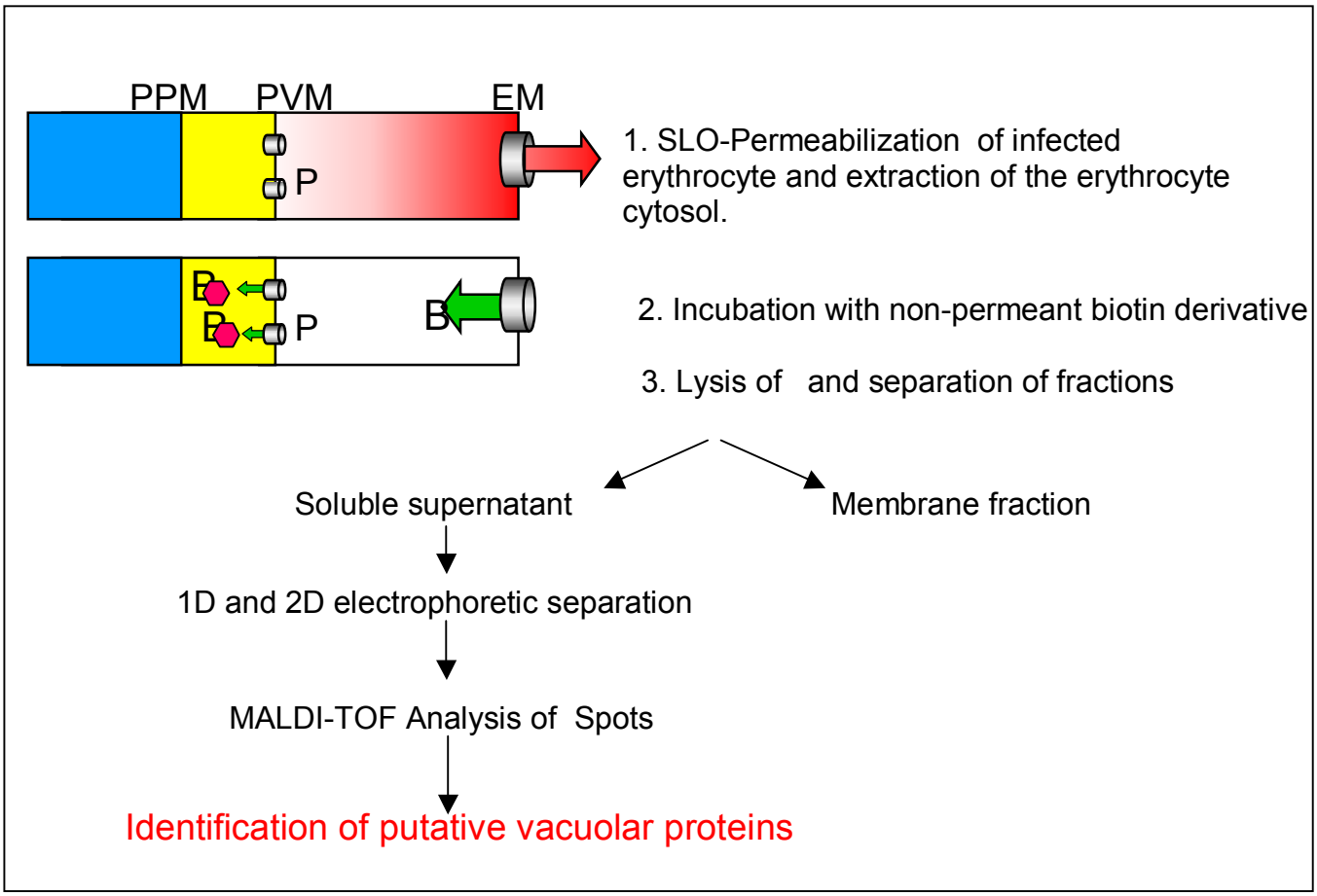


Figure 6. Experimental rationale. Infected erythrocytes are permeabilized with SLO, which forms pores on the erythrocyte membrane (EM). The erythrocyte cytosol is extracted before incubating the infected cell with a non-permeant biotin derivative (B) which penetrates the parasitophorous vacuole membrane (PVM) through the postulated proteinaceous pores (P) to selectively biotin label the proteins within the parasitophorous vacuole. The biotin derivative does not permeate the parasite plasma membrane (PPM) and the internal parasite proteins are not biotin labeled.

3.2 Permeabilization of infected erythrocytes with SLO preserves the integrity of the vacuolar membrane, and removes the erythrocyte cytosol.

The bacterial pore-forming toxin SLO has been shown to selectively permeabilize the erythrocyte plasma membrane and to preserve the integrity of the PVM (Ansorge *et al*., 1996). This is one prerequisite for the experimental strategy employed in this project. In initial experiments a careful study based on two parasite marker proteins, SERP and aldolase, was carried out to reproduce and assess this experimental approach. Aldolase is an abundant glycolytic enzyme localized in the parasite cytosol and SERP is located in the parasitophorous vacuole.

In this study $2x10^{8}$ infected erythrocytes were SLO permeabilized and the supernatant and pellet fractions, each equivalent to 10^{7} IRBC processed and subjected to SDS-PAGE. The proteins were transferred onto nitrocellulose membranes and the filters detected for SERP and PfAldolase. The results from the fractionation after SLO treatment show that the two proteins are exclusively found in the pellet fraction (Figure 7) confirming that the PVM remains intact during SLO lysis as previously reported by Ansorge *et al*., (1996). When 0.15% saponin was used to lyse infected erythrocytes SERP was exclusively detected in the supernatant while aldolase remained predominantly in the pellet (section 3.14). Some aldolase is released into the supernant after saponin treatment, demonstrating that the saponin treatment is more difficult to control than treatment with SLO. This clearly shows that during SLO lysis the PVM remains intact and the pellet contains intact parasites and the contents of the parasitophorous vacuole stilled enclosed by the PVM. Saponin lysis on the other hand results in a pellet containing intact cells without the contents of the parasitophorous vacuole. This confirms the suitability of using SLO to fractionate infected erythrocytes by extracting the host cytosol and creating a direct interface between the PVM and the external milieu. The results also confirm the suitability of using SERP as a PV marker and aldolase as a maker of the internal parasite proteins.

The quantitative removal of the erythrocyte cytosol is important in this experimental approach since this eliminates contamination by host proteins. Hemoglobin is the most abundant erythrocyte protein and the SLO pellet fraction was analysed spectrophotometrically for the presence of the protein. No detectable levels of hemoglobin were present in the permeabilized erythrocytes. These results not only confirm that the PVM remains intact during the permabilization process but also that the erythrocyte cytosol is quantitatively removed during the process.

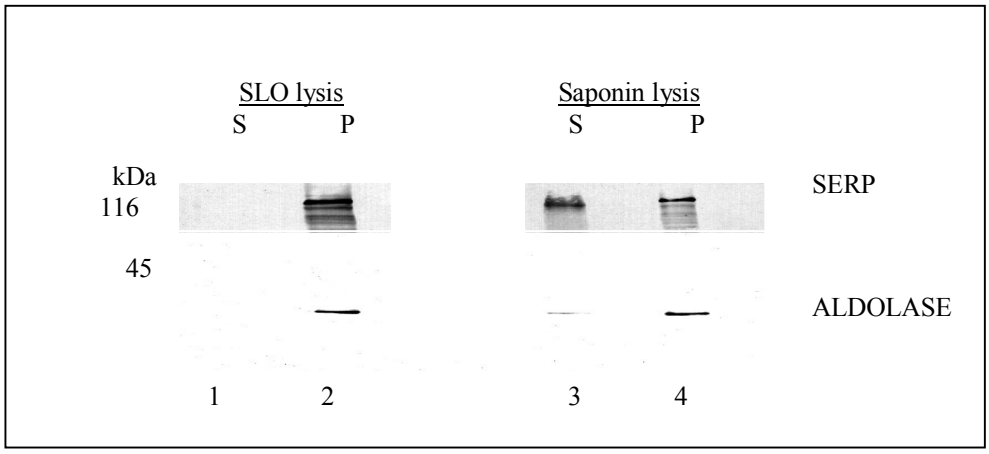


Figure 7. The PVM remains intact during SLO lysis but not during saponin lysis. Infected erythrocytes were SLO permeabilized (*lanes 1 and 2*) or lysed with 0.15% saponin (*lanes 3 and 4*). The pellet (P) and the fraction of released proteins (S) were analyzed by SDS-PAGE and immunoblotting using antisera against SERP a vacuolar space marker and aldolase an internal parasite protein. The primary antibodies were visualized by using alkaline phosphatase conjugated secondary antibody.

3.3 Biotin accumulates in the parasitophorous vacuole in SLO permeabilized infected erythrocytes.

The vacuolar membrane forms a barrier between the parasite surface and the host cell cytosol from which it must acquire nutrients that are essential for its survival. Therefore it is almost certain that the PVM is adapted to allow the transportation of these nutrients. Indeed Desai *et al*., (1993) reported the presence of high conductance channels that are permeable to organic and inorganic cations, using a patch-clamp technique. In bilayers, this channel allows passage of molecules up to 1,400 Da (Desai and Rosenberg 1997), which is similar to the non-selective pores discovered in the PVM of the taxonomically related parasite *Toxoplasma* (Schwab *et al*., 1994). The existence of these pores in *Plasmodium*-infected erythrocytes has not been verified, but if they exist, they should allow access of membrane-impermeable biotin derivatives that are ~600 Da in size.

Initially it was investigated at the morphological level whether biotin derivatives gain access to the PV. Infected erythroyctes were permeabilized using SLO to completely remove the host cell cytosol before incubation with the membrane-impermeable sulfo-NHS-LC-biotin. Confocal immunofluorescence microscopy was used to localize the biotin derivative using a fluorescent conjugated streptavidin. The fluorescence clearly showed a distinct ring around the periphery of the intracellular parasite (Figure. 8). The biotin co-localizes with the vacuolar marker SERP. This location is clearly different and distinct from the location of the erythrocyte membrane protein

band 3 and from the location of the parasite cytosolic protein aldolase. In some parts, the streptavidin and the antibody to band 3 co-localize; this is due to the fact that proteins of the erythrocyte plasma membrane also react with the biotin. Little if any biotin was detectable in the erythrocyte cytosol and parasite cytosol respectively. The ring-like staining around the periphery of the parasite can be attributed to a reaction of the biotin with the membrane proteins of the PVM and with the soluble vacuolar proteins.

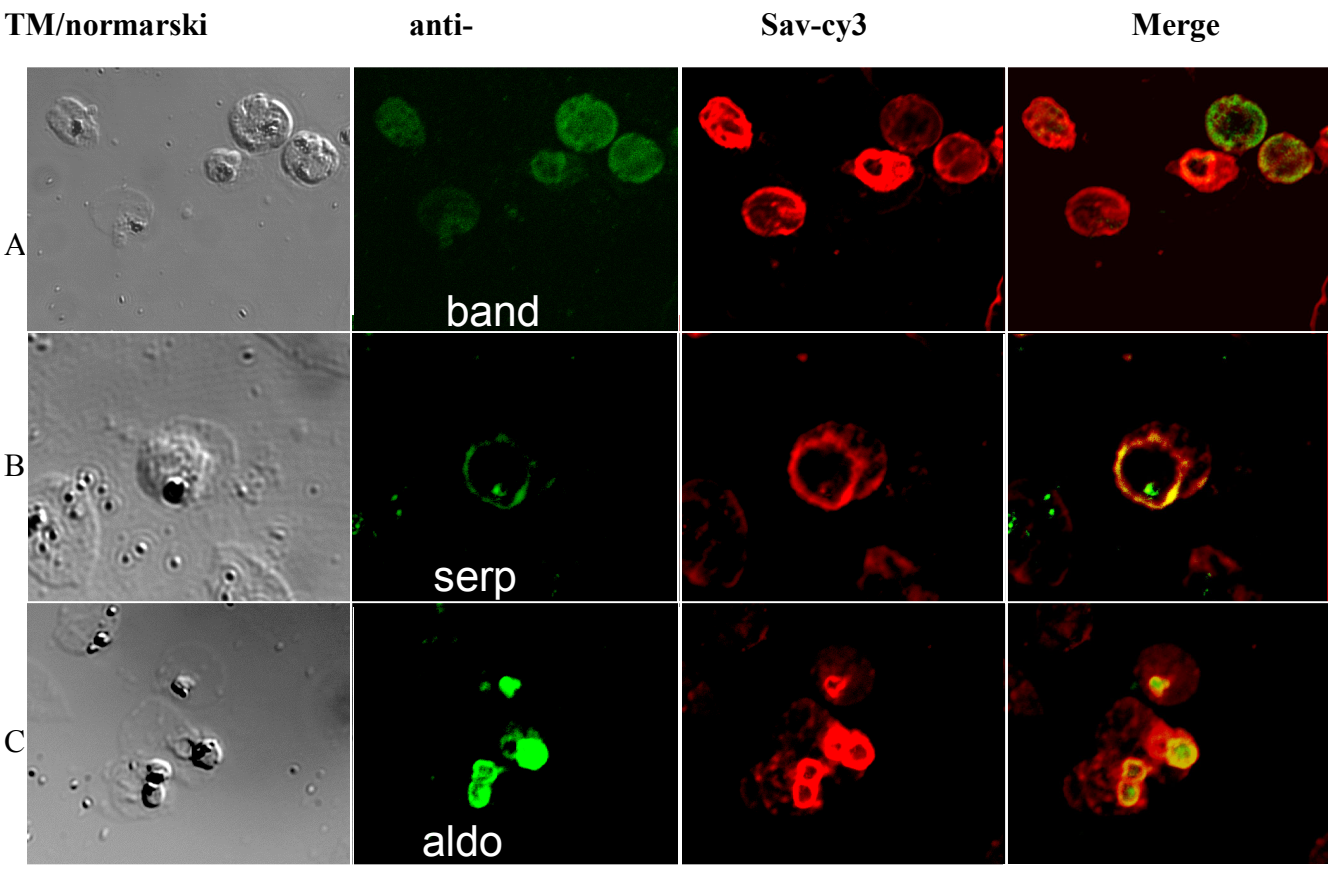


Figure 8. Biotin accumulates in the parasitophorous vacuole in SLO permeabilized infected erythrocytes. Trophozoite-infected erythrocytes were permeabilized with SLO. Biotin labeled and spread onto microscope slides. Cells were fixed and incubated with antibodies to SERP (*A*), a vacuolar resident protein. Conjugated secondary antibodies (*green colour*) colocalize with Sav-Cy3 (*red colour*). *B* and *C* show dual labeling of biotin and the erythrocyte membrane protein band 3 (B) or of biotin and the parasite cytosolic protein PfALD (*C*). Cells were imaged using a laser scanning confocal microscope. The *left panels* (TM/nomarski) show the transmission images of the respective fields, and the *right panels* (*merge*) show an overlay of fluorescence images for each of the antibodies used.

3.4 Streptavidin agarose beads specifically bind biotinylated parasite proteins.

To determine the suitability of streptavidin agarose beads for the affinity purification of biotinylated proteins, infected erythrocytes were cultivated for 30 min in the presence of L-[^{35} S] methionine and subsequently permeabilized with SLO to release the soluble contents of the host cytosol. The integrity of the PVM was confirmed as described in section 3.1. Since mammalian cells do not synthesize proteins *de novo*, the radiolabeled amino acids in this experiment are incorporated exclusively into parasite proteins. The soluble proteins from the parasite-containing vacuoles were obtained by centrifugation at 13,000 x g after three cycles of freezing and thawing in distilled water containing protease inhibitors. One aliquot of the lysate was treated with 1 mg ml^{-1} sulfo-NHS-LC-biotin at 4 °C for 30 min and another aliquot remained untreated after which both samples were incubated with SAv-agarose beads at 4 °C overnight. The beads were washed and the bound proteins eluted in SDS sample buffer and analysed by SDS-PAGE and autoradiography (Figure 9). No radiolabeled proteins from the non-biotinylated sample bound to SAv-agarose (Figure 9, lane 2), whereas after biotinylation, most radiolabeled proteins were found in the fraction of bound proteins (Figure 9, lane 4). The overall separation pattern was different between the two samples with the bands in the biotinylated sample appearing to be less focused.

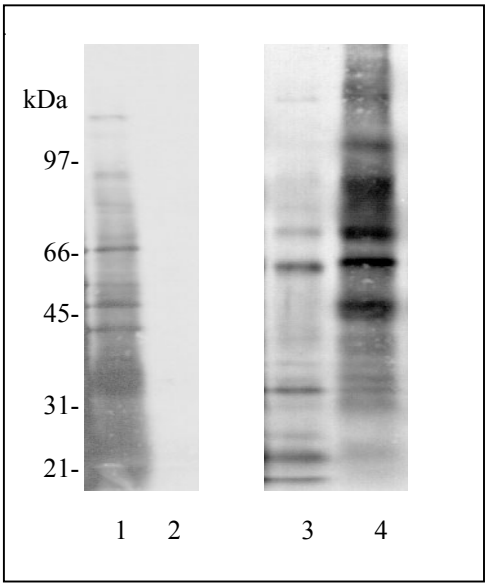


Figure 9. Streptavidin agarose beads specifically bind biotinylated proteins. Infected erythrocytes were metabolically labeled with L-[^{35}S] methionine and permeabilized with SLO, and the erythrocyte cytosol was extracted and the cellular fraction consisting of intact parasites and the intact PVM and PPM was then lysed and the soluble fraction obtained was divided into two aliquots. One aliquot was treated with sulfo-NHS-LC biotin a *(lanes 3* and *4*) and the other was untreated (*lanes 1* and *2*). Proteins that bound to SAv –agarose beads (*lanes 2* and *4*) were analyzed by SDS-PAGE and autoradiography.

The poor focusing of the biotin labeled proteins is due to the differential degrees of biotinylation of individual polypeptide chains. Since a given polypeptide may have several possible biotinylation sites it is possible that the degree of biotinylation varies within a protein species leading to differential migration of the identical peptides in the gel. Therefore the effects of biotin incorporation on electrophoretic mobility of proteins was studied using BSA as described below

3.5 Electrophoretic mobility of BSA is affected by biotinylation.

To assess the effects of biotin incorporation on the electrophoretic mobility of proteins, BSA was incubated with different molar ratios (12:1 and 1:1 respectively) of sulfo-NHS-LC-biotin, for 30 minutes at room temperature. The excess unreacted biotin was removed by size exclusion centrifugation and the mobility of the treated samples was compared to a non- treated sample by SDS-PAGE. As shown below (Figure 10) the electrophoretic mobility of BSA is altered depending on the degree of biotinylation. Increased biotinylation leads to a decrease in the mobility of the protein. This is due to the increase in the molecular size since each biotinylation with the sulfo-NHS-LC-biotin derivative increases the total mass by 339 Daltons. The poorly focused protein bands seen in Figure 9 lane 4 above is therefore consistent with differential biotinylation of individual polypeptide chains leading to polypeptides of different masses.

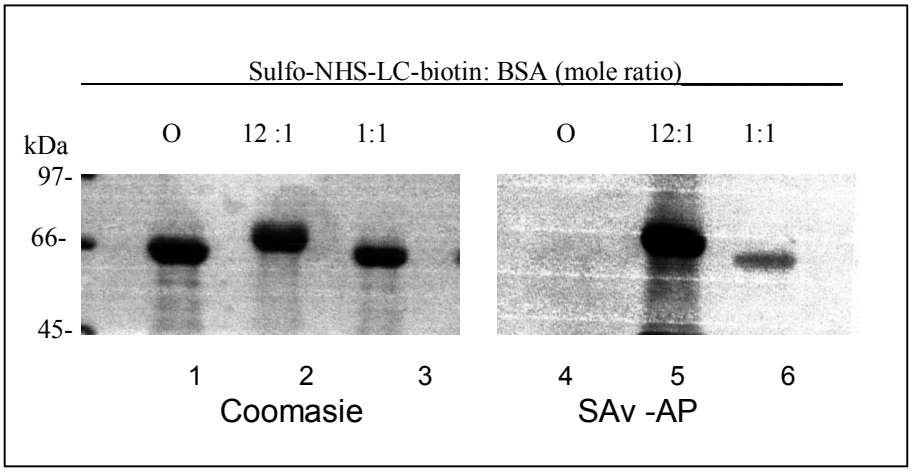


Figure 10. Extensive biotin labeling increases the molecular size of proteins. BSA was treated with sulfo-NHS-LC biotin at different molar ratios and analyzed by SDS-PAGE and Coomassie staining (*lanes 1-3*) or by reaction with alkaline phosphatase-conjugated SAv on nitrocellulose filters (*lanes 4*-6).

3.6 The marker proteins SERP, GBP and PfALD can be biotinylated.

The biotinylation of soluble marker proteins of the parasite was determined under different conditions to demonstrate that they are biotinylatable and that there is compartment specificity of biotinylation as shown by immunofluorescence microscopy in section 3.2. The soluble parasite proteins GBP, SERP and aldolase are located in different compartments of the infected erythrocytes and were used as markers. Aldolase is restricted to the parasite cytosol (Benting *et al*., 1994), SERP is restricted to the vacuole (Knapp *et al*., 1989), and GBP is found both inside the vacuole and within the erythrocyte cytosol (Ansorge *et al*., 1996). If the biotin derivatives have access to these proteins then they should be biotinylated and subsequently bind to SAv-agarose beads. In this experiment infected erythrocytes were SLO permeabilized and, after the complete removal of the host cytosol, a lysate containing soluble proteins of the parasite cytosol and the PV was prepared after freezing/ thawing and centrifugation. One aliquot of soluble proteins was treated with sulfo-NHS-LC-biotin and the other aliquot remained untreated. The samples were incubated overnight with SAv-agarose, and the bound and unbound proteins were analysed separately by SDS-PAGE and immunoblotting using antisera to the respective marker proteins (Figure 11). In the sample of non-labeled proteins, the three marker proteins were recovered in the fraction of unbound proteins (Figure 11, compare lanes 1 and 2). After biotin treatment, the three marker proteins SERP, GBP and PfALD were recovered in the fraction of bound proteins (Figure 11, compare lanes 3 and 4). In the case of GBP and SERP, recovery was almost complete and quantitative. The recovery of PfALD was not complete, however these results demonstrate that the marker proteins can be biotinylated and can be isolated by affinity chromatography on SAv-agarose. These experiments also further confirm that the marker proteins do not bind to the SAv-agarose unless they are biotinylated.

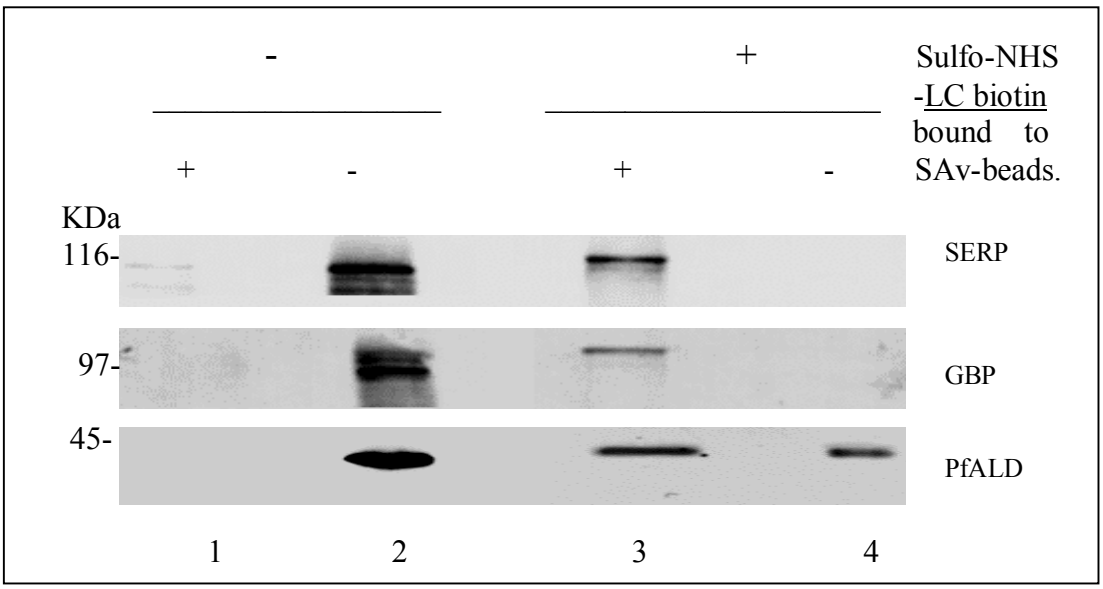


Figure 11. The marker proteins SERP, GBP and aldolase are biotinylatable. To confirm that the marker proteins can be biotin labeled when they are in solution, infected erythrocytes were permeabilized with SLO and the erythrocyte cytosol was extracted. The remaining fraction consisted of intact parasites including an intact PVM and parasite plasma membrane. The PVM and the parasite plasma membrane were then lysed, and membrane proteins and soluble proteins were separated. One aliquot of soluble proteins corresponding to 2 x 10^8 cells was treated with sulfo-NHS-LC-biotin (*lanes 3* and *4*), and one aliquot remained untreated (*lanes 1* and *2*). Proteins that bound to SAv-agarose beads (*lanes 1* and *3*) and unbound proteins (*lanes 2* and *4*) were analyzed by immunoblotting for the presence of the marker proteins SERP, GBP and PfALD.

3.7 Biotinylation of SLO permeabilized infected erythrocytes is compartmentalized.

The results from immunofluorescence microscopy in section 3.2 suggested a compartment-specific biotinylation. However it does not provide evidence that cytosolic proteins of the parasite are not biotinylated. Neither does it provide positive evidence that soluble vacuolar proteins are biotinylated. Therefore after the confirmation that all the marker proteins are biotinylatable a biochemical approach was used to ascertain the specific biotinylation of the vacuolar proteins and non-biotinylation of internal proteins. IRBC were SLO permeabilized and washed to completely remove the contents of the host cell cytosol before incubating with sulfo-NHS-LC-biotin. The excess biotin was removed and the soluble fraction obtained after centrifugation. The soluble proteins were immunoprecipitated using antisera against SERP and GBP respectively. The precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose filters. Initially, the filters were probed with SERP and GBP antibodies, and their binding was detected using an alkaline phosphatase-conjugated secondary antibody (Figure 12a, *lanes 1*and *3*). Subsequently, the filters were reprobed with peroxidase-conjugated streptavidin and analysed by

chemiluminescence (Figure 12a*, lanes 2* and *4*). The same analysis was carried out for PfALD, but because the antiserum against aldolase does not immunoprecipitate the protein, the total lysate was separated by SDS-PAGE and analyzed (Figure 12, *lanes 5* and *6*). The antisera specifically recognized the marker proteins. The bands that were detected with SAv were of identical sizes as GBP and SERP, indicating that under these conditions both proteins were biotinylated. However no biotin-labeled band corresponding in size to aldolase was detectable; a protein band of a smaller size reacted with SAv, but not with the aldolase-specific antibody (Figure 12a, *lane* 6).

To further analyze the biotinylation status of the marker proteins the biotinylated proteins in the soluble fraction obtained above were isolated by affinity chromatography using streptavidin-agarose beads and subsequently analysed by immunoblotting. In permeabilized cells, PfALD was not biotin labeled and did not bind to the streptavidin beads. The other two proteins, GBP and SERP, were found in the bound fraction. GBP was quantitatively labeled with biotin and the protein was recovered bound to the beads; on the other hand the labeling of SERP was incomplete. All the biotin labeled SERP was bound to SAv-agarose beads (figure. 12b) but a substantial proportion of the SERP molecules remained unbound, obviously because these molecules were not labeled with biotin as shown by the chemiluminescence using SAv-HRP (figure 12b, *lane* 2). This is different from the results obtained with total lysates in which SERP is released. Under these conditions the protein was labeled and precipitated quantitatively. These results confirm that the biotin derivative gains access only to the PV does not enter the parasite. Therefore there is specific biotinylation of the soluble proteins of the PV but internal parasite proteins are not biotinylated.

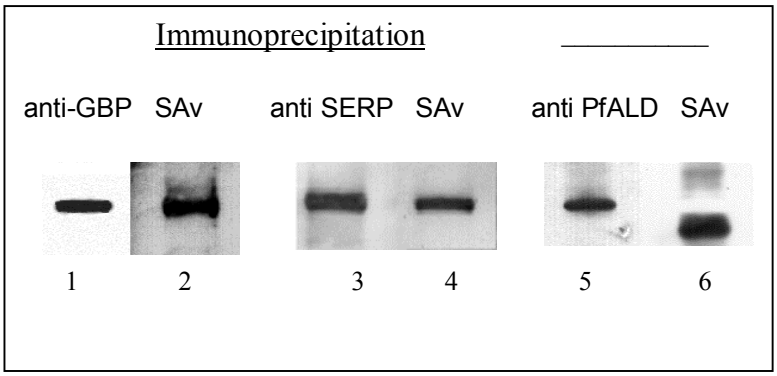


Figure 12a. Selective biotinylation of vacuolar marker proteins. Infected erythrocytes were permeabilized with SLO. After complete extraction of the erythrocyte cytosol, the cellular fraction consisting of intact parasites and the intact PV was incubated with sulfo-NHS-LC biotin and lysed after the removal of free biotin. Proteins were immunoprecipitated using antisera against GBP and SERP, respectively, separated by SDS-PAGE and transferred to nitrocellulose filters (*lanes* 1-4). The filters were first probed with antisera that were detected in a color reaction using alkaline phosphatase-conjugated secondary antibodies (*lanes 1 and 3*). The same filters were reprobed with peroxidase-conjugated SAv, which was subsequently detected by a chemiluminescence reaction (*lanes 2 and 4*). The incorporation of biotin into aldolase was analysed in the same way (*lanes 5 and 6*), except that soluble proteins were separated electrophoretically without prior immunoprecipitation.

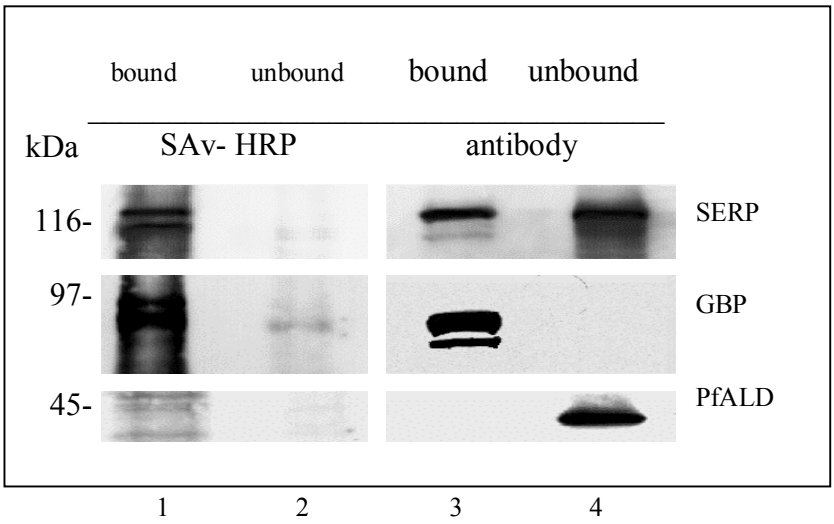


Figure 12b. Selective biotinylation of vacuolar marker proteins. Infected erythrocytes were permeabilized with SLO. After complete extraction of the erythrocyte cytosol, the cellular fraction consisting of intact parasites and the intact PV was incubated with sulfo-NHS-LC biotin and lysed after the removal of free biotin. The lysate was incubated with SAv-agarose beads. Fractions of bound (lanes 1and 3) and unbound (lanes 2 and 4) were analyzed for the incorporation of biotin (lanes 1 and 2) and the presence of the respective maker proteins (lanes 3 and 4).

3.8 The molecular chaperone PfBip interacts with biotinylated proteins.

After biotinylation of proteins in permeabilized cells, the streptavidin agarose bound fraction of soluble proteins was analyzed for the presence of PfBip, the plasmodial homologue of immunoglobulin binding protein, a molecular chaperone and endoplasmic reticulum resident protein in eukaryotic cells (Kumar *et al*., 1991). This was done with the view of having another abundant internal marker protein in addition to aldolase for which an antiserum was available. Secondly, since the vacuole is a protein sorting compartment it is conceivable that the PV contains molecular chaperones required for protein translocation across the PVM. Therefore it was investigated whether PfBip is biotinylated. Infected erythrocytes were permeabilized, one aliquot was treated with sulfo-NHS-LC-biotin and the other remained untreated. Lysates of cells were prepared and incubated with SAv-agarose beads. The fraction of unbound proteins and bound proteins were analyzed for the presence of PfBip using a specific antiserum. The results show that the protein does not bind non-specifically to the SAv agarose beads in the absence of biotin and it is detected only in the unbound fraction (Figure 13a *lanes 1* and *2*). However in the biotin treated sample the protein was present in both the unbound and bound fractions suggesting that the protein was biotin labeled (Figure 13a *lanes 3* and *4*). This observation was unexpected since Bip is an ER resident protein with the SDEL retention signal. It is possible that some of this protein may not be retained within the parasite's secretory pathway and hence may be transported into the vacuole, for example as a complex with other proteins that are translocated in the classical secretory pathway. Indeed there is a recent report (Akampong *et al*., 2002) suggesting that Bip can be translocated to the food vacuole in a complex together with other proteins such as such as plasmepsin I which has a signal sequence and is transported through the ER via the classical secretory pathway. To verify its biotinylation status, the protein was immunoprecipitated from the biotin treated lysate using specific anti-PfBip antisera. The precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose before incubation with an antiserum against PfBip. In a first step the binding of the anti-PfBip antiserum was determined using an alkaline phosphatase-conjugated secondary antibody (Figure 13b, *lane* 1). In a second reaction biotin was detected by chemiluminescence using peroxidase-conjugated SAv (Figure 13b, *lane* 2). Under these conditions, biotin was not detected. This suggests that the recovery of Bip in the fraction of proteins that bound to SAv-agarose was due to an interaction of the chaperone with other biotin labeled proteins. PfBip did not bind to SAv-agarose beads in the absence of biotin (figure 13) confirming that the protein does not directly interact with the streptavidin or the agarose.

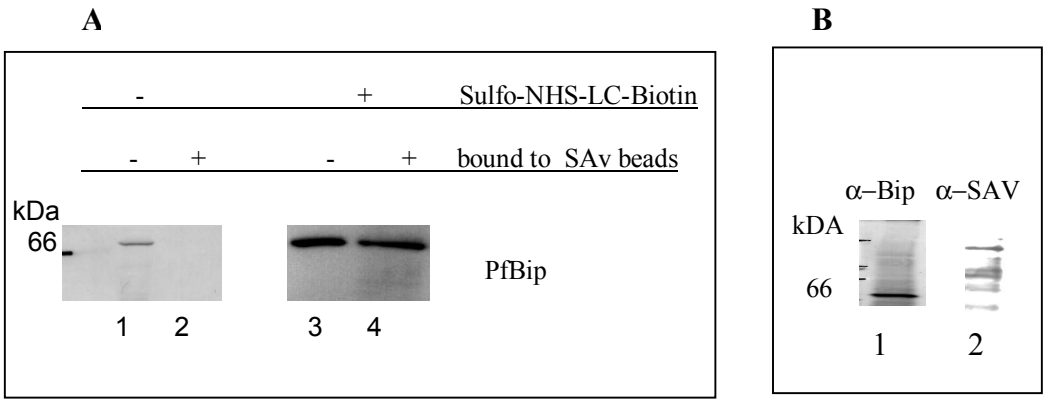


Figure 13. Molecular chaperone Bip interacts with biotinylated proteins. Infected erythrocytes were permeabilized, and one aliquot of the cells was treated with sulfo-NHS-LC-biotin the other was not treated. Lysates of cells were prepared and incubated with SAv-agarose beads. The fraction of unbound proteins (*lanes 1* and *3*) and the fraction of bound proteins (*lanes 2* and *4*) were analyzed for the presence of PfBip using a specific antiserum. *Lanes 1* and *2,* untreated sample; *lanes 3* and 4. In **B**, infected erythrocytes were SLO permeabilized and treated with sulfo-NHS-LC-biotin. PfBip was immunoprecipitated from the sample and analyzed by immunoblotting using either the specific the specific antiserum A, (*lane 1*) or the peroxidase-conjugated SAv (*lane 2*).

Experiments were further done to characterize the location of Bip in infected erythrocytes by subjecting the cells to saponin lysis and SLO permeabilization. Infected erythrocytes were SLO permeabilized or lysed with 0.15% saponin, after sedimentation the soluble proteins were collected and the pellets were divided into two one aliquot of each was trypsin treated and the other one remained untreated. Proteins contained in the soluble fraction and pellet, respectively, were separated by SDS-PAGE and subsequently transferred to nitrocellulose filters. The filters were incubated with anti-PfBip antiserum. While SLO preserves the integrity of the PVM saponin lysis disrupts the integrity of the PVM and exposes the vacuolar contents to proteolysis by trypsin. These results clearly show that when iRBC are subjected to lysis by both agents Bip is retained within the parasite and since it is not released by the lysis procedure it is not degraded by trypsin. Therefore the protein is not likely to be present in the vacuolar space (Figure 14 *lanes 1* and *4*). It is most likely that Bip and possibly other intraparasite chaperones associate with the SAv-agarose beads through interactions with misfolded proteins including biotinylated proteins of the vacuole.

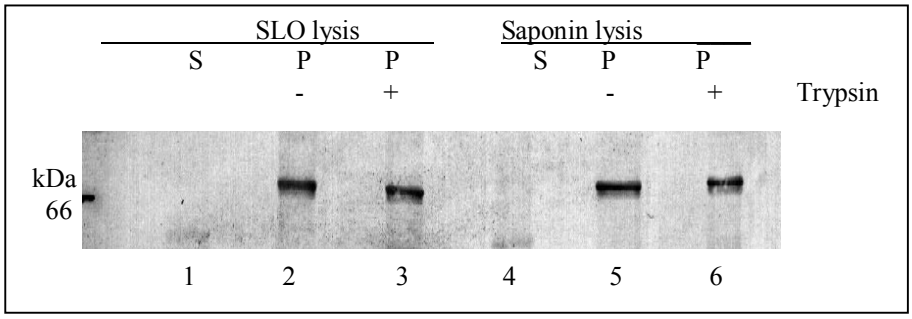


Figure 14. Localization of Bip in infected erythrocytes. Infected erythrocytes were SLO permeabilized or lysed with 0.15% saponin and the soluble proteins were collected and one aliquot of each pellet was trypsin treated and the other remained untreated. The lysates (*lanes 1* and *4*) and the pellets without trypsin treatment (*lanes* 2 and *5*) and with trypsin treatment (*lanes* 3 and *6*) were analyzed by SDS-PAGE and immunoblotting using antisera against PfBip; and the bound antibody were visualized by using alkaline phosphatase conjugated secondary antibody.

Chaperones are released from their substrates in a process that requires the hydrolysis of ATP (Young and Hartl 2000)..To investigate whether PfBip was associating with the biotinylated proteins due to its chaperonic activity iRBC were SLO permeabilized and treated with sulfo-NHS-LC-biotin. The soluble fraction after lysis was affinity purified using SAv beads. The beads were washed with either HEPES buffer, HEPES buffer containing Mg^{2++} or HEPES buffer containing Mg2++ and 5 mM ATP. The bound and unbound fractions were analysed for the presence of SERP and PfBip. SERP was consistently detected in the bound fraction in contrast PfBip was released from the beads in the presence of ATP (Figure 15). The addition of ATP led to the elimination of the of the co-isolation of Bip and possibly other non-biotinylated molecular, with the biotin labeled proteins bound to the SAv-agarose beads. The inclusion of ATP did not have an effect on the binding of SERP and/or possibly other biotin labeled proteins that are bound to the SAv beads. (Figure 15 *lane* 6).

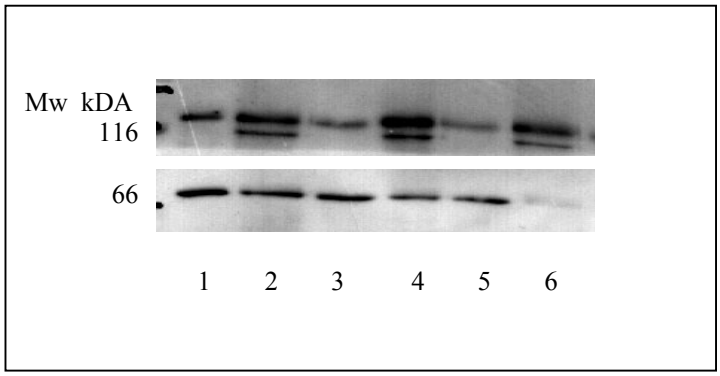


Figure 15. ATP releases Bip from streptavidin beads. Infected erythrocytes were SLO permeabilized and treated with sulfo-NHS-LC-biotin. Biotinylated proteins were bound to SAv beads and the beads were washed with HEPES buffer (*lanes 1*and *2*), HEPES buffer containing Mg^{2++} (*lanes 3* and *4*) and HEPES buffer containing Mg2++ and 5 mM ATP (*lanes 5* and *6*). Proteins that remained bound to beads (*lanes 2, 4,* and *6*) and unbound proteins (*lanes 1,3* and *5*) were analysed for the of SERP and PfBip.

Two important conclusions can be drawn from these experiments; first there is no positive evidence for vacuolar location of PfBip, which is shown by the lack of biotin label in the immunoprecipitated Bip after biotinylation of SLO permeabilized cells.Secondly PfBip is not released by treatment with either SLO or saponin. The inaccessibility of the protein to trypsin after both treatments also confirms the internal localization of the protein. The experiments also show that Bip, an ER localized chaperone, binds to the putative vacuolar proteins in lysates. Therefore extreme care must be taken to avoid binding of chaperones to other proteins after preparation of cell lysates. To avoid mistaking the identification of chaperones as vacuolar located, ATP was included in subsequent experiments.

3.9 Immunoelectronmicroscopy of SLO permeabilized and biotinylated IRBCs.

The localization and distribution of biotin was investigated using non-biotinylated iRBC as a control. Thin sections of SLO permeabilized iRBC were incubated with gold conjugated streptavidin and the localization of the gold particles observed under electron microscopy. The plasma membrane of the infected erythrocyte was clearly delineated with electron dense regions characteristic of the knobs clearly visible (Figure 15). The erythrocytic cytosolic space showed no evidence of the presence of hemoglobin which is usually observed in electronmicrographs of infected erythrocytes as a dark staining region (Dluzewski., 1989). The presence of an apparently intact erythrocyte plasma membrane but with the total absence of hemoglobin provides further evidence that the host cell cytosol, which predominantly comprises hemoglobin, is extracted after SLO permeabilization and the subsequent washing steps

Biotinylation of the compartments in the permeabilized IRBC was evaluated by the localization of the gold label particles as shown in the electron micrograph (Figure 14). The gold particles localized on both the external and internal surfaces of the erythrocyte plasma membrane. There was also labeling on the surface of the PVM and the vacuolar space. However, the PVM and PPM were in most areas in very close apposition to one another making the visualization of the vacuolar space difficult. Most importantly there were no gold particles visualized inside the parasites observed, in few cases a very small number of the gold particles localized within the parasites, probably non-specifically. These results confirm that after SLO lysis, the erythrocyte cytosol is extracted and the biotin derivative gains access to the erythrocyte cytosolic space, the PVM and the vacuolar space. Biotin labeling of the PVM and vacuolar space occurs but there is no obvious internal biotinylation.

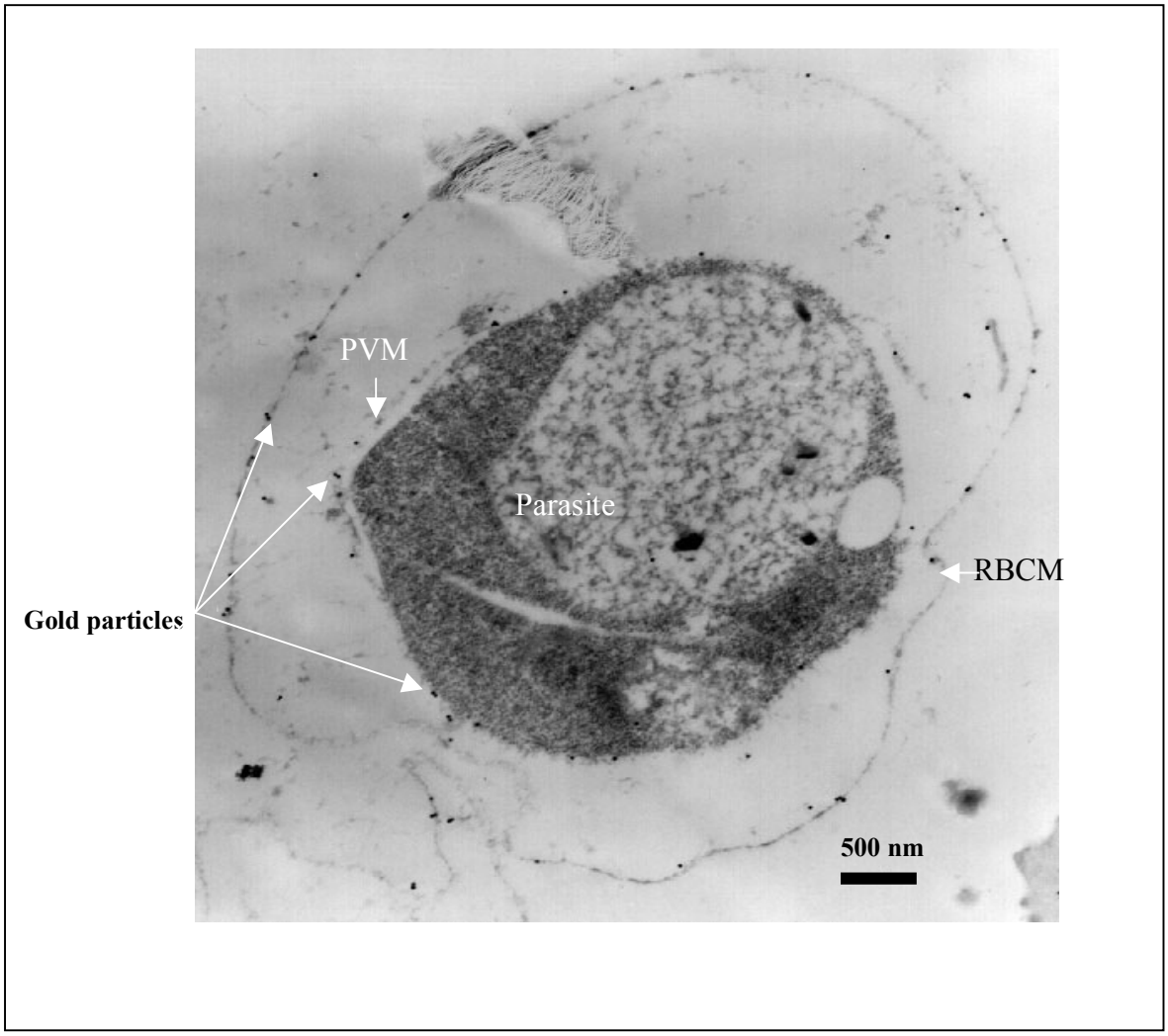


Figure 16. Electron micrographs of Streptavidin-immunogold-labelled sections of SLO permeabilized and biotinylated infected erythrocytes. IRBC, permeabilized with SLO and biotinylated with sulfo-NHS-LC-biotin embedded in Lowicryl and labeled with gold conjugated Streptavidin.

3.10 Analysis of biotinylated soluble proteins by SDS-PAGE.

The previous experiments showed selective biotin labeling of vacuolar proteins as shown by the vacuolar markers SERP and GBP. To analyze the abundance and diversity of the biotin labeled, putative vacuolar proteins, iRBC were incubated with L [^{35}S] methionine for 2 hours before SLO permeabilization and incubation with sulfo NHS-LC- biotin. Since the host erythrocytes do not synthesize proteins, the L [^{35}S] methionine is incorporated only into parasite protein and it is possible to identify them in autoradiographs. Cell lysates were prepared by freezing and thawing. Subsequently, the fraction containing soluble proteins was incubated with SAv agarose beads. Proteins bound to the beads and unbound proteins were separated by SDS-PAGE. The gel was stained with colloidal Coomassie, and subsequently dried and exposed to X-ray film to visualize the separated protein bands by autoradiography. There is a difference between the protein bands in the two fractions as shown by the autoradiography (Figure 17).

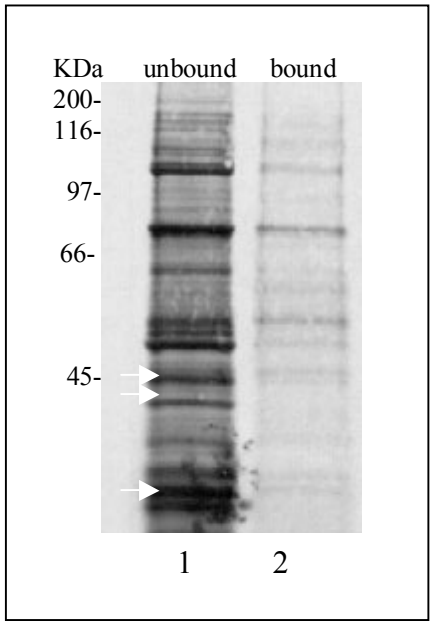


Figure 17. Analysis of biotinylated soluble proteins by SDS-PAGE. Infected erythrocytes were metabolically labeled with L-[^{35}S] methionine and permeabilized with SLO. The erythrocyte cytosol was extracted and the cellular fraction consisting of intact parasites and the intact PVM and PPM was treated with sulfo-NHS-LC biotin and then lysed by freezing and thawing. The soluble fraction obtained was incubated with SAv agarose beads and the unbound (lane 1) and bound fractions (lane 2) were analyzed by SDS-PAGE. Some of the proteins that are abundant in the unbound fraction but absent in the bound fraction are marked with arrowheads.

As one would expect fewer proteins are detectable in the bound fraction than in the eunbound fraction. However there was no protein band that was exclusively in the bound fraction, by definition an exclusively vacuolar space resident protein. There were proteins that are not

bound at all to the SAv-agarose beads, these are indicated by the closed arrows. The proteins that are abundant in the unbound fraction were also the most prominent in the bound fraction. It is important to note that since the vacuolar space is a sorting compartment it contains at least two categories of proteins, the vacuolar resident proteins such as SERP, and the transit proteins which are outward bound such as GBP and KAHRP (Ansorge *et al.,* 1996; Wickham *et al*., 2001) and also possibly the proteins that are re-imported into the parasite (Cheresh et al., 2001).

A second experiment was designed to demonstrate that the proteins that are bound are actually biotinylated and that there are no chaperonic protein-protein interactions that may lead to non-biotinylated proteins being co-isolated with biotin labeled proteins. Three batches of IRBC were prepared for analyses as follows. The first aliquot was incubated for with L [^{35}S] methionine for 2 hours before SLO permeabilization. The parasite and the vacuolar contents were subjected to 3 cycles of freezing and thawing, before sedimentation to obtain the soluble fraction for further analysis. The second aliquot was prepared in a similar way but the soluble proteins were biotinylated with sulfo NHS-LC-biotin at a concentration of 1mg ml^{-1} for 30 min at 4°C. The third aliquot was treated as the second but without the initial labeling with L [^{35}S] methionine.

The three aliquots were analyzed as follows; equal amounts of the first and second aliquots were incubated with SAv-agarose beads overnight at 4 °C. The bound and unbound fractions were analyzed by SDS-PAGE Coomassie staining and autoradiography. The results (Figure 18a *lanes 1, 2, 3* and *4*) clearly show that non-biotinylated proteins do not bind to the SAv-agarose beads. In a second analysis, an aliquot of non-biotinylated radiolabeled soluble proteins was mixed with an equal amount of biotinylated non-radiolabeled soluble proteins. The mixture was incubated overnight with SAv-agarose beads and the bound and unbound fractions were analyzed by SDS-PAGE and autoradiography. No radioactive signal was detected on the bound fraction clearly indicating that the non-biotinylated proteins do not interact with the SAv-agarose beads nor with the bound biotinylated proteins (Figure 18a lanes 3 and 4) since a coomasie staining showed the presence of biotinylated but non-radiolabeled proteins in the bound fraction (Figure 18b).

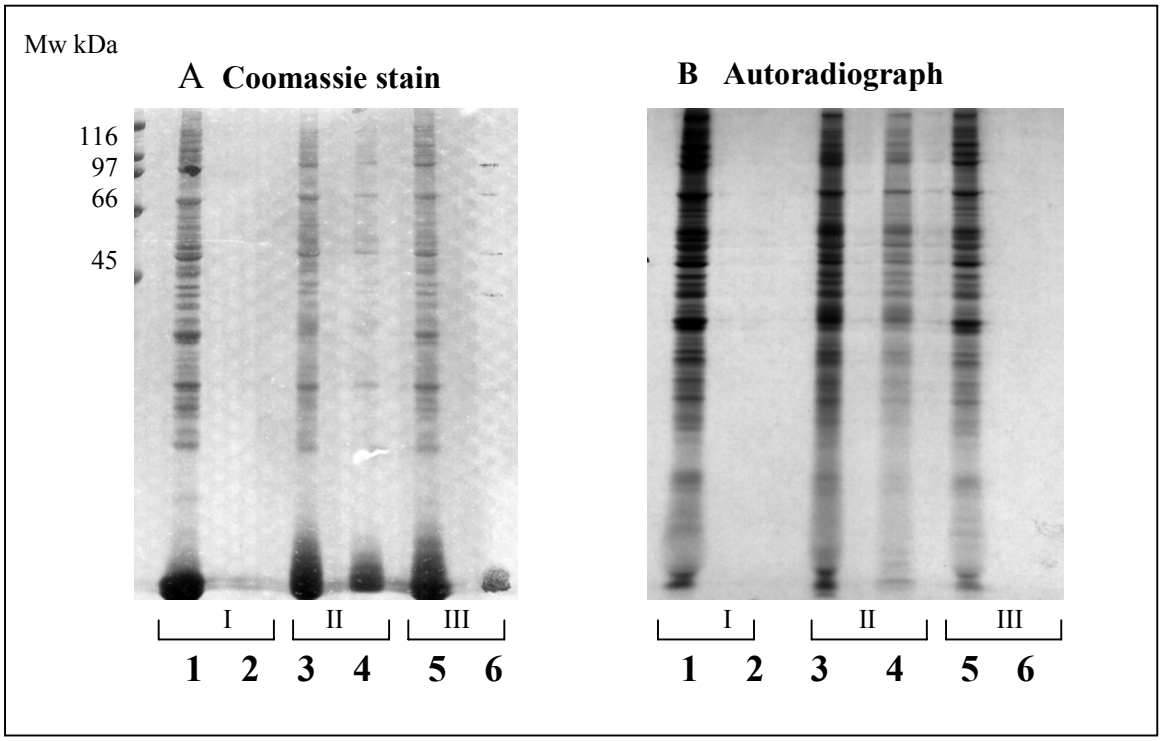


Figure 18. The streptavidin beads specifically bind biotin labeled proteins. Infected erythrocytes were metabolically labeled with L- [^{35}S] methionine and permeabilized with SLO. The cellular fraction consisting of intact parasites and the intact PV was either I, mock treated or incubated with sulfo-NHS-LC biotin, II. In a third analysis, III, an aliquot of non-biotinylated radiolabeled soluble proteins was mixed with an equal amount of biotinylated non-radiolabeled soluble proteins. The soluble fractions each corresponding to 2 x10^8 cells were incubated separately with SAv-agarose beads. The samples were then processed for SDS-PAGE and the separation patterns analysed after Coomassie staining (panel A). The unbound fractions are in *lanes1,3* and *5*. The bound fractions in *lanes* 2, 4 and 6. The gel was dried before exposing it to X-ray film to obtain an autoradiograph (panel B).

8M urea is a strong chaotropic agent that dissociates most non-covalent protein-protein interactions. In an additional experiment 8M urea was used to wash the beads and any dissociated proteins analyzed as follows. IRBCs were incubated for 2 hours with L-[^{35}S] methionine before SLO permeabilization and biotinylation. The soluble fraction was obtained by centrifugation after 3 cycles of freezing and thawing. Two aliquots, each containing the equivalence of 2x10^8 IRBC werw incubated overnight with SAv-agarose beads. In one aliquot, the beads were sequentially washed in buffers (A 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% NP40, 2 mM EDTA; B 10 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.2% NP40, 2 mM EDTA; C 10 mM Tris-HCl pH 7.5 and finally with PBS^{++}. The beads were then washed with 10 mM HEPES buffer pH 7.4 containing 20 mM $MgCl_2$ and 5 mM ATP. In the second the beads were washed in the same way

but with a final wash using 8M urea. The bound proteins were eluted with SDS-PAGE sample buffer and analyzed together with the unbound and the urea wash by SDS-PAGE and autoradiography (Figure 19). The urea does not release the proteins that are bound to the SAv-agarose beads, and there is no detection of radiolabeled protein bands thus confirming that there is no noticeable release of proteins by washing the SAv-agarose beads with 8M urea.

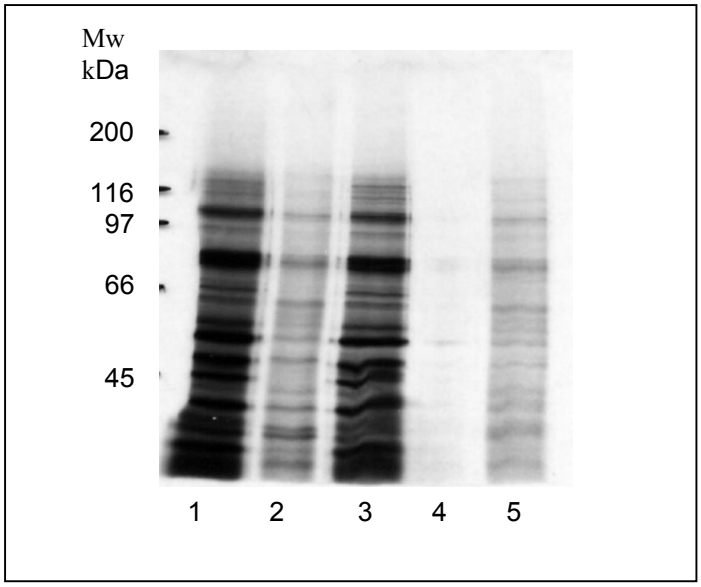


Figure 18. The bound proteins are specifically biotin labeled and are resistant to urea extraction from the beads. Infected erythrocytes were metabolically labeled with L-[^{35}S] methionine. IRBC were permeabilized with SLO. After complete extraction of the erythrocyte cytosol, the cellular fraction consisting of intact parasites and the intact PV was incubated with sulfo-NHS-LC biotin and lysed after the removal of free biotin. Two aliquots of the lysate each corresponding to 2 x10^8 cells were incubated separately with SAv-agarose beads. The samples were then processed and for SDS-PAGE and analysed by autoradiography. One aliquot was analysed directly for the unbound (*lane1*) and bound fraction (lane 2). The second aliquot was analysed for the unbound fraction (lane 3) and the proteins that are eluted after a 8M Urea wash (lane 4) and the bound fraction after the urea wash.

These results show that bond formed between avidin and biotin is resistant to 8M urea. This is consistent with their high affinity (K_a ~10^{15} M^{-1}) which can only be disintegrated using 8M guanidine chloride (Pierce Product Catalogue). This further confirms the suitability of the biotin-avidin system in affinity purification when using the sulfo-NHS-LC-biotin which has a non-cleavable spacer between the biotin molecule and the reactive NHS group.

3.11 Two-dimensional Gel electrophoretic analysis of Biotin labeled proteins.

The results from the analysis of the soluble proteins in the preceding sections show that there is selective biotin labeling of the putative vacuolar proteins. However the separation by SDS-PAGE was not sufficient to clearly resolve proteins of slightly different molecular masses. Some of the very prominent bands observed could be different polypeptides with the same molecular mass. Therefore a clearer and more accurate picture of the protein abundance and diversity could only be ascertained by using 2D gel electrophoresis where the proteins are separated on the basis of two different properties, pI in the first dimension and molecular mass in a second dimension. The net result is the resolution of individual proteins as single spots with a definitive pI and molecular mass.

The isolation and identification of novel vacuolar proteins required a high resolution of the separated and biotinylated proteins. Since the interaction between streptavidin and biotin is very tight ($K_a \sim 10^{15}\ M^{-1}$) and since isoelectric focussing does not allow boiling of sample in SDS to release the biotin labeled proteins from SAv agarose beads (as in the 1-D analysis) the most rational strategy is to use sulfo-NHS-SS-Biotin and cleave biotinylated proteins from the beads before 2D gel electrophoresis. Infected erythrocytes were radiolabeled with L-[^{35}S] methionine before SLO permeabilization and treatment with the sulfo-NHS-SS-Biotin. After lysis of cells the soluble fraction obtained was incubated with SAv-agarose beads to bind the biotin labeled proteins. The bound proteins were traeted with incubating the beads with two reducing agents, DTT and TCEP before electrophoresis. These compounds act by reducing the disulphide bonds (S-S) in the spacer arm between the protein and the biotin derivative thus releasing the protein from the biotin avidin complex.

Numerous experiments with varied concentrations of these reducing agents were used but it was not possible to efficiently elute the proteins from the beads without the addition of SDS. This was apparent in 1D analysis where the biotin labeled proteins remained bound to the SAv agarose beads after incubation with the reducing agents. The bound proteins could only be eluted in a second step after boiling the beads in SDS sample buffer. The addition of SDS, a highly negatively charged detergent to the elution buffer proved to be highly disadvantageous in the 2D-gel analysis. The SDS ´envelopes` all the polypeptides with a high negative charge which is much greater than their net intrinsic charge. The result was that the polypeptides could not be effectively used in 2D analysis because of their net negative charge and also due to the disruption of the pH gradient in the IPG strips by the detergent. Under these conditions isoelectric focussing does not occur. The resulting 2D gels showed very extensive horizontal streaking. It was not possible to use either dialysis or even higher concentrations other zwitterionic detergents such as TritonX-100 or

CHAPs to replace SDS in the elution assays. Competitive inhibition assays using low concentrations of D-biotin (5 mM) to elute biotin labeled proteins from the SAv agarose were also done but the amount of protein obtained was not sufficient to allow analysis by 2D-gel electrophoresis. Therefore the experimental approach was slightly changed, and sulfo-NHS-LC-biotin was used instead of the sulfo-NHS-SS-Biotin. The affinity purification step using the reducing agents was left out altogether. Instead the total soluble fraction was used in 2D-gel electrophoresis and biotin labeled spots as identified in western blots were selected for further analysis.

IRBCs were labeled metabolically labeled with L- [^{35} S] methionine, permeabilized with SLO and treated with sulfo-NHS-LC-biotin. After cell lysis, soluble proteins were separated in triplicate on different two –dimensional gels using a pH gradient of 4-7 in the first dimension. Initial experiments were done using IPG strips with pH gradient 3-10. The results showed very few proteins in the alkaline regions, confirming that most of the *P. falciparum* proteins have a low pI and they focus in the acid regions of the gradient. Subsequent separations were done using 13cm pH 4-7 IPG strips. To estimate the relative abundance of proteins in permeabilized erythrocytes, one gel was silver stained (figure 18A), and another was stained with Coomassie (figure 20B) The silver staining, which has a sensitivity of up to 1ng per spot revealed the more complex protein pattern. The sensitivity of the Coomassie is down to 20 ng and the most abundant proteins were well detected. There were fewer protein spots in the higher molecular weight regions, this is probably due to the fact that because of their large size, the high molecular weight proteins do not move into the IPG strip since they do not penetrate the pores in the IPG gel during the rehydration step. Alternatively this could also be due to inefficient transfer of the bigger polypeptides from the IPG strip to the second dimension SDS-PAGE. In an attempt to improve on this, the rehydration time was increased from 12 hours to 24 hours and the SDS concentration in the equilibration buffer after the first dimension was increased from 2% to 4%. The second dimension run was also started and maintained at low voltage (70 volts) for 2 hours to improve on the sample entry. The reproducibility of sample preparation, gel electrophoresis and gel staining was investigated by running sequential replicate gels with protein prepared from different batches of parasites on different days. The majority of protein spots could be resolved with a high degree of reproducibility.even though there are slight differences as indicated with the circles.The outcome of a typical experiment is shown in figure 21, each panel (i-iii) represents a magnified inset of Coomassie stained gels from 3 different batches of parasites run on different days, thus introducing the maximum number of potential variables. To compare biotinylated proteins to the total parasite proteins, proteins of a third gel were transferred to a nitrocellulose filter. The filter

was exposed to X-ray film to analyze the pattern of radiolabeled proteins (figure 20C). Subsequently, biotin-labeled proteins were detected using alkaline-conjugated SAv (Figure 20D). A comparison of the biotin-labeled proteins and the radiolabeled proteins clearly demonstrates that the pattern of biotin-labeled proteins is less complex and that it does not simply reflect the pattern of the most abundant radiolabeled proteins.

In contrast, the most intensely radiolabeled proteins appear not to be biotin labeled. Most non-biotin labeled protein spots appear as single spots in the two-dimensional pattern. In contrast, most biotin-labeled protein species appeared as horizontal spot series, which could represent the same protein with slight modifications that alter the pI. This was later confirmed for some of the identified spots (see section 3.13.2). Probably, a variable number of amino groups react with the sulfo-NHS-LC-biotin derivative resulting in a differential loss of protonable residues and stepwise decreasing pI values. This shift was also accompanied by a slight increase in molecular size since every biotin labeling at a free amino groups lead to a mass increase of 339 Daltons. A detailed comparison reveals 12 prominent spots (circled in Figure 19B), which represent highly abundant parasite proteins that are detectable by Coomassie Blue staining the same spots were also identified on the autoradiograph (20C) In the western blot analysis of biotinylated proteins, 9 of these spots appeared as a negative stain (Fig 20D) presumably because of the high local concentration of non-biotinylated protein. Thirty-nine protein spots were identified that were both biotinylated and radiolabeled and therefore fulfill the criteria expected for vacuolar proteins. Some of these spots may represent identical polypeptides that are biotinylated differentially.

Initial MALDI-TOF experiments, as described in more detail in section 3.13.2, identified the two most abundant protein spots (I and II) (Figure 20 A-D) as *P. falciparum* enolase (EC 4.2.1.11) 2-phosphoglycerate dehydratase (I) a glycolytic enzyme and (II) *P. falciparum* ornithine amino transferase (EC 2.6.1.13) an enzyme that catalyzes the transfer of an alpha-amino group from an amino acid to an alpha-keto acid. These are cytosolic housekeeping enzymes that are highly abundant and the fact that they are non-biotinylated confirms that the biotin derivarive has no access to the parasite cytosol.

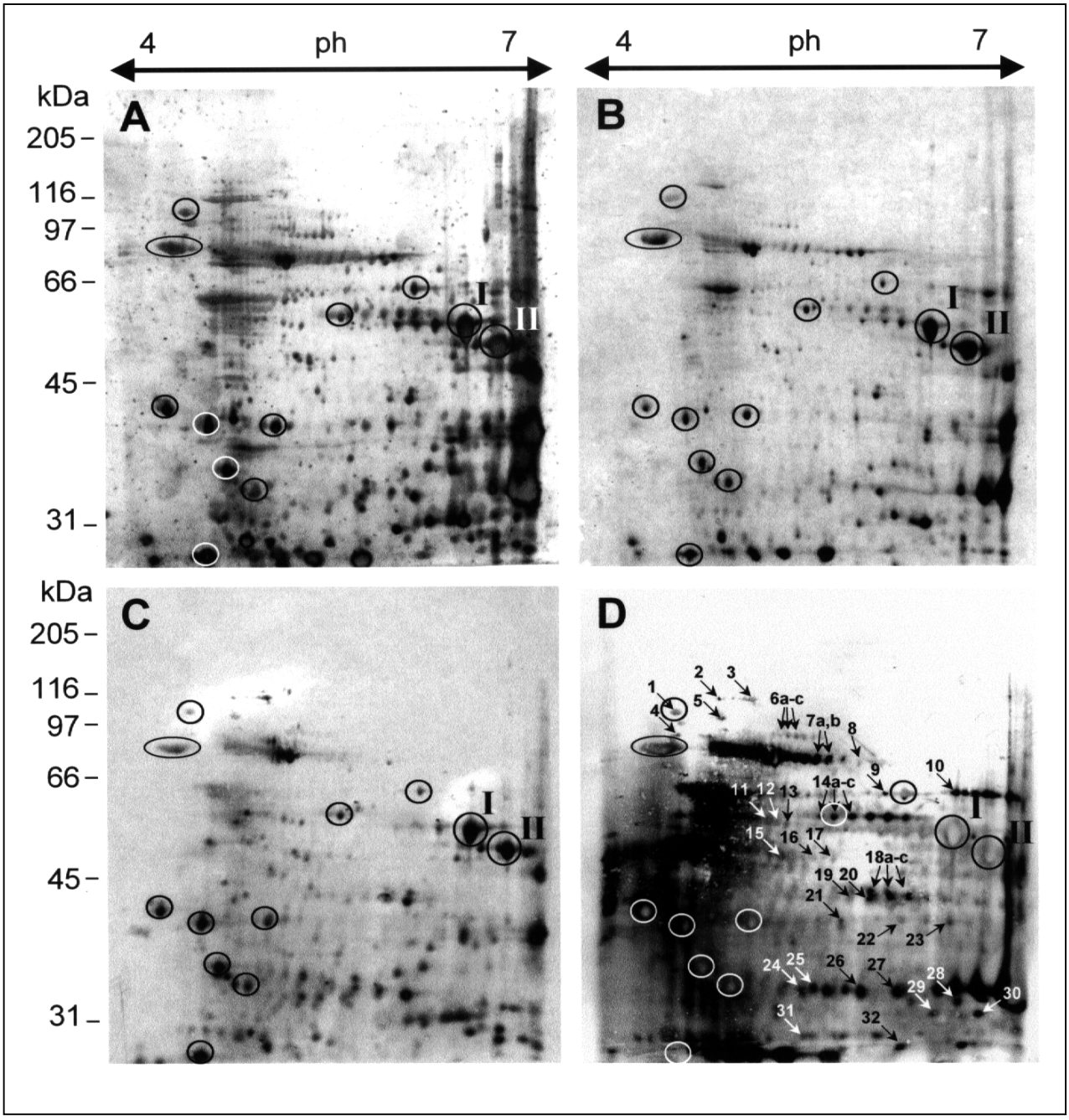


Figure 20. Protein pattern of putative vacuolar proteins. Proteins in intact infected erythrocytes were metabolically labeled, and cells were permeabilized and treated with sulfo-NHS-LC-biotin. The fractions of soluble proteins. Each corresponding to 2 x 10^9 infected erythrocytes was subjected to two-dimensional gel electrophoresis using a pH gradient of 4-7 in the first dimension and 12% SDS-PAGE in the second dimension. *A*, silver-stained gel. *B*, Coomassie Blue stained gel. *C* and *D*. proteins from a third gel were transferred to a nitrocellulose filter. The filter was first exposed to x-ray film to visualize metabolically labeled parasite proteins and subsequently reacted with alkaline phosphatase-conjugated SAv to visualize biotinylated proteins (*D*). The most prominent spots that were identified as parasite proteins are marked with *circles*. Proteins that are both biotinylated and radiolabeled are indicated by *Arabic numerals*; spots that may represent *identical peptides* at various degrees of biotinylation are marked with lowercase letters. *I, P. falciparum* enolase; *II P. falciparum* ornithine aminotransferase.

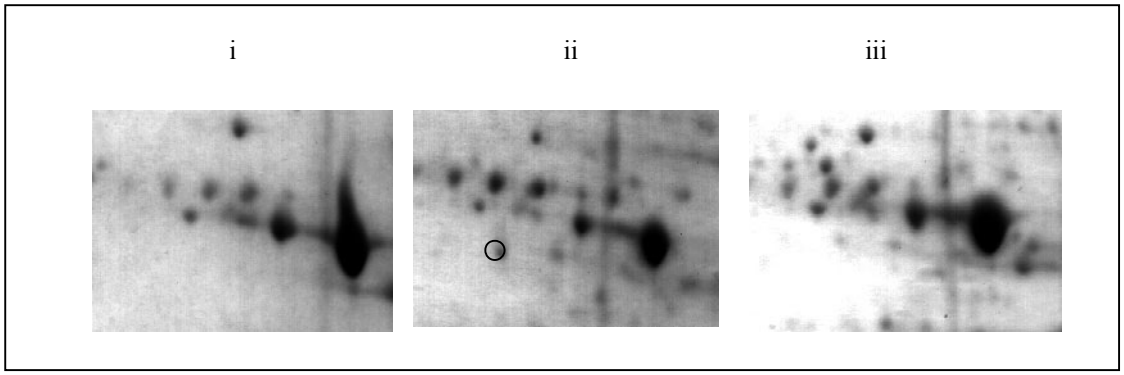


Figure 21. Reproducibility of 2-DE gels. Reproducibility of sample preparation, gel electrophoresis and staining. The panels (*i-iii*) represent the same enlarged region of Coomassie-stained gels. Each of the three gels were run independently on different days using different batches of parasites after SLO permeabilization and biotinylation and using different batches of poured second dimension gels.

3.12 Biotinylation pattern of the membrane proteins.

In infected erythrocytes the parasite is enclosed by 3 different membranes, the erythrocyte membrane, the parasitophorous vacuole membrane and the parasite plasma membrane. Other than the pores that are formed during SLO permeabilization, the erythrocyte membrane probably remains structurally stable surrounding the intact PVM, the vacuolar contents and the parasite. The erythrocyte membrane is clearly discernable in electron micrographs of SLO permeabilized cells (section 3.8) and band 3 an integral membrane protein of the erythrocyte was shown to be biotin labeled by immunofluorescence assay (Figure 5A). Other than the erythrocyte membrane proteins, with experimental approach the biotin should label parasite proteins that are present on the PVM and on the external face of the parasite plasma membrane. Figure 20 shows the expected biotinylation pattern of membrane proteins.

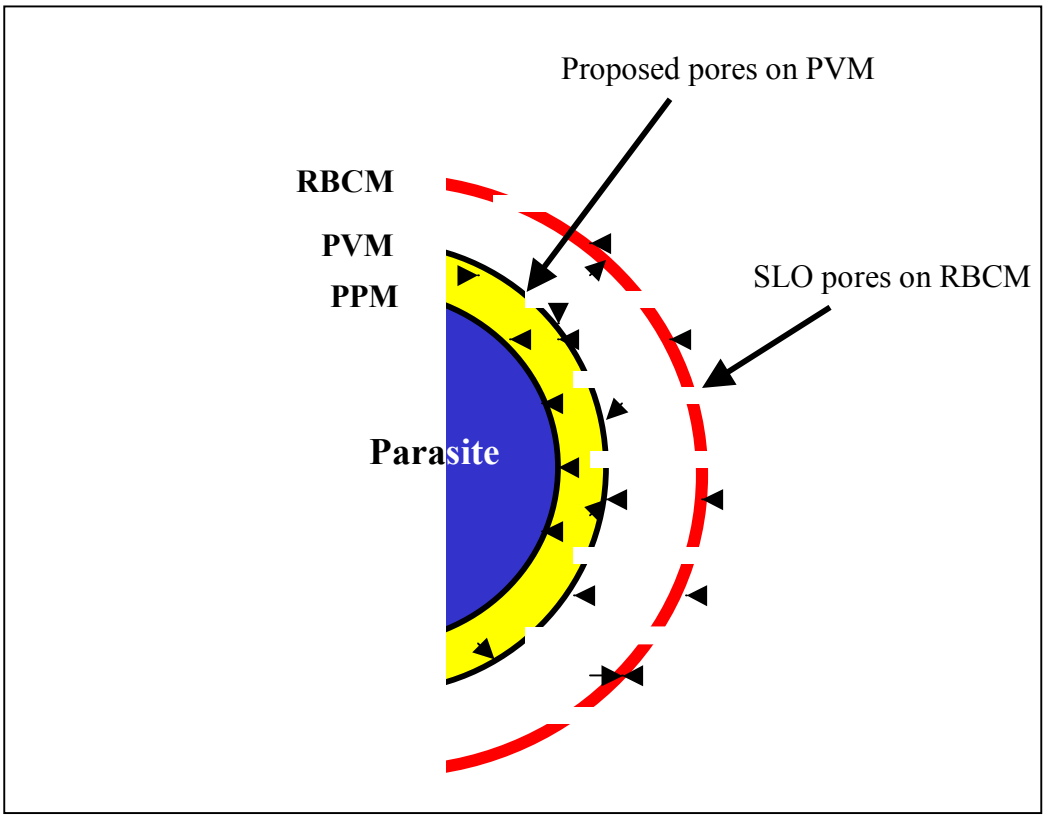


Figure 22. Schematic diagram of the membranes surrounding SLO permeabilized infected erythrocytes. RBCM, erythrocyte membrane protein, PVM parasitophorous vacuole membrane and PPM, the parasite plasma membrane. The parasite is shown in blue color and the vacuolar space in yellow. The biotin is readily accessible to the proteins of the EMP and both the internally and externally exposed proteins can be biotinylated after SLO permeabilization. The biotin derivative is depicted by closed arrow heads. Biotin should access the PVM after the extraction of the erythrocyte, and the proteins in both sides of the membrane can be biotinylated if the biotin access the vacuolar lumen through the proteinaceous pores are postulated to be on the PVM. The proteins on the external face of the erythrocyte membrane can also be biotinylated. However the non-permeant biotin derivatives do not penetrate the membrane and the internal proteins are not biotinylated.

To study the extent of biotin labeling of membrane proteins, iRBC were labeled metabolically labeled with L- [^{35}S] methionine, permeabilized with SLO and treated with sulfo-NHS-LC-biotin. After cell lysis, insoluble membrane proteins were obtained by sedimentation at 10,000 x g and separated in duplicate on different two-dimensional gels using a pH gradient of 4-7 in the first dimension. One gel was stained with Coomassie (figure 22A) and proteins of the second gel were gel were transferred to a nitrocellulose filter. The filter was exposed to x-ray film to analyze the pattern of radiolabeled proteins (Figure 22B). Subsequently, biotin-labeled proteins were detected using alkaline-conjugated SAv (Figure 22C) after which the filters were incubated with antisera against the marker proteins of the PVM exp-1 and exp-2 before visualization by ECL (Figures 22D i and D ii). A comparison of the biotinylation pattern with the antibody localization of the two proteins indicated a stronger biotin label for exp-1 compared to exp-2. This is expected since exp-1 is an integral membrane with the C-terminus exposed on the outer face of the PVM (Ansorge *et al*., 1997) and since the protein is therefore accessible to the biotin derivative after SLO lysis. In contrast exp-2 lacks a typical hydrophobic transmembrane domain and it has been suggested that the protein associates with the vacuolar membrane via an amphipathic helix located in the N-terminal half of the protein (Fischer *et al*., 1998). The tight association of EXP-2 with the PVM as demonstrated by its resistance to extractions with high salt or alkaline solutions (Johnson *et al*., 1994) would imply that there is a very strong and close interaction between the protein and the PVM. Since the protein does not have a transmembrane domain it is possible that the tight association the protein has with the PVM may be due strong electrostatic interactions that cause a helix orientation parallel to the to the membrane (Johnson *et al*., 1994). This may limit the access of biotin molecules to the protein resulting in its poor biotinylation as seen in the western blots. Since it has been shown that SERP, a soluble PV resident protein is biotin labeled (Figure. *5* and *10*) it is possible that the conformation of exp-2 in the vacuole does not have many free accessible amino groups to allow more extensive biotin labeling to occur. Electron micrograph studies showed a very close association between the parasite plasma membrane and the PVM (Figure 16). It has been previously suggested that the two membranes form regions of very close association. Taken together with the possibility of biotinylation blocking the proposed proteinaceous pores on the PVM, the consequence could be a reduction in the efficiency in the biotinylation of the vacuolar space proteins and the PVM associated proteins that are exposed to the lumen of the vacuole.

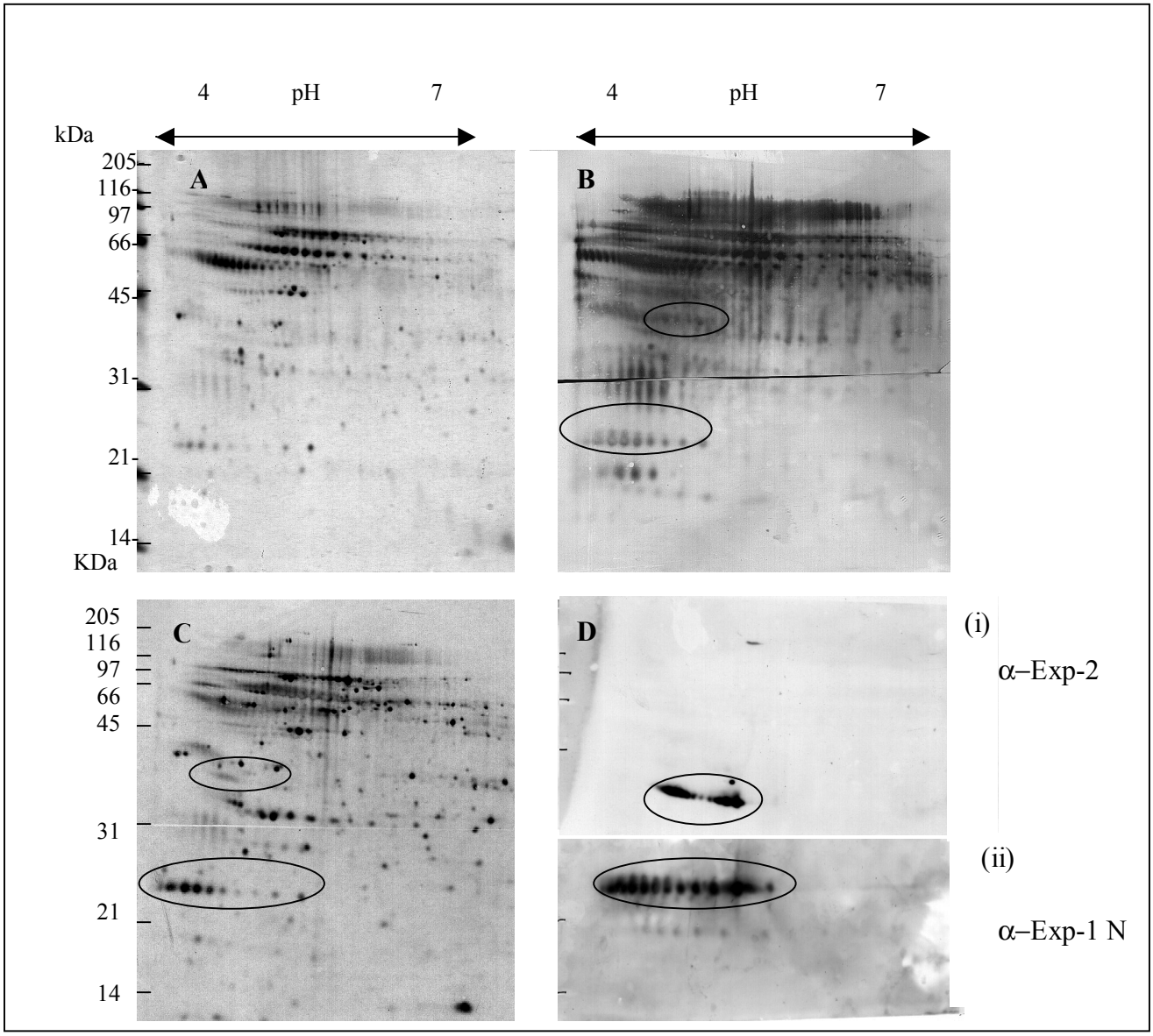


Figure 23. Biotinylation pattern of the membrane proteins. Proteins in intact infected erythrocytes were metabolically labeled, and cells were permeabilized and treated with sulfo-NHS-LC-biotin. The fractions of membrane proteins, each corresponding to 2 x 10^9 infected erythrocytes were subjected to two-dimensional gel electrophoresis using a pH gradient of 4-7 in the first dimension and 12% SDS-PAGE in the second dimension. *A*, Coomassie Blue stained gel. *B* and *C*, proteins from a second gel were transferred to a nitrocellulose filter. The filter was first exposed to x-ray film to visualize metabolically labeled parasite proteins (*C*) and subsequently reacted with alkaline phosphatase-conjugated SAv to visualize biotinylated proteins (*B*). The filter was divided into two parts (*D*) the upper portion was probed with antisera against exp-2 and the lower with antisera against exp-1 and subsequently reacted with horseradish-peroxidase secondary antibody to visualize the protein spots. The spots corresponding to the two marker proteins as identified after immuno detection are marked with the ellipses.

3.13 Mass spectrometry analysis results.

3.13.1 Comparison of biotin labeled and non-biotin labeled bovine serum albumin.

MALDI-TOF analysis relies on a highly accurate molecular mass determination of the peptides generated after enzymatic treatment of proteins. The method allows to differentiate between peptides on the basis of their molecular masses in the Dalton range. The modification of amino acids by biotinylation leads to differences of the expected masses that would make accurate assessments impossible. Therefore the accurate mass added on to a peptide during the biotinylation reaction as shown in figure 5 and as described for BSA in section 3.5 was determined by mass spectrometry.

The effect of biotinylation on the mass of peptides was experimentally determined as follows, one portion of fraction V of bovine serum albumin BSA was biotin labeled using 12-fold higher mole ratio of biotin. The other portion was not biotin labeled. The two aliquots were separated by SDS-PAGE and the protein bands were cut and processed for the MALDI-TOF analysis. A section of the spectra obtained are shown in figure 24 below. Both samples of protein were identified by peptide mass finger printing as BSA, however a comparison of the spectra clearly showed some differences, with some peaks present only in the biotin labeled sample. In the biotin labeled BSA sample, a peptide with mass of 1156.6593 Dalton and a high peak intensity is detected but it is absent in the non-biotin labeled sample. This peptide was further analyzed by post source decay fragmentation. The peptide was subjected to laser shots at a higher wavelength resulting in the fragmentation of the peptide into its constituent amino acids. The analysis determined the peptide sequence to be –(R)SLGKVGTR(C)- with a theoretical mass of 817.4896 compared to the observed mass of 1156.6507 Dalton as obtained by mass spectrometry (Figure 25). The mass increase was 339 Daltons, and analysis of the ions obtained by post source fragmentation identified a lysine amino acid with a similar increase in mass after modification by the biotin derivative as shown in figure 2. This mass can be accounted for by the biotin molecule and the spacer arm with a molecular formula ($C_{16}H_{25}N_3O_3S$) that is incorporated into the peptide. This can be described to have a sequence –LG**B**VGT- with a mass of 817.4896 Da where the B represents a lysine residue that is bonded to biotin through an amide bond at its epsilon amino group. The biotin molecule and spacer arm is added to a free amino group of a lysine when the biotinylation reaction occurs.

The increase in mass is responsible for the mass shift that is observed in figure 8, where there is reduced mobility for the biotin labeled BSA compared to the unlabeled. The biotin labeled BSA migrates at a higher molecular mass compared to the non-biotin labeled sample because of the incorporation of the biotin. Further analysis showed that the trypsin digestion did not occur

when the lysine residue was biotin labeled. The protease trypsin cleaves peptides at the carboxyl end of lysine and arginine residues and it is evident that biotinylation blocks its activity at these sites.

These results confirm that the biotinylation of proteins with the sulfo NHS-LC-biotin biotin derivative introduces an additional mass of 339 Daltons. Therefore this mass must be included as a possible modification when performing database searches during peptide mass finger printing using peptides that have been labeled with the derivative. The biotinylation did not prevent the identification of the protein and it was possible to identify both non-biotin labeled BSA and biotin labeled BSA by MALDI-TOF-MS. However the lack of trypsin cleavage at biotinylated lysine residues leads to reduction in the number of generated peptide peaks. The accuracy and probability of identification of proteins is greatly enhanced by having a high number of generated peptides. Therefore identification of highly biotinylated proteins may be impeded by the reduction of the number of peptides generated by trypsin digestion.

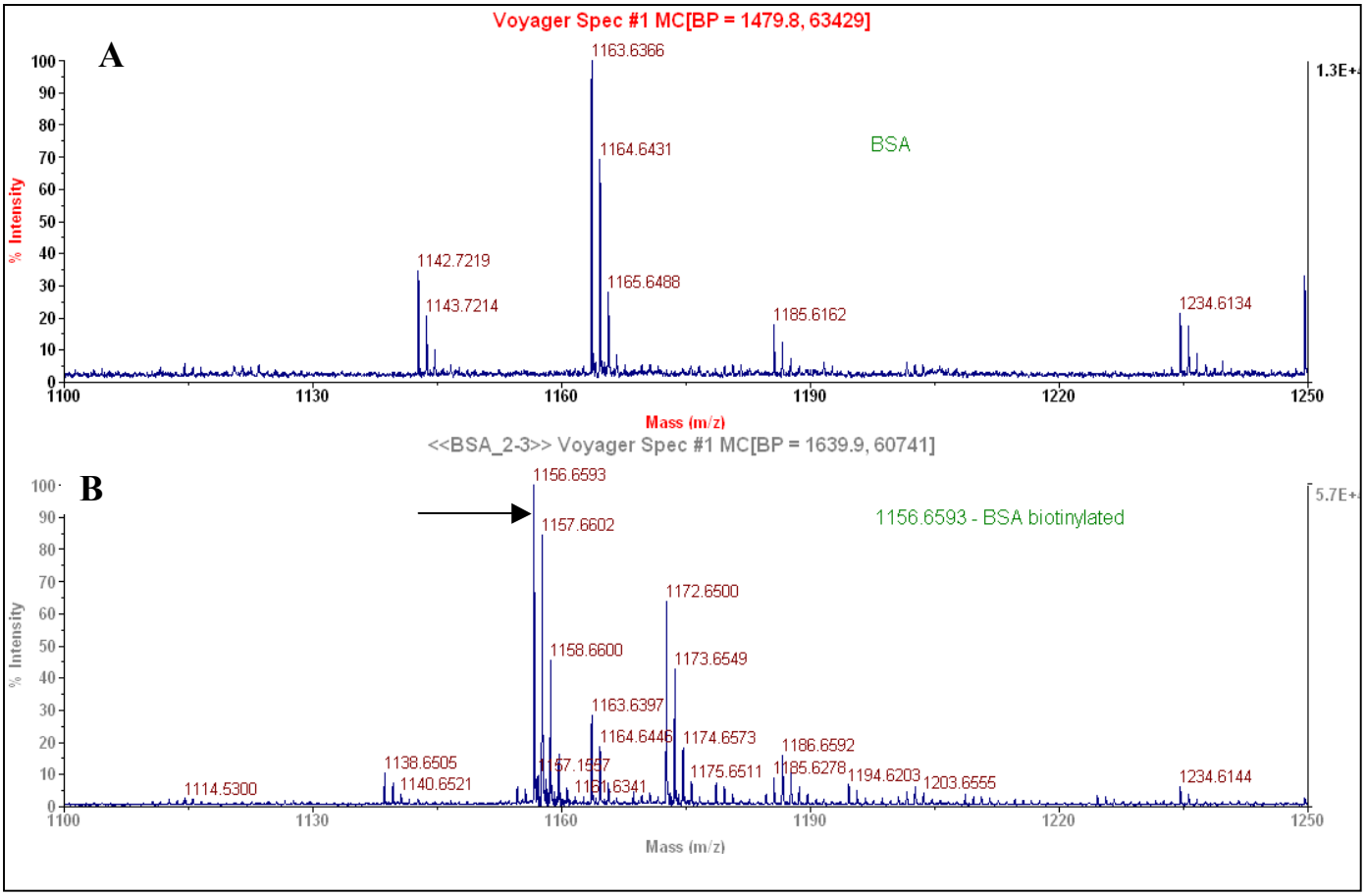


Figure 24A and B. Comparison of biotinylated and non-biotinylated BSA samples. One portion of fraction V of bovine serum albumin BSA was biotin labeled using 12-fold higher mole ratio of biotin. The other portion was not biotin labeled. The two aliquots were separated on a SDS-PAGE and the protein bands were cut and processed for the MALDI-TOF analysis. A shows a section of the spectra obtained for the non-biotinylated protein, the spectra B is from the biotinylated BSA sample. The marked peak (mass 1156.6593) is present in the biotinylated sample but absent in the non-biotinylated sample.

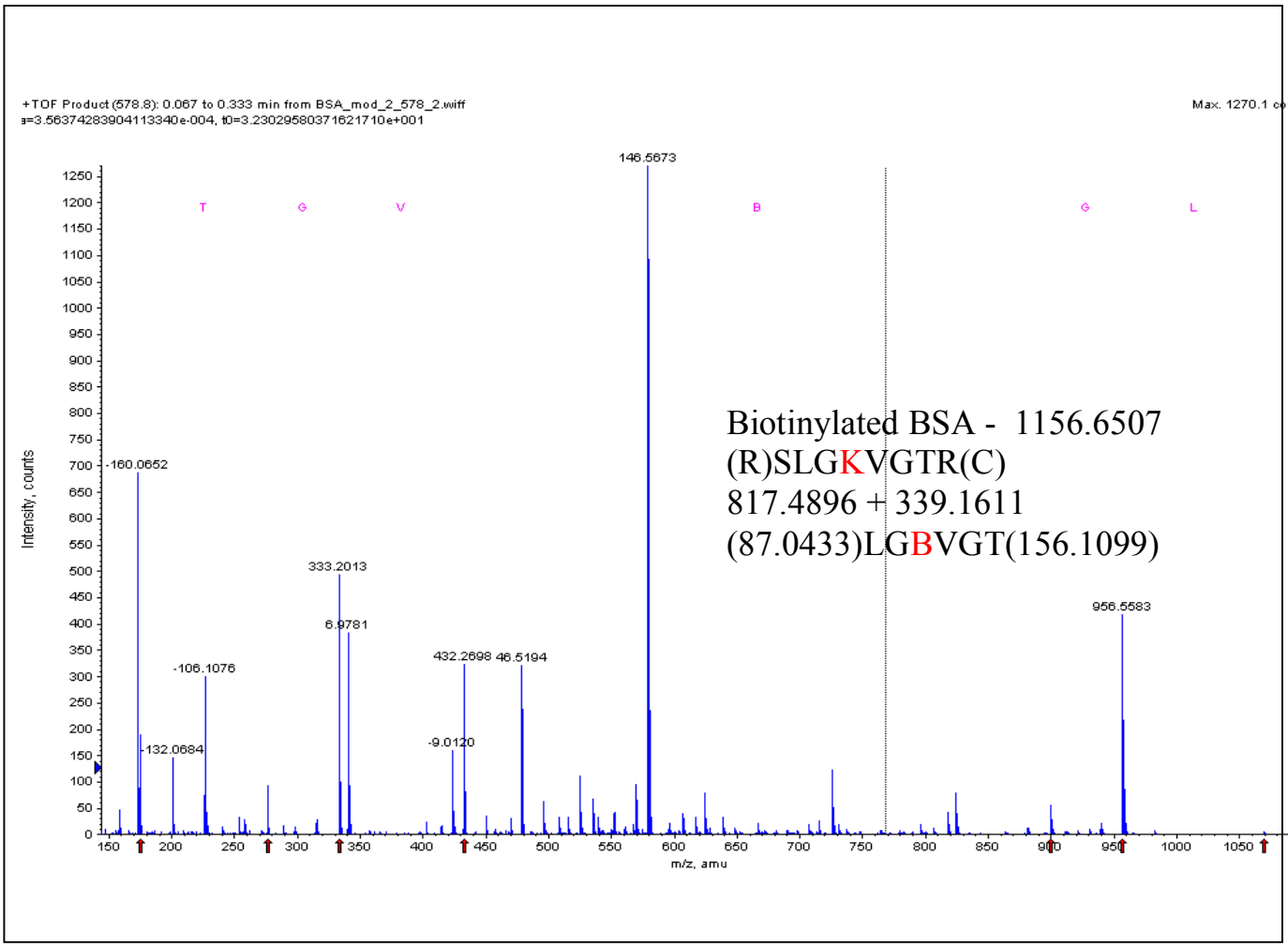


Figure 25. MALDI-MS-MS identification of biotin label in BSA peptides. The peptide peak with mass 1156.6507 was subjected to post source decay fragmentation and the resulting ions were analysed to identify the amino acid residues. The parent peptide fragmented to generate ions that were analysed and found to correspond to the amino acid sequence -LGBVGT- where B corresponds to a lysine residue modified with the group ($C_{16}H_{25}N_3O_3S$) from the biotinylation reaction. The peaks show the masses of the generated ions and their relative intensities with the identity of the respective amino acids above the ions.

3.13.2 Identification of *P. falciparum* proteins using peptide mass fingerprint data from MALDI-TOF mass spectrometry to search protein databases.

The proteins analysed by 2D-gelelectrophoresis as described in section 3.12 can be classified as follows, proteins detectable by Coomassie staining and Silver staining but not by autoradiography. These could be host cell proteins or proteins that are synthesized before the metabolic labeling with L-[^{35} S] methionine or alternatively parasite proteins that do not havea high number of methionine residues in their polypeptides. There are proteins that are metabolically labeled and those that are are metabolically labeled and biotinylated.

In order to assess the identities of a represenative sample of proteins, several protein spots including the most abundant were excised the 2D gels and used in MALDI-TOF analysis. The searching of the NCBI protein database with the generated peptide masses enabled 75 % of the total number of samples analyzed to be characterized from their peptide masses and full gene sequences. For example enolase, ornithine amino transferase, actin I, and 1-cys- peroxidoxin were readily identified along with other proteins from the NCBI and PlasmoDb databases (table 2). In the majority cases, theoretical molecular weight and isoelectric points obtained in the database searches matched to the position of the protein spots on the 2-DE gels. For example a protein spot was identified to be actin by peptide mass finger printing. The protein has a theoretical mass of 41870.9 Daltons and a calculated pI of 5.21. These values correspond closely to the position of the identified spot in the 2D gel. However in some samples the calculated pI values obtained in the database search and observed pI values from the spot position in the 2D gels were slightly different. The difference between the observed and calculated isoelectric focusing point may indicate the modification by the biotin derivative for the biotin labeled proteins and may also indicate co- and/or post-translational modification of the polypeptide within the parasite.

There were two prominent spots for both enolase and ornithine amino transferase, both abundant cytosolic proteins. Enolase (2-phospho-D-glycerate hydrolase, E.C.4.2.1.11) an enzyme that catalyzes the reversible dehydration of D-2-phosphoglycerate (PGA) to phosphoenolpyruvate (PEP) as a key step in both glycolysis and gluconeogenesis was the most abundant protein as shown by the Coomassie staining and autoradiographs of the 2D-gels. Ornithine amino transferase catalyses the transfer of an alpha-amino group from an amino acid to an alpha-keto acid was the second most abundant protein (figure 24). In the case of ornithine amino transferase there are two variants in the database with slightly different pIs and molecular masses. This is consistent with the results obtained in this study since two protein spots in the 2-D gel were positively identified by MALDI-TOF-MS to be ornithine amino transferase. On the other hand there is only one expressed enolase gene described in the PlasmoDb database and the two identified spots may be

due to modifications of the enzyme within the parasite since the spots are not biotinylated. The observed difference in pI between the two spots is too and large it may be due to other post translational modifications but not to biotinylation since each biotinylation reaction brings a pH change of approximately 0.05 units. Experiments done using non-biotinylated soluble parasite proteins showed the same result thus confirming that the two spots were not due to biotin modification. Indeed, experiments in yeast have shown that enolase complexes with Mg^{2+} for its enzymatic activity and further analysis demonstrated that an unprotonated histidine and a protonated lysine are important for substrate binding and catalysis. In the yeast enzyme the lysine 396 and histidine 159 are proposed to be the catalytic acid –base pair in the yeast enolase (Vinarov and Nowak, 1998). Therefore it could be possible that the different populations of enolase as seen in the 2-D gel could be due to the different reaction states of the enzyme or due to other post translational modifications.

The other abundant proteins that were identified include actin I, guanine nucleotide-binding protein and1cys-peroxidoxin. Actin is an abundant protein that forms actin filaments in all eukaryotic cells. The monomeric form is sometimes called globular or G-actin; the polymeric form is filamentous or F-actin which is formed by polymerization of globular actin molecules. Since the samples analyzed by 2-DE were the soluble fraction obtained after centrifugation at 100,000 x g it is most likely the soluble globular form is what was identified. The actin was identified as a single prominent spot which was not biotinylated as confirmed by western blot analysis and tandem mass spectroscopy. The two other proteins, guanine nucleotide-binding protein and 1cys-peroxidoxin were also not biotin labeled.

On the basis of the experimental rationale, the results propose the vacuolar presence of the following proteins, a cysteine protease (spot 20), Hsp90 (spot 19.0), Hsp70 (spot 18) and hypothetical proteins (spots 5,7 and 8). The identified cysteine protease is most likely PfSERP; sequence analysis revealed 97% identity and their molecular weight and pI of the protein were also identical. The N-termini of all identified proteins were analyzed to ascertain the presence of hydrophobic residues which could constitute putative signal sequences. Internal hydrophobic motifs that could also serve as signal sequences were also analysed. Table 4 shows the results obtained after subjecting the proteins to the Psort program (Nakai and Kanehisa 1992: http://psort.nibb.ac.jp/) for locating signal motifs and the Plasmodium data base at www.PlasmodB.org which uses the SignalP www server at http://www.cbs.dtu.dk/services/SignalP/output.html for signal peptide location and prediction. Analysis of these proteins for signal peptides revealed a N-terminal signal sequence for the cysteine protease, Hsp90 and the hypothetical protein spot 5 using the Psort program while the

Hsp90 was predicted not to have a signal peptide when using the SignalP server. No 'internal' signal sequences were detected for these proteins.

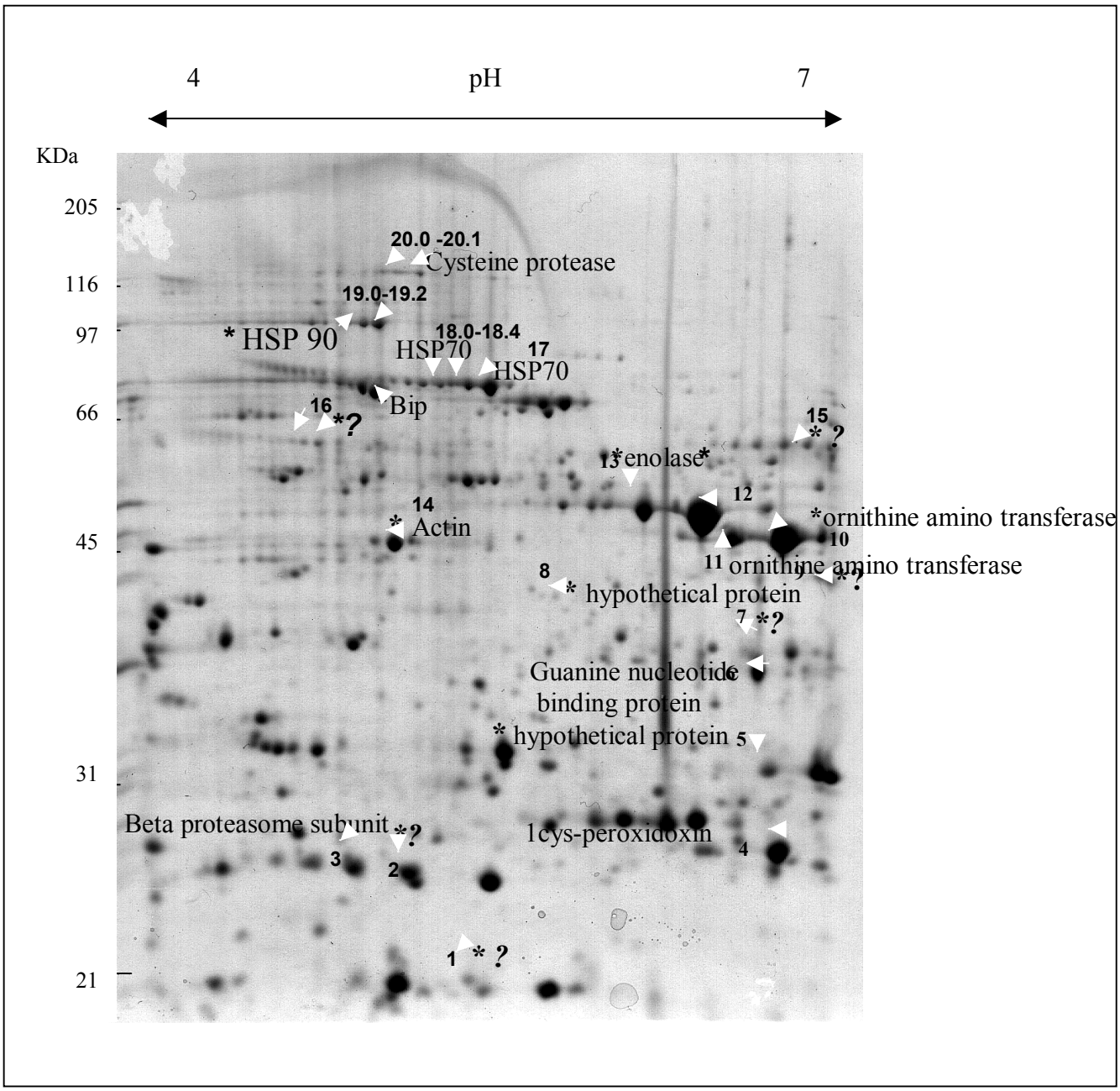


Figure 26. Pattern of proteins identified by MALDI-TOF analysis. Proteins in intact infected erythrocytes were metabolically labeled. Cells were permeabilized and treated with sulfo-NHS-LC-biotin. The fractions of soluble proteins, each corresponding to 2 x 10^9 infected erythrocytes was subjected to two-dimensional gel electrophoresis using a pH gradient of 4-7 in the first dimension and 12% SDS-PAGE in the second dimension and Coomassie Blue stained gel. A parallel gel was transferred to a nitrocellulose filter. The filter was first exposed to X-ray film to visualize metabolically labeled parasite proteins and subsequently reacted with alkaline phosphatase-conjugated SAv to visualize biotinylated proteins. Spots were excised and analysed by MALDI-TOF. The spots are numbered 1-20. The spots that are possible isoforms are numbered together with decimal and the ones that were biotin labeled as assessed by western blot analysis are marked with an asterix* and those that could not be identified are marked with a question mark **?**.

The spectra shown in Figure 25 were generated in the MALDI-TOF analysis for spot 11. The peptide mass peaks were processed using DATA Explorer Software ® and the masses used to interrogate the NCBI database using the search program at searchhttp://prowl.rockefeller.edu/cgi-bin/ProFound. The protein was unambigously identified as ornithine amino transferase (Figure 26). The detailed results for the matched peptides and sequence coverage are shown in figure 27.

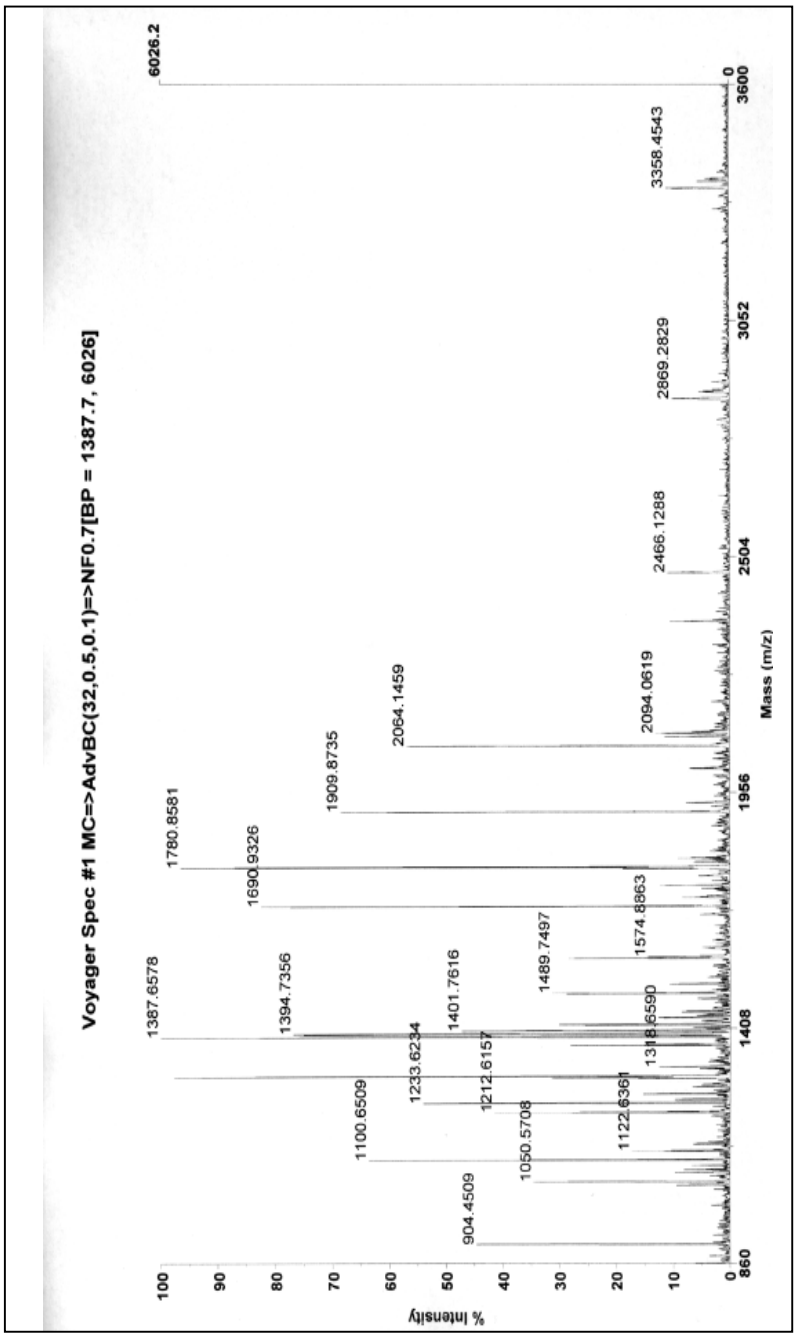
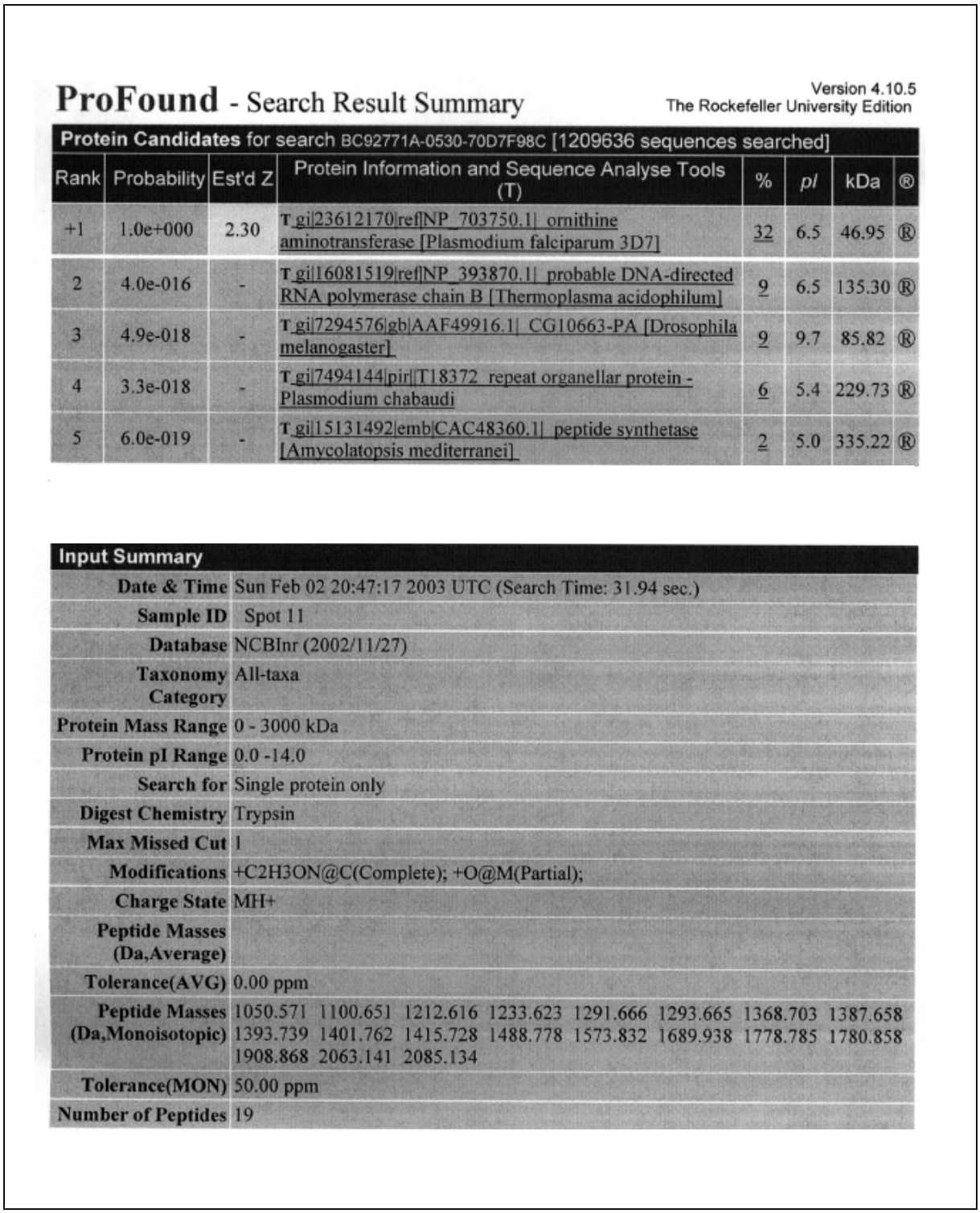


Figure 27. MALDI-TOF-MS spectra of tryptic digests of protein spot number 11. A piece of gel was excised from spot 11 (figure 23) and trypsin treated and analysed by MALDI-TOF. The spectra shows the peaks obtained, their m/z and intensity. The peptide peaks such as 904.4509, 1296.6936 and1570.7087 are molecular mass standards which were included in the analytes to be used in internal mass calibration.



ProFound - Search Result Summary

Version 4.10.5
The Rockefeller University Edition

Protein Candidates for search BC92771A-0530-70D7F98C [1209636 sequences searched]

Rank	Probability	Est'd Z	Protein Information and Sequence Analyse Tools (T)	%	*pI*	kDa	®
+1	1.0e+000	2.30	T gi|23612170|ref|NP_703750.1| ornithine aminotransferase [Plasmodium falciparum 3D7]	32	6.5	46.95	®
2	4.0e-016	-	T gi|16081519|ref|NP_393870.1| probable DNA-directed RNA polymerase chain B [Thermoplasma acidophilum]	9	6.5	135.30	®
3	4.9e-018	-	T gi|7294576|gb|AAF49916.1| CG10663-PA [Drosophila melanogaster]	9	9.7	85.82	®
4	3.3e-018	-	T gi|7494144|pir||T18372 repeat organellar protein - Plasmodium chabaudi	6	5.4	229.73	®
5	6.0e-019	-	T gi|15131492|emb|CAC48360.1| peptide synthetase [Amycolatopsis mediterranei]	2	5.0	335.22	®

Input Summary

Date & Time	Sun Feb 02 20:47:17 2003 UTC (Search Time: 31.94 sec.)
Sample ID	Spot 11
Database	NCBInr (2002/11/27)
Taxonomy Category	All-taxa
Protein Mass Range	0 - 3000 kDa
Protein pI Range	0.0 -14.0
Search for	Single protein only
Digest Chemistry	Trypsin
Max Missed Cut	1
Modifications	+C2H3ON@C(Complete); +O@M(Partial);
Charge State	MH+
Peptide Masses (Da,Average)	
Tolerance(AVG)	0.00 ppm
Peptide Masses (Da,Monoisotopic)	1050.571 1100.651 1212.616 1233.623 1291.666 1293.665 1368.703 1387.658 1393.739 1401.762 1415.728 1488.778 1573.832 1689.938 1778.785 1780.858 1908.868 2063.141 2085.134
Tolerance(MON)	50.00 ppm
Number of Peptides	19

Figure 28. Identification of ornithine amino transferase. The masses of the generated peptides were used in peptide mass finger printing. The masses of the peptides were submitted to the Profound site at Rockefeller University (http://prowl.rockefeller.edu/cgi-bin/ProFound) and searched against the NCBInr data base. The protein was identified unambiguously to be ornithine amino transferase. The theoretical pI and molecular mass of the protein are similar to the observed values for spot number 11 in the 2D gel.

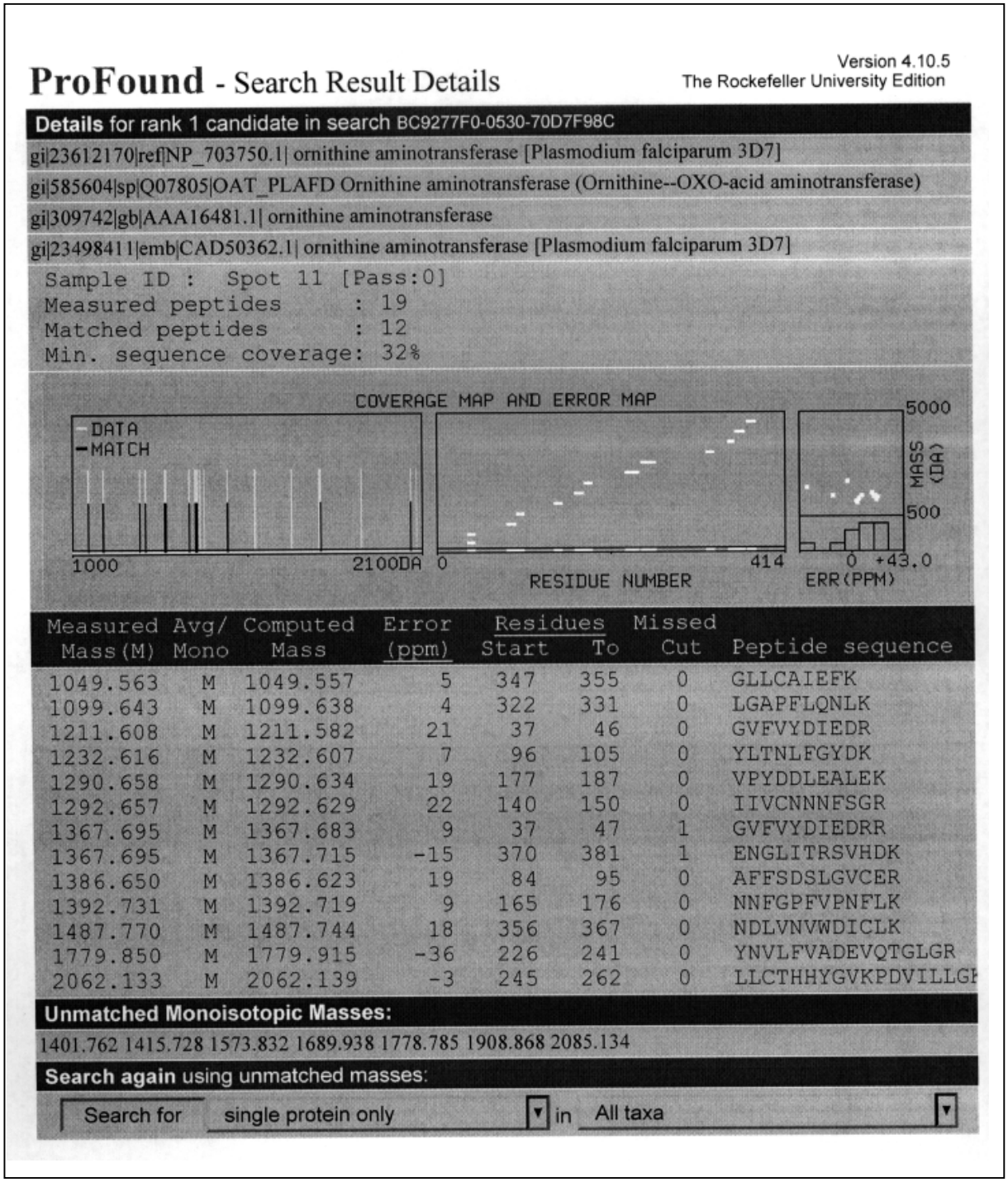


Figure 27. Detailed database search results for ornithine amino transferase. In this analysis 19 peptides were submitted for peptide match finger printing at http://prowl.rockefeller.edu/cgi-bin/ProFound and searched against the NCBInr database. 12 peptides were matched to ornithine aminotransferase and these peptides covered 32% of the amino acid sequence of the protein.

Spot No.	masses matched	sequence coverage %	Mw (Da) pI	protein match	Accession No. NCBInr P. falciparum annotated	Mowse Score E
1	not identified					
2	not identified		-		-	-
3	8/50	50	23080.7 /5.08	Beta 3 proteasome subunit	PFA0400c	1.69E+05
4	17/82	74	25164.2 / 6.31	1 cys peroxidoxin	Pf08_0131	2.16E+10
5	7/43	29	38834.7 / 6.06	Hypothetical protein	PFD0955w	478
6	9/ 47	52	35686.6 / 6.24	Guanine nucleotide binding protein	PF08_0019	1.50E+07
7	11/79	34	37117.5 / 6.06	Hypothetical protein	MAL8P1.96	151
8	7/17	25	39688.9 /5.62	Hypothetical protein	PF_0457	177
9	not identified					
10	17 /71	48	46055.4 / 6.47	Ornithine amino transferase	MAL6P1.91	1.79E+12
	13/33	38	46055.4 / 6.47	Ornithine amino transferase	MAL6P1.91	
	13/71	37	44844.2 /6.28	S-adenosylmethionine synthetase	PFI1090w	4.96E+05
11	13/33	38	46055.4 / 6.47	Ornithine amino transferase	MAL6P1.91	1.51E+10
		38	46055.4 / 6.47	Ornithine amino transferase	MAL6P1.91	1.51E+10
12	22/48	55	48678.0 / 6.21	Enolase	PF10_0155	3.81E+16
13	22/48	55	48678.0 / 6.21	Enolase	PF10_0155	9.09E+11
14	12/47	37	41870.9 / 5.21	Actin	PFL2215w	8.43E+09
15	not identified					
16	8/ 66	15	64574.3 /5.05	Hypothetical protein	PFB0775w	1.41E+03
	7/ 54	17	55067.3 / 4.73	Hypothetical protein	PF08_0109	93.8
17	21/50	30	73915.3 /5.50	Heat shock protein 70 kda protein	PF08_0054	1.91E+11
18_0	21/40	30	73915.3 /5.50	Heat shock protein 70 kda protein	PF08_0054	2.64E+11
18_1	not identified					
18_2	not identified					
18_3	6/ 40	13	73915.3 /5.50	Heat shock protein 70 kda protein	PF08_0054	526
18_4	8/ 29	13	73915.3 /5.50	Heat shock protein 70 kda protein	PF08_0054	5.50E+04
19_0	18/53	30	86166.8 / 4.94	Heat shock protein 90	PF07_0029	1.68E+12
19_1	14/44	22	86166.8 / 4.94	Heat shock protein 90	PF07_0029	1.34E+08
19_2	10/44	21	86166.8 / 4.94	Heat shock protein 90	PF07_0029	4.35E+06
20	9/111	10	111768.8/ 5.26	Cysteine protease	PFB0340c	1.51E+06
21		16	72460/5.1	Bip	gi 311895	

Table 2. Summary of results of proteins identified by MALDI-TOF-MS. The proteins were identified by peptide mass finger printing against the NCBInr database using the program MS-FIT at http://www.prospector.ucsf.edu/;

Spot No.	Mw (Da) pI	protein match	Biotin label by SAv western blot detection	Biotin label by MALDI-TOF MS
1	Not identified		-	-
2	Not identified		-	-
3	23080.7 /5.08	Beta 3 proteasome subunit	No	No
4	25164.2 / 6.31	1 cys peroxidoxin	No	No
5	38834.7 / 6.06	Hypothetical protein	Yes	Yes
6	35686.6 / 6.24	Guanine nucleotide binding protein	No	No
7	37117.5 / 6.06	Hypothetical protein	Yes	Yes
8	39688.9 /5.62	Hypothetical protein	Yes	Yes
9	not identified		Yes	Yes
10	46055.4 / 6.47	Ornithine amino transferase	No	Yes
	46055.4 / 6.47	Ornithine amino transferase		
	44844.2 /6.28	S-adenosylmethionine		
11	46055.4 / 6.47	Ornithine amino transferase	No	No
	46055.4 / 6.47	Ornithine amino transferase		
12	48678.0 / 6.21	Enolase	No	No
13	48678.0 / 6.21	Enolase	No	Yes
14	41870.9 / 5.21	Actin	No	No
15	not identified		-	-
16	64574.3 /5.05	Hypothetical protein	Yes	Yes
	55067.3 / 4.73	Hypothetical protein	Yes	Yes
17	73915.3 /5.50	Heat shock protein 70 kda protein	No	Yes
18_0	73915.3 /5.50	Heat shock protein 70 kda protein	Yes	Yes
18_1	not identified		Yes	Yes
18_2	not identified		Yes	Yes
18_3	73915.3 /5.50	Heat shock protein 70 kda protein	Yes	Yes
18_4	73915.3 /5.50	Heat shock protein 70 kda protein	Yes	Yes
19_0	86166.8 / 4.94	Heat shock protein 90	Yes	Yes
19_1	86166.8 / 4.94	Heat shock protein 90	Yes	Yes
19_2	86166.8 / 4.94	Heat shock protein 90	Yes	Yes
20	111768.8/ 5.26	Cysteine protease	Yes	No
21	72460/5.1	Bip	No	not done

Table 3. **Summary of the results of biotin label analysis by western blot and by MALDI-TOF MS.** The biotinylation status of the proteins was ascertained by western blot analysis of parallel gels and by mass spectroscopy. analysis of the peptides generated after trypsin treatment .

Spot No.	Protein match	N- terminal secretory signal	"Internal" secretory signal	other motifs
1	Not identified	-		
1	Not identified	-		
2	Beta 3 proteasome subunit	No	No	
4	1 cys peroxidoxin	No	No	
5	Hypothetical protein	Yes	No	No ER retention signal
6	Guanine nucleotide binding protein	No	No	
7	Hypothetical protein	No	No	
8	Hypothetical protein	No	No	
9	Not identified	-		
10	Ornithine amino transferase	No	No	
	S-adenosylmethionine	No	No	
11	Ornithine amino transferase	No	No	
12	Enolase	No	No	
13	Enolase	No	No	
14	Actin	No	No	
15	not identified	-		
16	Hypothetical protein	No	No	
	Hypothetical protein	No	No	
17	Heat shock protein 70 kda protein	No	No	ATP binding domain
18_0	Heat shock protein 70 kda protein	No	No	ATP binding domain
18_1	not identified	-		
18_2	not identified	-		
18_3	Heat shock protein 70 kda protein	No	No	ATP binding domain
18_4	Heat shock protein 70 kda protein	No	No	
19_0	Heat shock protein 90	Yes	No	ATP binding domain
19_1	Heat shock protein 90	Yes	No	ATP binding domain
19_2	Heat shock protein 90	Yes	No	ATP binding domain
20	Cysteine protease	Yes	No	No ER retention signal
21	Bip	Yes	No	ER retention signal

Table 4. **Signal peptide analysis of the identified proteins and other functional motifs.** The identified proteins were analysed for hydrophobic domains which can serve as signal peptides http://psort.nibb.ac.jp/) for locating signal motifs and the Plasmodium data base at www.PlasmodB.org which uses the SignalP www server at http://www.cbs.dtu.dk/services/SignalP/output.html for signal peptide. The peptides were also analysed to establish the presence of other functional motifs.

3.14 Analysis of western blots from two-dimensional gels.

The translocation of proteins across the parasite plasma membrane into the PV and then across the PVM into the erythrocyte cytosol is comparable to the import of proteins into the mitochondria. Most mitochondrial proteins are encoded in the nucleus and are synthesized as precursor proteins on cytosolic polysomes. The study of post-translational translocation of precursor proteins across mitochondrial membranes show that unfolding of most proteins is necessary before they are inserted into membrane (Eilers and Schatz 1986). It has been shown that some of the parasite proteins that are exported are secreted in two steps (Ansorge *et al*., 1996; Wickham *et al*., 2001). The proteins are first secreted in to the vacuole and then in a second step they are translocated across the PVM. The proteins that are released into the vacuolar space in the first step are most likely to be having a folded conformation. Lingelbach (1997), proposed the presence of chaperones within the PV. These chaperones are postulated to assist in unfolding the proteins before they are translocated across the PVM possibly through a membrane bound translocator. The translocation of proteins across the PVM inasfar as portein unfolding is concerned could be mediated by a process similar to that used in the import of proteins into the mitochondria.

The possibility of vacuolar location of known plasmodial chaperones, HSP 90, HSP 70, and Bip for which antibodies were available, was analysed as follows. A section of the western blot in Figure 19 D was used to detect and analyze the biotinylation patterns of these proteins. The filter in which the biotin labeled spots were previously detected using alkaline phosphatase conjugated streptavidin was stripped with western blot stripping buffer before blocking the filter again with 2% BSA and incubating it with the antisera against PfHsp90. The bound antibody was visualized by ECL after incubation with the secondary antibody conjugated to horseradish peroxidase. The PfHsp90 antiserum detected a protein spot that was biotin labeled confirming that indeed the protein is biotin labeled (Figure 30C). The filter was then stripped and the analysis repeated in a similar procedure but using an antiserum against Bip. The ER marker protein was detected as a clearly non-biotinylated spot, (Figure 30B) confirming the previous results obtained from the precipitation of biotinylated proteins using streptavidin beads which show that the protein interacts with the biotinylated proteins though it is not biotinylated and it can be washed out from the fraction by using ATP. This shows that the biotin derivative does not have access to Bip which is localized in the ER. The procedure was repeated for PfHSP70 and unlike the other two. Several protein spots were identified some which had a biotin label and others without (Figure 30D). There were also several spots detected as a chain with slightly differing pIs. This suggests that

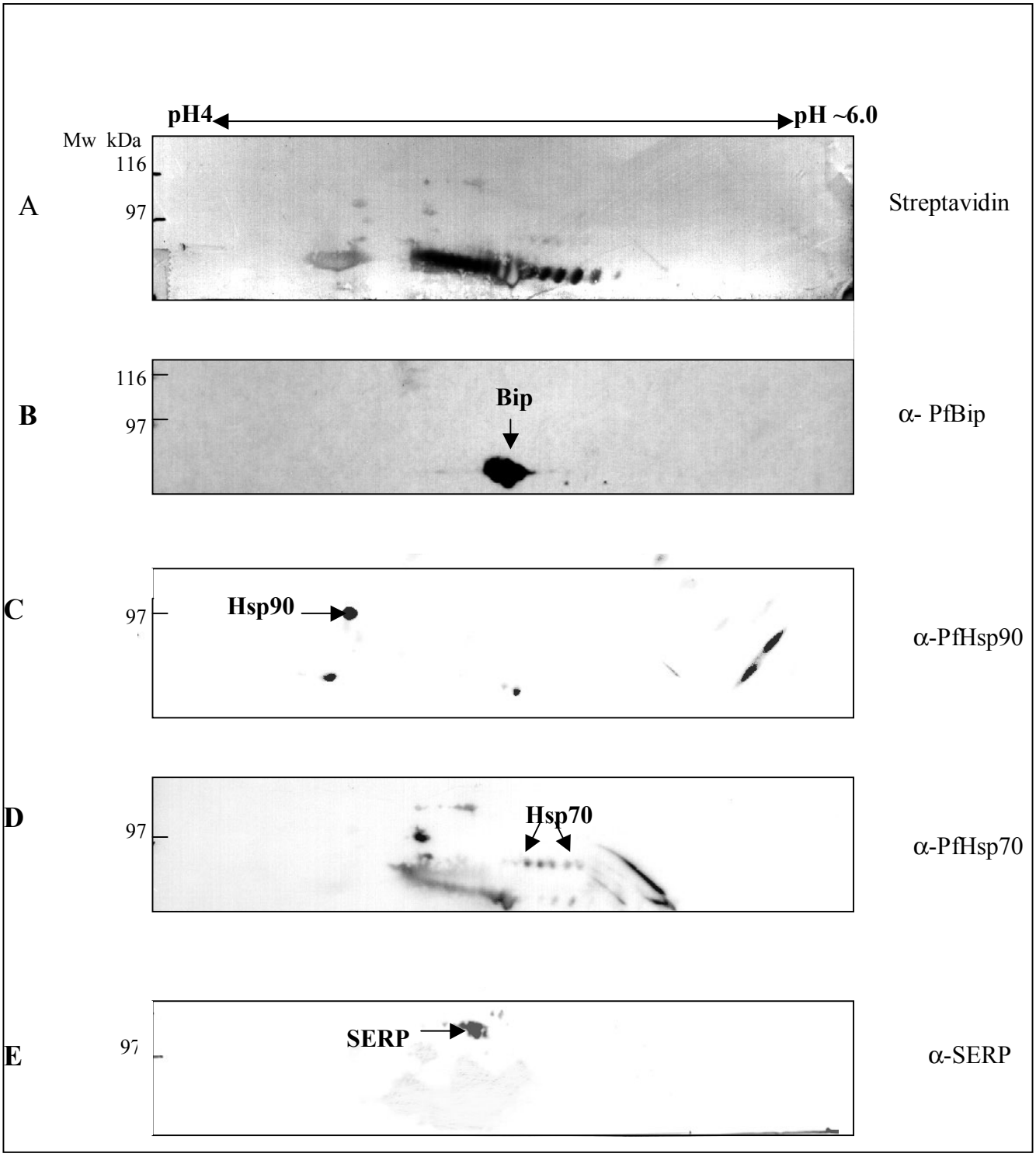


Figure 30. Western blot analysis of two-dimensional gels. A section of the nitrocellulose filter described in figure 17D was probed sequentially with antisera to PfHsp90 then with PfBip and finally with PfHsp70. *Panel A,* SAv –AP detection of biotin labeled protein; *Panel B*; detection of PfBip with horseradish–peroxidase *Panel C* detection of PfHsp90 with horse radish –peroxidase; *Panel D* detection of PfHsp70 with horseradish – peroxidase. In **E** the same filter was incubated with antisera to SERP and the protein detected using secondary antibody conjugated to horse radish –peroxidase.

unlike PfHsp90 which appears to be completely biotinylated and to the same degree (shown by migration as a single biotinylated spot in this experiment). The biotinylation PfHsp70 is either partial or alternatively there could be different pools of this protein, some of which is biotinylated and part of which is not accessible to the biotin. This could be the case since the Hsp70 protein family consists of a number of different proteins which are highly conserved in amino acid sequence but have different subcellular localization (Bianco *et al*., 1986; Adeshir *et al*.,1987 and Kumar *et al*.,1991). However only one Hsp70 species of the protein PF08_0054 was identified for all the spots that were analysed.

In subsequent experiments the same blot was incubated with antisera against the vacuolar space marker SERP. The results (Figure 30E) show that SERP is biotinylated and this providing further evidence that the protein is within the parasitophorous vacuole.

The western blot analysis data suggested biotinylation of Hsp90, in the case of Hsp70 the antibody reacted with a string of spots some of which also reacted with streptavidin conjugated to alkaline phosphatase implying that the protein is also biotinylated. These protein were analysed in more detail by MALDI-TOF-MS to identify any peptides modified by biotin labeling.

The detailed results in the following page show the masses of the peptides obtained by MALDI-TOF analysis, these were used in searches against the NCBInr data base. The matched peptides are shown, together with the matching amino acid sequences and the modifications. The observed modifications include biotinylation and other modifications that occur during the 2D-electrophoresis these include oxidized methionine residues and carbamidomethylated cysteines. The peptides which were not matched are shown at the bottom of each set of results.

For spot number 17, which was identified to be *Plasmodium falciparum* Hsp70, 21 out of the 50 peptide masses that were generated and submitted to the databases for peptide mass finger printing were matched. The matched peptides represented 209 amino acids covering 30 % of the protein that has a total of 677 amino acids. Three of the matched peptides were biotin labeled with one peptide (amino acids 125-140) being labeled by two biotin molecules. Therefore the protein was labeled with a total of 4 biotin molecules. Spot number 19 was identified as *Plasmodium falciparum* PfHsp90. 18 out of the 53 peptide masses that were generated and submitted to the databases for peptide mass finger printing were matched. The matched peptides represented 226 amino acids also covering 30 % of the protein which has a total of 745 amino acids. Three of the matched peptides were biotin labeled with one peptide (amino acids 669-676) being labeled by two biotin molecules. This protein was also labeled with a total of 4 biotin molecules. The schematic diagram (Figure 31) shows the identified peptides and the modifications that were detected.

Spot 17. 1. 21/50 matches (42%). 73915.3 Da, pI = 5.50

>Pfa3D7|chr8|PF08_0054|Annotation|Sanger (protein coding) **heat shock 70 kDa protein**

m/z submitted	MH+ matched	Delta ppm	start	end	Peptide Sequence	Modifications
1217.6346	1217.6312	2.7664	172	183	(K)DAGTIAGLNVMR(I)	
1219.6640	1219.6588	4.2906	472	482	(K)FHLDGIPPAPR(K)	
1233.6468	1233.6261	16.7424	172	183	(K)DAGTIAGLNVMR(I)	1Met-ox
1270.6263	1270.6142	9.5351	249	258	(R)LVNFCVEDFK(R)	
1339.7422	1339.7334	6.5771	343	355	(K)SVHEVVLVGGSTR(I)	
1346.7716	1346.7571	10.7342	586	596	(K)TITTILEWLEK(N)	
1391.6337	1391.6306	2.2543	315	324	(R)FEELCIDYFR(D)	
1405.6796	1405.6462	23.7521	315	324	(R)FEELCIDYFR(D)	1Cys-am
1405.6796	1405.6893	-6.9291	333	341	(K)VLKDAMMDK(K)	1Bio 1Met-ox
1426.7249	1426.7153	6.7318	249	259	(R)LVNFCVEDFKR(K)	
1487.7136	1487.7018	7.9225	48	60	(R)TTPSYVAFTDTER(L)	
1593.7718	1593.7617	6.3664	609	622	(K)QKEAESVCAPIMSK(I)	1Met-ox
1618.7740	1618.7688	3.2199	313	324	(R)ARFEELCIDYFR(D)	
1657.8814	1657.8624	11.4884	125	138	(K)LFHPEEISSMVLQK(M)	
1673.8697	1673.8573	7.4269	125	138	(K)LFHPEEISSMVLQK(M)	1Met-ox
1681.9523	1681.9277	14.6075	184	199	(R)IINEPTAAAIAYGLHK(K)	
2627.3066	2627.3110	-1.6579	125	140	(K)LFHPEEISSMVLQKMK(E)	2Bio 2Met-ox
2628.2974	2628.2946	1.0760	438	460	(K)SQIFTTYADNQPGVLIQVYEGER(A)	
2640.2307	2640.3094	-29.7916	315	332	(R)FEELCIDYFRDTLIPVEK(V)	1Bio 1Cys-am
2663.3027	2663.3676	-24.3685	575	596	(K)LQPAEIETCMKTITTILEWLEK(N)	1Met-ox
2756.4576	2756.3895	24.6936	437	460	(K)KSQIFTTYADNQPGVLIQVYEGER(A)	
3004.4749	3004.5202	-15.0878	6	33	(K)GSKPNLPESNIAIGIDLGTTYSCVGVWR(N)	

29 unmatched masses: 1368.7488 1384.7465 1392.1814 1413.6456 1429.6793 1544.7315 1679.8415 1695.4531 1703.9541 1787.9373 2153.0605 2221.1629 2225.1421 2298.2367 2408.0720 2634.1994 2651.2635 2678.3110 2733.2913 2757.3195 2831.5277 2914.5947 2917.3554 2921.4347 2939.3859 3349.6849 3350.8931 3366.4200 3516.7389

Spot 19_0 18/53 matches (33%). 86166.8 Da, pI = 4.94

>Pfa3D7|chr7|PF07_0029|Annotation|Sanger (protein coding) **heat shock protein 90**

m/z submitted	MH+ matched	Delta	start	end	Peptide Sequence	Modifications
1142.491	1142.4941	-2.5115	366	374	(R)APFDMFENR(K)	1Met-ox
1204.6159	1204.6366	-17.2122	197	206	(K)HSEFISFPIK(L)	
1208.6219	1208.6315	-7.9835	56	65	(K)LSAEPEFFIR(I)	
1282.5872	1282.6003	-10.1869	365	374	(K)RAPFDMFENR(K)	
1298.5994	1298.5952	3.2494	365	374	(K)RAPFDMFENR(K)	1Met-ox
1313.7023	1313.7177	-11.7517	87	98	(K)NDLINNLGTIAR(S)	
1313.7023	1313.6862	12.2221	312	318	(K)QKPLWMR(K)	1Bio 1Met-ox
1313.7023	1313.7074	-3.8619	651	658	(K)KIMEINAR(H)	1Bio
1413.6472	1413.6650	-12.6155	1	13	(-)STETFAFNADIR(Q)	Acet N
1470.7196	1470.6609	39.9298	439	450	(K)CLDMFSELAENK(E)	1Cys-am
1504.7019	1504.7019	0.0178	277	289	(K)VEDVTEELENAEK(K)	
1513.7656	1513.7862	-13.6162	406	419	(K)GVVDSEDLPLNISR(E)	
1527.7363	1527.7443	-5.2673	334	346	(K)SLTNDWEDHLAVK(H)	
1527.7363	1527.7187	11.5034	669	676	(K)ADADKSDK(T)	2Bio
1671.8659	1671.8780	-7.2397	14	27	(R)QLMSLIINTFYSNK(E)	
1671.8659	1671.8933	-16.3647	196	206	(K)KHSEFISFPIK(L)	1Bio
1687.8651	1687.8729	-4.6322	14	27	(R)QLMSLIINTFYSNK(E)	1Met-ox
1833.8434	1833.8547	-6.1540	319	333	(R)KPEEVTNEEYASFYK(S)	
2317.9998	2318.0213	-9.2795	140	160	(K)NNDDEQYVWESAAGGSFTVTK(D)	
2399.0706	2399.1012	-12.7369	611	631	(R)ITDSPCVLVTSEFGWSANMER(I)	
2415.1082	2415.0961	5.0219	611	631	(R)ITDSPCVLVTSEFGWSANMER(I)	1Met-ox
2645.3726	2645.3350	14.1987	509	532	(K)DIYYITGESINAVSNSPFLEALTK(K)	

35 unmatched masses: 1036.1055 1049.4787 1207.6062 1209.1255 1222.6383 1311.6575 1314.7018 1316.7245 1361.7226 1371.6605 1407.7314 1410.7099 1411.7094 1414.1948 1427.6880 1474.7466 1538.7055 2084.0185 2183.0433 2221.1053 2225.1013 2301.1765 2353.4161 2356.0020 2368.0036 2409.0176 2420.2220 2431.0598 2647.3469 2663.4157 2811.2282 2915.5519 2916.3741 2923.2410 3072.4651

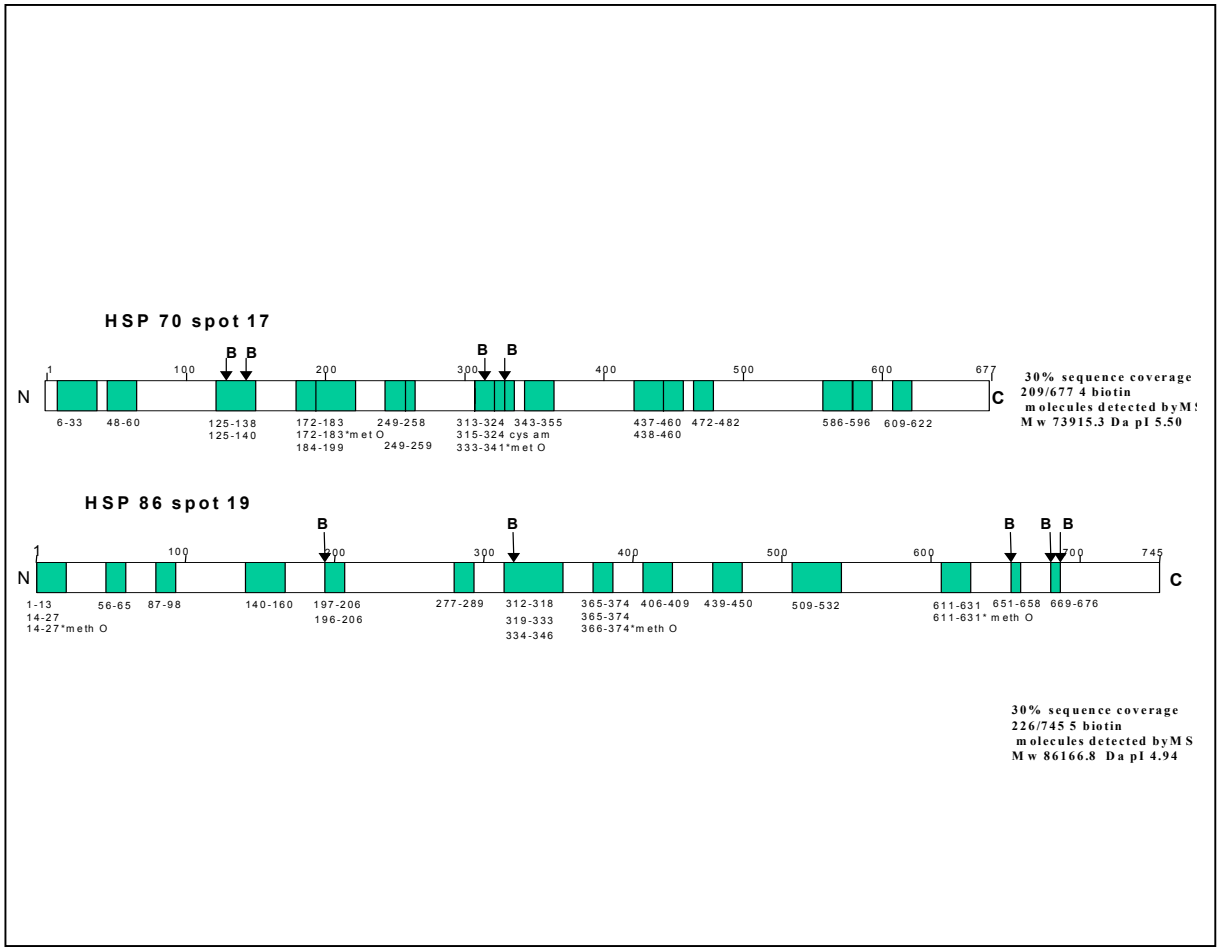


Figure 31. Schematic diagram of the heat shock of the proteins identified in the vacuole, their sequence coverage and biotin labeled peptides. The shaded sections depict the peptides that were identified by MALDI-TOF analysis and the biotin labeled peptides are indicated with a B and the other modifications are also indicated.

3.15 Localization of the heat shock proteins after cell fractionations.

The mass spectrometry data showed that two parasite chaperones Hsp90 and Hsp70 were biotin labeled. Since the PV is protein sorting compartment (Lingelbach *et al*.1997; Wickham *et al*., 2001) and with previous postulations about the possible vacuolar localization of Hsp90 (Jendoubi *et al*., 1985) the localization of these heat shock proteins in infected erythrocytes was analyzed. IRBC were fractionated using either saponin or SLO, and the distribution of the heat shock proteins, PfHsp70, PfHsp90 and PfBip compared to the marker proteins SERP and Pf aldolase. Following treatment with either saponin or SLO and subsequent centrifugation, the pellet fraction were divided into two for each preparartion. One aliquot of each was trypsin treated and the other was not. When the supernatant and pellet fractions were analyzed by western blot, none of the marker proteins was detected in the fraction of proteins released with SLO, all the proteins were in the pellet fraction and were not sensitive to trypsin treatment (Figure 32). After the saponin treatment, Hsp90 and SERP were released and detected in the supernatant, however considerable amounts of these proteins remained in the pellet fraction.. Upon trypsin treatment of the pellet fraction it seems Hsp90 is protected from trypsin while SERP is not. PfBip and Hsp70 were exclusively in the pellet fraction after saponin treatment and they were protected from trypsin digestion. In the case of aldolase, after saponin treatment a very small proportion was detectable in the supernatant. However the protein was insensitive to trypsin treatment. This result suggests that Hsp90 is in the vacuolar space, it does not rule out a dual location with a proportion of the protein within the parasite. Since Hsp70 was shown to be biotinylated it would be expected to be in the fraction of the proteins that are released after saponin treatment. However there are no detectable amounts of the protein in this fraction. Since a major spot that was analyzed and identified to be Hsp70 was not biotin labeled in the western blot it is possible that only a limited amount of the protein is released into the vacuole. It is probable that the amounts are not sufficient to be detected by western blot in a SDS-PAGE. The incomplete biotinylation of Hsp70 needs further investigation, there are several homologues of Hsp70 that have been reported in *P.falciparum* (Bianco *et al*., 1986; Adeshir *et al*., 1987 and Kumar *et al*.,1991) and it is possible that they have different cellular localization. Since the identified Hsp70 does not seem to have a classical N-terminal signal sequence its suggested vacuolar location should be investigated further to conclusively ascertain its cellular localization.

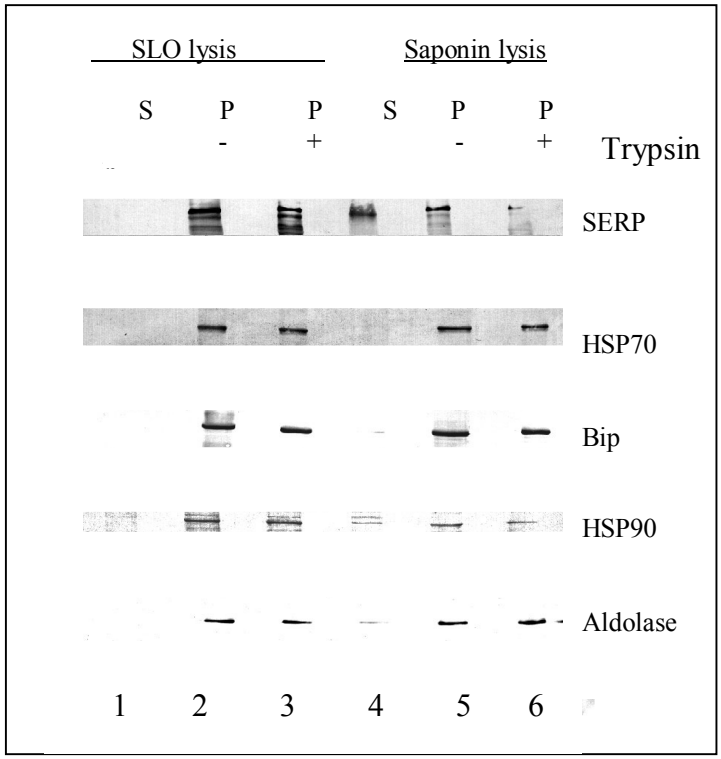


Figure 32. Localization of some *P. falciparum* proteins after fractionating of infected erythrocytes. Infected erythrocytes were SLO permeabilized or lysed with 0.15% saponin and the lysates obtained. The pellets were divided into two for each preparation; and one aliquot of each was trypsin treated and the other was not. The lysates (*lanes 1* and *4*) and the pellets without trypsin treatment (*lanes* 2 and *5*) and with trypsin treatment (*lanes* 3 and *6*) were analyzed by SDS-PAGE and immunoblotting using antisera against SERP, PfHsp70, PfBip, PfHsp90 and aldolase; and the protein visualized by using alkaline phosphatase conjugated secondary antibody.

4. Discussion.

Over the past 10 years ample evidence has been provided that the parasite modifies the properties its host erythrocyte by transporting some of its own proteins to specific destinations within the host cell. Some of these proteins may be involved in nutrient acquisition (Kirk *et al*., 1994) and others such as PfEMP1 are responsible for pathogenesis (Miller *et al*.,1994). What pathways are used to target the parasite products, particularly proteins, to their destinations?

The PV forms the interphase between the erythrocyte cytosol and the parasite. The PV has been postulated to be involved in various functions that are important for the parasite survival within the infected erythrocyte. These functions include nutrient acquisition from the erythrocyte cytosol and the possibly from the external milieu. Constituents of the PV are involved in proteolytic processes that lead to the release of PVM enclosed merozoites at the end of the erythrocytic cycle. The processing of merozoite surface proteins, e.g. MSP-1, that is important in merozoite reinvasion, also occurs within the vacuole (Blackman *et al*. 1991). The PVM and the contents of the vacuole presumably protect the parasite from potentially harmful substances in the host cytosol. Studies have also shown that some exported parasite proteins are sorted within the vacuolar space, some are retained and others that are destined for sites beyond the PVM undergo forward sorting to their respective destinations (Knapp *et al*.,1989; Ansorge *et al*., 1996;Wickham *et al*., 2001). Therefore the PV forms a distinct and invaluable compartment to the parasites within infected erythrocytes. Characterization of the proteome will provide more biochemical information about the proteins present and their importance to the physiology and development of the parasite. Such information may provide new insights into new approaches that can be used in the control of the disease.

4.1 Experimental strategy.

To facilitate the characterization of the vacuolar proteins an experimental strategy that allows the selective labeling and identification of vacuolar space proteins was adopted. Streptolysin O, a bacterial toxin was used to selectively permeabilize infected erythrocytes (Ansorge *et al*., 1996; Ansorge *et al*., 1997). The procedure allows the total removal of the host cell cytosol, the integrity of the PVM is maintained and the contents of the PV remain intact. After permeabilization infected erythrocytes were incubated with non-permeant biotin derivative. These access the surface of the PVM after passing through the SLO generated pores on the erythrocyte membrane. The PVM is proposed to have proteinaceous pores that allow free movement of molecules with size less than 1300 Daltons (Desai and Rosenberg,1997). The biotin derivatives used were of sizes less than 600 Daltons and they should access the vacuolar space through the

pores and react with the soluble PV resident proteins. Since the internal parasite proteins are restricted by the parasite plasma membrane that is not permeated by the highly hydrophilic biotin derivatives they should not be biotinylated. The selectively biotin labeled PV proteins should be isolated and characterized using molecular and biochemical approaches. The study had the following specific objectives.

4.2 Specific Objectives:

4.2.1 Removal of all of the cytosolic proteins of the erythrocyte.

The first objective of the project was to ensure that all the cytosolic proteins of the erythrocyte are removed. The SLO permeabilization procedure and the subsequent washing steps resulted in the total removal of hemoglobin, within the detection limits as assayed photometrically. Hemoglobin is the most abundant cytosolic protein of the host cell and its total extraction is a confirmation that the extraction of the host cell cytosol is complete. This observation was confirmed further in 2D gels where an absolute majority of the Coomassie stained protein spots were also radiolabeled by L-[^{35} S] methionine. Since erythrocytes do not have the ability to synthesize proteins *de novo* all the radiolabeled proteins are of parasite origin. The same is true for silver stained 2D gels which has a higher sensitive compared to Coomassie. None of the protein spots analyzed by peptide mass finger printing after MALDI-TOF-MS was identified as a human protein further confirming that the cytosolic proteins of the erythrocyte are removed completely.

4.2.2 Verification of the existence of non-selective pores within the PVM.

The second objective was to verify the existence of non-selective pores within the PVM that allow passive bi-directional diffusion of small molecules (Desai et al., 1993; Desai and Rosenberg 1997).These authors used electrophysiological data to propose the existence of these pores and has been no previous independent biochemical data that confirm their existence. This study provides the first independent biochemical data confirming the presence of pores on the PVM. These results show that a non-permeant biotin derivative that does not permeate plasma membranes gains access to the parasitophorous vacuole after SLO permeabilization. The biotin labels proteins within the vacuolar space. This was confirmed biochemically using two PV marker proteins SERP and GBP. SERP is a vacuolar resident protein whereas GBP is translocated to the erythrocyte in a two-step process that involves the PV as a transit compartment. Control experiments confirmed that the integrity of the PVM is maintained after the SLO procedure and the since the biotin derivative used sulfo-NHS-LC-biotin and sulfo-NHS-SS-biotin are highly hydrophilic it is certain that the biotin derivatives gain access to the vacuolar space through the non-selective pores that are

proposed to be in the PVM of *Plasmodium* spp together with other Apicomplexans (Desai *et al*., 1993; Desai and Rosenberg, 1997; Schwab *et al*.,1994). Since the access occurred at 4° C it is clear that the biotin labeling of the proteins in the PV is a passive process that is not energy dependent. This is the first independent biochemical evidence confirming the presence of the pores on the PVM of the parasites.

Several models have been proposed to try to explain how the parasite acquires nutrients from the external medium as reviewed in section 1.1. This study provides supportive evidence for the existence of non-selective pores within the PVM that allow passive bi-directional diffusion of small molecules (Desai et al., 1993; Desai and Rosenberg 1997). These results imply that in infected erythrocytes exchange of small molecules between the erythrocyte cytosol and the PV could be mediated through the pores.

4.2.3 Specific biotinylation of vacuolar proteins.

The third objective was to avoid the biotinylation of internal parasite proteins. Using affinity chromatography purification the study clearly showed that aldolase an abundant cytosolic protein was non-biotin labeled. This confirmed that the biotin derivatives used are non-permeant and they do not pass through the parasite plasma membrane. This observation was also true for Bip an ER resident protein. Although the protein associated with biotin labeled proteins after affinity purification with streptavidin beads, immunoprecipitation analysis clearly showed that the protein was not biotin labeled. Further analysis of the affinity purified biotin labeled proteins showed that the protein could be released from the immobilized binding matrix by washing with buffer containing ATP. This confirmed that there was chaperonic interaction between Bip some of the biotin labeled proteins. Analysis of 2D gels by immunoblotting also confirmed the non-biotinylation of Bip The non-biotinylation of Bip further confirmation that biotinylation of the internal parasite proteins does not occur and Bip which is an important component in the early events of the secretory pathway is retained within the parasite.

Several abundant internal parasite proteins were identified by MALDI-TOF-MS and peptide mass finger printing. These included ornithine aminotransferase, enolase, actin, guanine nucleotide binding protein, 1cys peroxidoxin and Beta proteasome sub-unit alpha. These were among the most abundant proteins as detected by Coomassie staining. The analysis of the peptides generated from these proteins show that they were not modified by biotin, providing further evidence at molecular level that internal labeling does not occur under the conditions used in the study.

The same conclusions can also be made from immunfluorescence microscopy results where a distinct circular biotin labeling on the periphery of the parasite is observed. This colocalized with the immunofluorescent labeling for SERP a vacuolar maker protein. Immunoelectron microscopy detected very little or no internal biotinylation. Taken together these results confirm the specific labeling of the vacuolar space in SLO permeabilized infected erythrocytes and the absence of internal biotinylation. Therefore, on the basis of these controls, it is conceivable to assume that the biotin labeled are vacuolar proteins.

4.2.4 2D Analysis and identification of biotinylated proteins.

The fourth specific objective of the study was to analyze biotin labeled proteins by 2D gel electrophoresis, a technique for separating proteins on the basis of two independent variables isoelectric point and molecular mass (pI and M_r) to give a comprehensive pattern of protein visualized as spots in a stained gel. The initial approach was to perform 2D gel analysis using the affinity purified biotin labeled vacuolar proteins. The proteins were labeled using the non-permeant sulfo-NHS-SS-biotin after which they were affinity purified using streptavidin fixed to a solid matrix. The biotin labeled proteins were to be analyzed after cleavage of the proteins from the bound avidin-biotin complex using reducing agents. However, it was not possible to obtain sufficient quantities of the bound proteins from the complex using reducing agents such as DTT, mercaptoethanol and TCEP. The reduction or elution of the proteins was only possible after the addition of the detergent SDS, which rendered the samples unsuitable for 2D analysis because the highly negatively charged SDS interfered with the isoelectric focussing during the 1st dimension.

As a consequence of the above technical problem the experimental strategy was slightly changed, the proteins SLO permeabilized IRBC were biotinylated with the non-cleavable sulfo-NHS-LC-biotin and the total soluble protein sample was analyzed without an affinity purification step. This allowed the detection of biotin labeled protein spots in by immunodetection in western blots after 2D electrophoresis. The resolution of the 2D gels was sufficient and reproducible to clearly identify a number of spots that were biotinylated and radiolabeled. These proteins could be confidently assigned to the parasite with vacuolar localization.

4.2.5 MALDI-TOF- MS analysis.

The fifth and final objective was to analyze the proteins by MALDI-TOF-MS and subsequently identify the proteins using bioinformatic tools and data base searches including the recently published *Plasmodium falciparum* complete genome (Gardner *et al*., 2002.). Despite the modification of the proteins in the biotinylation reaction prior to 2D gel electrophoresis, it was

possible to unambiguously identify several parasite proteins by peptide mass finger printing. These included both biotin labeled and non-biotin labeled proteins. The accuracy of the approach allowed the identification of some peptides containing a lysine residue modified in the biotin reaction. This provided evidence for the biotinylation status of the protein at molecular level. The identified proteins included the non-biotinylated house keeping enzymes present in the cytosol such as enolase and ornithine amino transferase. The other non-biotinylated proteins included actin, which is also an abundant protein in the cytosol and cytoskeleton of the parasite, guanine nucleotide binding protein, 1cys-peroxidoxin and beta proteasome sub unit alpha. The biotin labeled proteins that were identified included Hsp86, also commonly referred to as Hsp90, Hsp70, a cysteine protease with an identical sequence to the serine rich protein (SERP) and a number of hypothetical proteins that are described in the recently published *Plasmodium falciparum* genome (Gardner et al., 2002). The biotin labeled proteins fulfill the experimental criteria to conclude that they are localized within the parasitophorous vacuole.

4.3 Limitations and Expectations of the study.

One of the major limitations of the study was the low abundance of the proteins in the PV. Although SLO permeabilization led to the complete removal of the host cell cytosol, in the permeabilized cell the volume of the lumen of the PV compared to the entire infected erythrocyte is 1:10,000 (Burghaus and Lingelbach, 2001). It was not possible to isolate large amounts of the biotin labeled proteins by affinity chromatography for subsequent analysis by 2D gel electrophoresis. Therefore the experimental strategy had to be modified to use the total soluble protein fraction in the 2D analysis. The disadvantage of this approach is that due to high abundance of the internal parasite proteins at the maximum protein loading capacity of the IPG strips the amount of the PV proteins is very low. Therefore in this study only the most abundant biotinylated proteins were characterized.. A large majority of the proteins could not be identified by MALD-TOF-MS due to their low abundance and this remains a major problem which has to be overcome in order to fully characterize the proteome of the PV. In principal the experimental strategy is correct and it can be used to selectively isolate vacuolar proteins. However, biotin may not be the ideal molecule to use in labeling the proteins which are subsequently affinity purified in streptavidin columns. The same experimental approach can be utilized but using different cross-linkers that can be easily dissociated during elution thereby releasing the labeled proteins.

4.4 Characterization of novel vacuolar proteins and their possible functions.

Conceptually four main categories of proteins can be postulated to be localized in the PV. These include exclusively PV resident proteins e.g., SERP, proteins with a dual localization in both the parasite and the PV e.g., Sar1p and NSF, proteins that transit the PV and are outward bound e.g., GBP and proteins that are present within the PV but can be internalized by the parasite by endocytosis or other mechanisms (Cheresh *et al*., 2002). Translocation across the PVM requires unfolding and chaperones are also likely to be found in the vacuole.

The results have shown the presence of a chaperone HSP86 which is also referred to as HSP90 in the vacuolar space as confirmed by both immunoblotting and masspectroscopy. Heat shock proteins (Hsps) are families of proteins expressed in all prokaryotic and eukaryotic organisms which play a critical role in the development and adaptation of cells to different environments (Hartl *et al*., 1994). They are constitutively expressed as well as induced when cells are exposed to a variety of stressful conditions such as temperature, nutrient deprivation, etc. They show a high degree of sequence identity among species and function as molecular chaperones. By recognizing structural elements, predominantly hydrophobic residues exposed in unfolded and partially denatured proteins, they stabilize he non-native conformation and facilitate their correct folding (Hartl, 1996). Additionally, Hsps located inside cellular organelles play critical roles in assisting translocation of proteins from the cytosol into these organelles (Cheng *et al*., 1989).

P. falciparum has a complex life cycle alternating between a poikilothermic invertebrate vector and a warm-blooded vertebrate host and it is not surprising that the parasite abundantly expresses Hsps (Kumar *et al*., 1991), where they could play a significant role during the adaptation of the parasites to different environments. Genes of the Hsp70 (Bianco *et al*., 1986; Kumar *et al*., 1988), Hsp90 (Bonnefoy *et al*., 1994; Su and Wellems, 1994), and Hsp60 (Syin and Goldman, 1996) have been characterized in the parasite. Hsp70 has been the most extensively studied. It is expressed in all stages of the parasite life cycle (Kumar *et al*., 1993; Tsuji *et al*., 1994) and its expression is induced by high temperature during the erythrocytic stage (Kumar *et al*., 1991).

Previous analysis of the deduced protein sequence of PfHsp90 revealed a unusually large region of charged amino acids when compared to hsp90 from other species, with the region showing homology to the calcium binding domain of calreticulin, the major calcium binding protein of endoplasmic reticulum (Bonnefoy *et al*., 1994). In this study analysis of the N-terminus of the identified hsp90 protein using different algorithms to predict the cellular location of the protein gave inconsistent results. The Psort program at http://psort.nibb.ac.jp/ predicted a signal peptide at its N-terminus, however the program SignalP www server at

http://www.cbs.dtu.dk/services/signalP/output.html predicted that the protein does not have a signal peptide. Analysis of the tryptic digestion peptides by MALDI-TOF-MS detected the presence of peptides corresponding to amino acids 1-13 and 14-27 of the N-terminal region of the protein. This implies that the putative signal sequence is not cleaved as would be expected of a protein that is secreted via the classical secretory pathway. Therefore the mechanism of its translocation to the PV may be similar to that of proteins that exported out of the parasite despite the fact that they do not carry a typical N-terminus signal sequence. Alternatively the N-terminal sequence acts as an ER-targeting signal that enables the protein to be recruited into the classical secretory pathway, however the signal is not cleaved from the protein.

The plasmodial hsp90 is an ATP binding protein encoded by a single gene constitutively expressed in both asexual (trophozoite) and sexual (gametocyte) stage parasites. The hsp90 protein is homologous to a previously identified 90-kDa antigen strongly recognized by both sera from vaccinated monkeys and monoclonal antibody (Bonnefoy *et al*., 1994). In these vaccination trials using several identified antigens, the protected monkey sera had significantly higher antibody titers to the hsp90 protein. No correlation could be drawn with response to any other antigen investigated (Bonnefoy *et al*., 1994). The monoclonal antibody used in the identification of the hsp90 mAb XIV/7 has been reported previously to react with a protein located in the parasitophorous vacuole (Jendoubi *et al*., 1988). Recent studies appear to confirm this interpretation (Banumathy *et al*., 2002). The vacuolar localization of PfHsp90 in this study confirms the proposal that molecular chaperones may be present in the PV and that they are involved in protein sorting activities in this compartment (Lingelbach, 1997).

These results support the two-step model for the translocation of proteins to destinations in the host cell. . Most mitochondrial proteins are encoded in the nucleus and are synthesized as precursor proteins on cytosolic polysomes. The study of post-translational translocation of precursor proteins across mitochondrial membranes show that unfolding of most proteins is necessary before they are inserted into membranes (Eilers and Schatz 1986). Since proteins which are secreted from the parasite into the vacuolar space are likely to have a folded conformation, a chaperone like Hsp90 may assist in the unfolding of the proteins in order to facilitate translocation through a membrane-bound translocator. This process would require energy and indeed the translocation of GBP across the PVM has been shown to be ATP dependent (Ansorge *et al*., 1996).

The Hsp90 proteins share a common structural plan. The amino terminal domain contains a binding site for ATP and for geldanamycin a representative of the ansamycin drugs, which specifically target Hsp90 (Whitesell *et al*., 1994). A divergent charged sequence separates the

amino terminus from a highly conserved though structurally flexible middle domain and the carboxyl-terminal domain. Both the N-terminus and the C-terminus have been implicated in binding of substrate polypeptides and substrate binding at the N-terminus is affected by nucleotides, GA, and the adjacent charged sequence of Hsp90 (Young *et al*., 1997; Scheibel *et al*., 1998,1999). ATP binding and hydrolysis are necessary for the function of HSP90 in vivo as demonstrated in both yeast and *E. coli* (Prodromou *et al*., 1997; Oberman *et al*., 1998; Panaretou et al., 1998). Indeed the translocation of GBP across the PVM has been shown to be dependent of the availability of ATP (Ansorge *et al*., 2000).

At least five different Hsp70 P. *falciparum* genes have been identified (Peterson *et al*., 1988; Kumar and Zheng 1992). This study proposes the presence of Hsp70 in the parasitophorous vacuole as shown by biotin labeling and mass spectroscopy, however Bip an ER resident chaperone which is also a member of the Hsp70 protein family is not in the vacuole since it was not biotin labeled. Like grp78 in mammalian cells the PfBip (Pfgrp) has a hydrophobic signal sequence at the amino terminus and a tetra-peptide sequence of Ser-Asp-Glu-Leu (SDEL) which serves as a signal sequence for ER retention as compared to KDEL in mammalian cells (Munro and Pelham 1987) and HDEL in yeast (Rose *et al*., 1989; Pelham *et al*., 1988). These results confirm that Pfgrp is not present in the vacuolar space and therefore if there is a chaperone mediated protein-sorting activity then it is modulated by a different molecule. The possibility of having hsp70 in the vacuole is new since previous studies have localized the protein in the nucleus and cytoplasm of the parasite (Kumar *et al*., 1991) the cytoplasm (Bianco *et al*.,1986). The cytoplasmic hsp70 does not have an N-terminal signal sequence and the import into the vacuole must be depended on a different mechanism.

Despite the purported cytosolic location of the Hsp families as previously reported, Hsp70, in spite of being the most conserved protein, present in all organisms studied (Gupta and Golding 1993) is a major immunogen in infections caused by a number of organisms including *P. falciparum* (Adeshir *et al*., 1987; Behr *et al*., 1992; Bianco *et al*., 1986, Kumar *et al*., 1990). To elicit an immune response the protein must be exposed to B- and T-cells which mediate these immunological responses. Indeed a 75kd protein with a sequence homologous to the hsp70 protein family has being localized on the merozoite surface (Adeshir *et al*., 1987). Recent experiments however rule out the possibility that like, many parasite encoded proteins PfHsp70 may be exported to the host cytoplasm as shown by saponin lysis (Banumathy *et al*., 2002), this results were confirmed in the current study. A result that makes it difficult to conclusively ascertain the cellular location of the protein. However recent experiments that suggest the movement of

proteins into the vacuole and back into the parasite (Cheresh *et al*., 2002) may offer an explanation for the presence of the protein the vacuole.

The presence of Hsp90 in the vacuole may indicate the possibility of having Hsp70 also in the same compartment, since some cellular signaling proteins require coordinated activities of theHsp70 and Hsp90 molecular chaperones for their folding and conformational regulation. Many polypeptide chains interact cotranslationally with the chaperones of the hsp70 family which prevent misfolding and aggregation of the newly formed polypeptides. The newly synthesized polypeptides are either released for folding to their native state without the help of additional chaperones or are passed on to more specialized chaperone systems which includes the Hsp90 multichaperone system (Hartl, 1996; Johnson and Craig 1997). Hsp90 receives its substrates from Hsp70 in a reaction that is dependent on the function of Hop (Hsp70 and Hsp90 organizing protein) an adaptor protein that provides specific binding sites for these two chaperones (Chang *et al*., 1997).

This study has localized a cysteine protease in the vacuole, and amino acid sequence analysis showed that the protein is identical to the serine rich protein (SERP). Serp is localized in the vacuole and it contains domains that are identical cysteine proteases or related proteases. The result is significant and it supports previous data which suggest a role of vacuole resident proteases in the release of merozoites from the host cell at the end of the erythroyctic stage of development. Protease inhibitors have been shown to inhibit the exit of merozoites from the parasitophorous vacuole (Salmon *et al*., 2000). Proteases are also important in the processing of MSP-1 possibly within the vacuole (Holder *et al*., 1987). The processed form of the protein is localized on the membrane of the merozoite and is implicated in the invasion process. The identification and characterization of vacuolar localized proteases that are important in the parasite developmental cycle is significant since protease inhibitors are currently in use in the treatment of microbial infections.

The proteins that have been identified as putative vacuolar protein by this approach can be divide into two main categories; those with signal sequences at their N-terminus and those without. The proteins in the former category include Hsp90, cysteine protease and the hypothetical protein spot 5. These proteins would under normal circumstances be secreted in through the classical pathway. The spot 5 which was identified as a hypothetical protein with a signal sequence predicted to be located between amino acids 1-24 did not have any predicted metabolic activity or other functional motifs. However when its sequence was matched against other annotated proteins in the NCBI database the closest match was to a yeast gene YKR095w which codes for a protein accession NP_013021. The protein has high molecular mass with a total of

1875 amino acids. In yeast this protein is involved in translocation of macromolecules between the nucleoplasm and the nuclear pore complex. The other hypothetical proteins spots 7 and 8 were predicted not to have signal peptides or known functional properties.

Conclusions:

SLO permeabilized cells can be used to introduce cross-linking molecules in to the PV. Presumably, these molecules gain access via non-selective pores within the PVM. The abundance of some of the vacuolar proteins clearly is sufficient to allow their identification by MALDI-TOF analysis. among th enovel proteinsidentified within the PV are heat shock proteins. Their precise roles in the complex process involved in protein sorting within the vacuole need to be established.

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Summary

Plasmodium falciparum, the causative agent of the most severe form of human malaria, is a unicellular parasite which infects erythrocytes. Inside the erythrocyte the *P. falciparum* develops within a parasitophorous vacuole, the membrane of vacuole forming an interface between the parasite and the host cell cytosol. Although the volume of the vacuole is extremely small in comparison to the host and the parasite cells, respectively, it plays an essential role for parasite development. Putative functions of vacuolar proteins include nutrient acquisition, protein sorting and lytic properties required for parasite release at the end of the intraerythrocytic development. Although the significance of the vacuole as a separate compartment in infected erythrocytes was recognized many years ago, experimental attempts to selectively identify vacuolar proteins were limited because of the small volume of the vacuole and because the vacuole is an intermediate compartment between two separate cells (the parasite and the host erythrocyte) which complicates the use of standard cell fractionation methods. A major aim of this project was the development of a novel experimental strategy that would allow the selective identification of vacuolar proteins.

In a first step, infected erythrocytes were permeabilized with streptolysin O which allowed complete removal of the erythrocyte cytosol. Permeabilized cells were then treated with non-permeant biotin derivatives. These derivatives were shown to gain access to the vacuolar lumen, but they were excluded from the parasite cell. This was demonstrated by biochemical analyses of specific marker proteins located within the vacuole and within the parasite cytosol, respectively. The vacuolar proteins were labeled with biotin whereas the parasite cytosolic proteins, although they were present at higher abundance, were not labeled. These results provided the first biochemical evidence for the existence of non-selective pores within the vacuolar membrane that were postulated based on electrophysiological studies. Soluble vacuolar proteins that were biotinylated were isolated by affinity chromatography and analysed by SDS-PAGE.

Secondly, infected erythrocytes were cultivated in the presence of L-[^{35}S]- methionine to label parasite proteins metabolically. Following biotin treatment of permeabilized erythrocytes, protein extracts were subjected to reproducible 2D gelelectrophoresis. The comparison of biotin labeled proteins versus radiolabeled proteins allowed the identification of 17 protein spots that were both radiolabeled and biotinylated, and which therefore represent candidate vacuolar proteins. A detailed MALDI-TOF analysis of several of these spots revealed plasmodial heat shock protein 70, heat shock protein 90 as novel vacuolar proteins. In addition a cysteine protease, known to be a vacuolar protein from previous studies, was also identified. Out of the radiolabeled but non-biotinylated spots, MALDI-TOF analyses identified 8 cytoplasmic parasite proteins.

Together the results confirm the suitability of the experimental rationale used and they provide the basis for the full characterization of the vacuolar proteome.

Zusammenfassung

Plasmodium falciparum, Erreger der schwerste Form der menschlichen Malaria, ist ein einzelliger Parasit, welcher Erythrozyten befällt. Im Erythrozyten entwickelt sich *P.falciparum* innerhalb einer parasitophoren Vakuole, wobei die Membran der Vakuole eine Schnittstelle zwischen Parasit und Zytosol der Wirtszelle bildet. Obwohl das Volumen der Vakuole im Vergleich zur Wirtszelle und dem Parasiten sehr klein ist, spielt sie eine wichtige Rolle in der Entwicklung des Parasiten. Mögliche Aufgaben von Proteinen der Vakuole umfassen Nährstoffaufnahme, Proteinverteilung und lytische Eigenschaften, welche am Ende der intraerythrozytären Entwicklung für die Freisetzung der Parasiten notwendig sind. Obwohl die Bedeutung der Vakuole als separates Kompartiment in infizierten Erythrozyten bereits vor vielen Jahren erkannt wurde, waren experimentelle Ansätze zur Identifizierung von Proteinen der Vakuole aufgrund des sehr geringen Volumens limitiert. Weiterhin hat die Vakuole als Kompartiment zwischen zwei separaten Zellen (Parasit und Erythrozyt) den Einsatz von gebräuchlichen Methoden der Zellfraktionierung erschwert. Das Hauptziel dieser Arbeit war die Entwicklung einer neuen experimentellen Strategie, welche die selektive Identifikation von Proteinen der Vakuole ermöglicht.

In einem ersten Schritt wurden infizierte Erythrozyten mit Streptolysin O permeabilisiert, was zur vollständigen Entfernung des Erythrozytenzytosols führt. Die permeabilisierten Zellen wurden dann mit nicht membrangängigen Biotinderivaten behandelt. Diese Derivate gelangten in den Vakuolenraum, aber nicht in das Zytosol des Parasiten. Dieses wurde durch biochemische Analysen bestimmter Markerproteine gezeigt, die sich innerhalb der Vakuole und innerhalb des Parasitenzytosols befinden. Die Proteine der Vakuole wurden mit Biotin markiert, während die zytosolischen Proteine des Parasiten, obwohl sie in grösseren Mengen vorkommen, nicht markiert wurden. Diese Ergebnisse boten den ersten biochemischen Beweis für die Existenz nicht-selektiver Poren innerhalb der Vakuolenmembran, welche aufgrund von elektrophysiologischen Studien postuliert wurden. Lösliche Proteine der Vakuole, die biotinyliert wurden, konnten durch Affinitätschromatographie isoliert und mittels SDS-PAGE analysiert werden.

In einem nächsten Schritt wurden infizierte Erythroyzyten in der Anwesenheit von L-[^{35}S] Methionin kultiviert, um Proteine des Parasiten metabolisch zu markieren. Nach einer anschließenden Behandlung permeabilisierter Erythrozyten mit Biotin wurden die Proteinextrakte einer reproduzierbaren 2D-Gelelektrophorese unterzogen. Der Vergleich biotinylierter Proteine mit radioaktiv markierten Proteinen ermöglichte die Identifikation von 17 Proteinspots, die sowohl radioaktiv markiert, als auch biotinyliert waren und deshalb als Proteine der Vakuole in Frage

kamen. Eine detaillierte MALDI-TOF Analyse mehrerer dieser Spots enthüllte Hsp70 und Hsp90 von *Plasmodium falciparum* als neue Proteine der Vakuole. Zusätzlich wurde eine Cystein Protease, welche aufgrund früherer Studien als Vakuolenprotein bereits bekannt war, identifiziert. Von den radioaktiv markierten, nicht –biotinylierten Spots, identifizierte die MALDI-TOF Analyse 8 zytoplasmatische Parasitenproteine.

Zusammengefaßt bestätigen die Ergebnisse die Eignung des benutzten experimentellen Ansatzes und bieten die Basis fur die vollständige Charakterisierung des Vakuolenproteoms.

Erklärung

ich versichere, dass ich meine Dissertation

An experimental strategy towards the proteome analysis of the parasitophorous vacuole in *Plasmodium falciparum*-infected ertyhrocytes.

selbstständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg den 13 März 2003	Julius O. Nyalwidhe
(Ort/Datum)	(Unterschrift mit Vor- und Zuname)

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A Nonpermeant Biotin Derivative Gains Access to the Parasitophorous Vacuole in *Plasmodium falciparum*-infected Erythrocytes Permeabilized with Streptolysin O*

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In its host erythrocyte, the malaria parasite *Plasmodium falciparum* resides within a parasitophorous vacuole, the membrane of which forms a barrier between the host cell cytosol and the parasite surface. The vacuole is a unique compartment because it contains specific proteins that are believed to be involved in cell biological functions essential for parasite survival. As a prerequisite for the characterization of the vacuolar proteome, we have developed an experimental approach that allows the selective biotinylation of soluble vacuolar proteins. This approach utilizes nonpermeant biotin derivatives that can be introduced into infected erythrocytes after selective permeabilization of the erythrocyte membrane with the pore-forming protein streptolysin O. The derivatives gain access to the vacuolar lumen but not to the parasite cytosol, thus providing supportive evidence for the existence of nonselective pores within the vacuolar membrane that have been postulated based on electrophysiological studies. Soluble vacuolar proteins that are biotin-labeled can be isolated by affinity chromatography using streptavidin-agarose.

Plasmodium falciparum, the causative agent of the most severe form of malaria, invades human erythrocytes, where this unicellular parasite develops within the so-called parasitophorous vacuole (PV).[1] The parasitophorous vacuolar membrane (PVM) is formed during the process of invasion, and it contains lipids from the erythrocyte plasma membrane (1, 2) and, presumably, parasite-derived proteins and lipids (3). This membrane presents a barrier between parasite and host cell cytosol, although electrophysiological studies in *Plasmodium*-infected erythrocytes suggest the existence of nonselective pores within the PVM that allow passive bidirectional diffusion of small molecules (4, 5). With respect to biogenesis and protein contents, the PV differs significantly from endocytic vacuoles (6), with the most apparent difference being the almost cytosolic pH of the vacuole (7, 8). In recent years, many observations have indicated a number of cell biologically relevant processes within the vacuole that are important for parasite survival.

In the course of the infection, several parasite proteins are exported into the erythrocyte, and some of these proteins, such as the members of the PfEMP1 family, are key molecules involved in the pathogenesis of malaria (9). For at least some exported proteins, the PV is a transit compartment from which they are transported into the host cell cytosol (10–12). Translocation across the vacuolar membrane must be a selective process because other proteins are retained within the PV (13, 14). Recently, it has been suggested that proteins destined for the parasite apicoplast, a unique plastid-like intracellular organelle of apicomplexan parasites, also may be trafficked via the PV (15). Thus, the PV must contain a machinery involved in protein sorting. Upon completion of parasite development and multiplication, merozoites, the invasive stages, are released from the infected cell. Release of merozoites from the PV can be prevented by treatment of infected erythrocytes (IRBCs) with protease inhibitors (16). In addition, evasion of merozoites is accompanied by a specific sequential cleavage of the major merozoite surface protein 1 that is essential for the subsequent reinvasion of noninfected cells (17). These data suggest a role of vacuolar proteases in the release of parasites from the infected host cell. Collectively, the current observations underscore the notion that the PV is a unique intracellular compartment critical for parasite survival within the host cell and that it most likely contains novel proteins of elementary functions.

The volume of the vacuole compared with the volumes of the parasite cytosol and the erythrocyte cytosol, respectively, is very small. Estimates based on morphological data suggest a 1:10,000 ratio of vacuolar volume to erythrocyte cytosol in IRBCs infected with stages of the parasite that have completed ~30 h of the 48-h intraerythrocytic development (12). Cell fractionation experiments on IRBCs infected with the same developmental stages show that more than 70% of the total protein are erythrocyte cytosolic proteins, predominantly hemoglobin (18). Therefore, the isolation of vacuolar proteins is difficult; consequently, only a few vacuolar proteins have been identified thus far. In this report, we describe a novel strategy for the identification of vacuolar proteins as a prerequisite for a comprehensive proteome analysis of this compartment.

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[1] The abbreviations used are: PV, parasitophorous vacuole; GBP, glycophorin-binding protein; IRBC, infected red blood cell; PfALD, *P. falciparum* aldolase; PfBiP, *P. falciparum* immunoglobulin heavy chain-binding protein; PVM, parasitophorous vacuolar membrane; SAv, streptavidin; SERP, serine-rich protein; SLO, streptolysin O; PBS, phosphate-buffered saline; DTT, dithiothreitol; BSA, bovine serum albumin; sulfo-NHS-SS-biotin, sulfosuccinimidyl-2-(biotinamido) ethyl-1–3-dithiopropionate; sulfo-NHS-LC-biotin, sulfosuccinimidyl-6-(biotinamido) hexanoate; PfEMP1, *P. falciparum* erythrocyte membrane protein 1.

EXPERIMENTAL PROCEDURES

Biotin derivatives sulfosuccinimidyl-2-(biotinamido) ethyl-1–3-dithiopropionate (sulfo-NHS-SS-biotin), sulfosuccinimidyl-6-(biotinamido) hexanoate (sulfo-NHS-LC-biotin), and streptavidin (SAv)-conjugated agarose beads were obtained from Pierce. L-[^{35}S]Methionine was obtained from Amersham Biosciences. Streptolysin O (SLO) was kindly provided by S. Bhakdi and prepared as described elsewhere (10). Polyclonal rabbit antibodies directed against GBP, SERP, parasite aldolase, and PfBiP have been described previously (12, 19). The mouse monoclonal antibody to band 3, clone III-136, was obtained from Sigma. Secondary antibodies against rabbit IgG conjugated to alkaline phosphatase or to horseradish peroxidase were obtained from DAKO A/S. Cy2-conjugated goat anti-mouse IgG, Cy5-conjugated goat anti-rabbit IgG, and Cy3-conjugated streptavidin were from Jackson Immuno Research Laboratories, Inc. Protein A-Sepharose beads were from Amersham Biosciences.

Parasite Cultures and Permeabilization of Infected Erythrocytes—Parasites of the *P. falciparum* FCBR strain were cultivated in RPMI 1640 medium (Invitrogen) containing 10% human plasma and erythrocytes of blood group A$^+$ (Marburg Blood Bank) under standard conditions (20). Trophozoite-infected erythrocytes (IRBCs) were enriched to a parasitemia of >90% by gel floatation (21). For metabolic labeling of newly synthesized proteins, enriched IRBCs were washed twice in methionine-free RPMI 1640 medium and cultivated for 1 h in plasma-free, methionine-free RPMI 1640 medium, in the presence of 50 μCi ml^{-1} L-[^{35}S]methionine. IRBCs were permeabilized with SLO as described previously (10). Briefly, 2×10^8 IRBCs were incubated with 3–4 hemolytic units of SLO in RPMI 1640 medium at room temperature for 6 min. Samples were centrifuged at 10,000 $\times$ *g* for 15 s. The pellet containing intact parasites, the vacuolar contents, and membranes was washed with 200 μl of RPMI 1640 medium before biotinylation. Complete removal of the erythrocyte hemoglobin was monitored spectrophotometrically as described previously (10).

Biotin Labeling—For the biotinylation of intact erythrocytes, 10^8 parasitized or nonparasitized erythrocytes were washed three times in PBS containing 0.6 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.6 (PBS^{2+}), and then incubated in PBS^{2+} containing 1 mg ml^{-1} sulfo-NHS-SS-biotin for 30 min on ice. Cells were sedimented by centrifugation at 10,000 $\times$ *g* for 15 s at 4 °C. The supernatant was analyzed photometrically at 412 nm for the release of hemoglobin. To block and remove unbound biotin, cells were washed three times in PBS^{2+} containing 100 mM glycine. For cleavage of extracellularly exposed membrane proteins, an aliquot of the cells was treated with 1 mg ml^{-1} trypsin for 15 min at 37 °C. The protease was inactivated using soybean trypsin inhibitor at a concentration of 1 mg ml^{-1} for 15 min on ice. Cells were lysed by repeated freezing and thawing in water supplemented with a protease inhibitor mixture containing antipain, chymostatin, aprotinin, trypsin inhibitor, Na-EDTA, pepstatin, leupeptin, and elastatinal, each at a concentration of 1 μg ml^{-1}. A membrane fraction and a fraction of soluble proteins were prepared by centrifugation.

For the biotinylation of internal parasite proteins and vacuolar proteins, IRBCs permeabilized with SLO were washed with PBS^{2+} and then resuspended in an optimal volume, routinely 800 μl, of distilled water containing the above-mentioned protease inhibitors. Lysates were prepared by three cycles of freezing and thawing. Soluble proteins contained in the supernatant after centrifugation at 10,000 $\times$ *g* for 15 min were biotin-labeled in PBS^{2+} containing 1 mg ml^{-1} sulfo-NHS-LC-biotin for 30 min at 4 °C with frequent shaking. The solution was centrifuged at 3,000 $\times$ *g* using a microconcentrator (Millipore Corp.) with a size exclusion of 5 kDa to remove any free biotin. The sample was diluted with water containing the protease inhibitors. This step was repeated twice.

For the labeling of soluble vacuolar proteins, permeabilized cells were incubated with sulfo-NHS-LC-biotin as described above. The solution was centrifuged at 4 °C at 1,300 $\times$ *g* for 5 min, and the sediment of parasite-containing vacuoles was washed three times in 100 mM glycine in PBS^{2+} to block the unreacted biotin derivative. After a final wash in PBS^{2+}, cells were lysed and processed as above.

Affinity Purification of Biotin-labeled Proteins—Biotin-labeled proteins from 2×10^8 IRBCs were incubated with streptavidin-conjugated agarose beads at 4 °C for 2 h under constant shaking. The beads were sedimented by centrifugation at 10,000 $\times$ *g* for 15 s. The supernatant was collected and stored at −20 °C until further use. Beads were washed sequentially in buffer A (10 mM Tris-HCl, pH 7.5, 0.2% Nonidet P-40, 2 mM EDTA, and 150 mM NaCl), buffer B (10 mM Tris-HCl, pH 7.5, 0.2% Nonidet P-40, 2 mM EDTA, and 500 mM NaCl), buffer C (10 mM Tris-HCl, pH 7.5), and, finally, PBS^{2+}. The beads were then washed with 10 mM HEPES buffer, pH 7.4, containing 20 mM $MgCl_2$ and 5 mM ATP. Bound proteins were eluted by boiling the beads in SDS-PAGE sample buffer before analysis.

Immunoprecipitation—The marker proteins SERP and GBP were precipitated from lysates of IRBCs as follows. After permeabilization of IRBCs and complete removal of the erythrocyte cytosol, parasite-containing vacuoles were lysed osmotically. Soluble proteins in a volume of 100 μl, corresponding to 2×10^7 IRBCs, were diluted with 400 μl of distilled water and 500 μl of solubilization buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, and 4 μg ml^{-1} phenylmethylsulfonyl fluoride. The respective antisera were added, and the mixture was incubated at room temperature for 30 min. Subsequently, 15 μl of protein A-Sepharose beads were added and incubated for 1 h at 4 °C. The beads were sedimented by centrifugation, and the supernatant was collected. Proteins were precipitated from the supernatant with 10% trichloroacetic acid on ice for 10 min. The samples were centrifuged at 4 °C, and the precipitate was washed with ice-cold acetone and dissolved in SDS-PAGE sample buffer. The protein A-Sepharose beads were washed sequentially with buffers A, B, and C and PBS^{2+} as described above, and bound proteins were eluted by boiling the beads in SDS-PAGE sample buffer.

Gel Electrophoresis—Routinely, proteins were dissolved in SDS sample buffer and separated on 10% or 12% gels under reducing conditions. When proteins were labeled with the cleavable biotin derivative sulfo-NHS-SS-biotin, separation was on 5–20% gradient gels under nonreducing conditions. Protein bands were visualized by Coomassie Blue staining before exposure to x-ray film for the detection of radiolabeled proteins. For two-dimensional PAGE, soluble parasite and vacuolar proteins from 2×10^9 permeabilized IRBCs were precipitated using 3 volumes of 10% trichloroacetic acid in acetone containing 20 mM DTT for 1 h at −20 °C. The proteins were collected by centrifugation, washed with ice-cold acetone containing 20 mM DTT, and dried. Protein extracts were dissolved in 300 μl of a rehydration solution (8 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 4% DTT, 2% ASB (Calbiochem), and 2% immobilized pH gradient buffer pH 4–7 (Amersham Biosciences)) and incubated at room temperature for 1 h. After rehydration for 24 h under low viscosity paraffin oil, 13-cm immobilized pH gradient strips (Amersham Biosciences) covering a pH range of 4–7 were subjected to isoelectric focusing for 28,000 V-h. After isoelectric focusing, the individual strips were consecutively incubated in equilibration solution A and B (50 mM Tris-HCl, pH 6.8, 8 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, complemented with 3.5 mg ml^{-1} DTT (solution A) or 45 mg ml^{-1} iodoacetamide instead of DTT (solution B)), each for 15 min. The equilibrated strips were transferred to 12% slab gels and separated in the second dimension. After drying of the gels, radiolabeled bands were visualized by autoradiography.

Immunoblot Analysis—Proteins separated by SDS-PAGE or two-dimensional PAGE were transferred to nitrocellulose membranes. For the detection of biotin-labeled proteins, membranes were blocked using 2% BSA in PBS, pH 7.4, for 1 h at room temperature. Filters were incubated for 20 min with alkaline phosphatase-conjugated streptavidin (Pharmingen) diluted 1:10,000 in 2% BSA in PBS, pH 7.4. The membranes were washed three times in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl for 5 min. Biotin-labeled proteins were stained with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate following standard procedures. The same membranes were then incubated with specific rabbit antisera at a dilution of 1:500 for 16 h at 4 °C. Specific marker proteins were detected after incubation with horseradish peroxidase-conjugated anti-rabbit IgG for 1 h at room temperature and development of the filters using the ECL system (Amersham Biosciences) following standard procedures.

Indirect Immunofluorescence Assay—Permeabilized and biotin-treated IRBCs were spread on glass slides and air-dried. Subsequently, they were fixed in acetone-methanol (1:1) for 10 min at −20 °C. The slides were incubated with the primary antibodies for 2 h at room temperature. The monoclonal anti-band 3 IgG was diluted 1:20, and the polyclonal rabbit antisera specific for SERP and aldolase, respectively, were diluted 1:10 in PBS, pH 7.2. After three washes with PBS, pH 7.2, slides were probed with a mixture of the corresponding secondary antibody (1:100) and Cy3-conjugated streptavidin (1:200) for 30 min at room temperature in the dark. Autofluorescence and nonspecific fluorescence levels were determined by viewing control samples of either biotin-treated or untreated IRBCs not incubated with Cy3-conjugated streptavidin and secondary antibodies. The cells were mounted in glycerol containing 0.1% of the antifade reagent 1,4-diazobicyclo(2,2,2)-octane. Fluorescence microscopy was performed using a Leica TCS SP2 laser scanning confocal microscope.

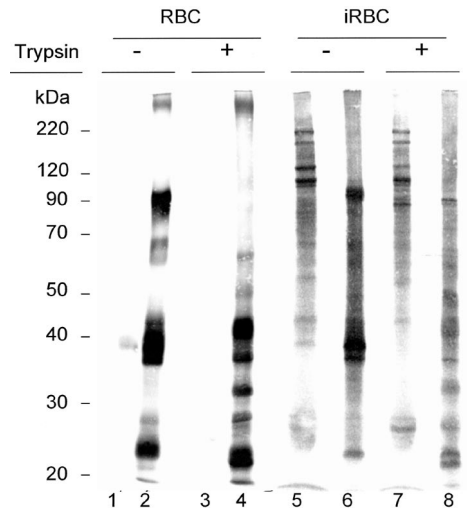

FIG. 1. **Infected erythrocytes are permeable for a nonpermeant biotin derivative.** Noninfected (*RBC*) and infected (*iRBC*) erythrocytes were incubated with sulfo-NHS-SS-biotin, and a sample from each reaction was subsequently treated with trypsin (*lanes 3, 4, 7,* and *8*) to cleave externally exposed proteins. Cells were lysed and separated into a fraction of soluble proteins (*lanes 1, 3, 5,* and *7*) and into a membrane fraction (*lanes 2, 4, 6,* and *8*). Proteins, corresponding to 2×10^7 cells, were electrophoresed through a 5–15% nonreducing SDS-polyacrylamide gel, transferred to a nitrocellulose filter, and probed with alkaline phosphatase-conjugated SAv.

RESULTS AND DISCUSSION

A Membrane-impermeable Biotin Derivative Gains Access into Intact Infected Erythrocytes—The intracellular parasite depends on the acquisition of nutrients from the extracellular milieu and from the host cell cytosol. Extracellular nutrients must be taken up across three membranes, the erythrocyte plasma membrane, the PVM, and the parasite plasma membrane. In the course of infection, the erythrocyte plasma membrane undergoes major alterations. Most notably, the transport properties of the membrane change, increasing the influx rates for a number of low molecular weight solutes. The identification of the proteins that mediate these novel transport properties is being pursued intensely (for current reviews, see Refs. 22–24). In preliminary experiments designed to study transport of small solutes across the erythrocyte membrane, we noticed that the membrane-impermeable sulfo-NHS-SS-biotin gained access to the interior of erythrocytes infected with trophozoite-stage parasites, but not to noninfected erythrocytes. Intact noninfected and infected erythrocytes were incubated with sulfo-NHS-SS-biotin. Cells were lysed in water by repeated freezing and thawing, and soluble proteins were separated from the membrane fraction by centrifugation. Whereas in noninfected cells, no biotin labeling of soluble proteins was detectable (Fig. 1, *lanes 1* and *3*), soluble proteins were found to be biotin-labeled in infected cells (Fig. 1, *lanes 5* and *7*). When intact erythrocytes were treated with trypsin after the biotin reaction, the pattern of biotin-labeled proteins in the membrane fraction changed, due to proteolytic cleavage of externally exposed protein domains (Fig. 1, *lanes 2, 4, 6,* and *8*). More importantly, trypsin treatment had little effect on the pattern of biotin-labeled soluble proteins in infected cells (Fig. 1, *lanes 5* and *7*). This result demonstrates that the protease, unlike the biotin derivative, was excluded from the erythrocyte cytosol. Most likely, access of biotin into intact erythrocytes is due to the novel transport properties of the erythrocyte membrane, and we are currently investigating the pathways involved in this process. We never observed that biotin gained access to vacuolar proteins in infected erythrocytes using these experimental conditions (data not shown). We anticipate that internalization of biotin across the intact host cell plasma membrane is limited and that the excess of erythrocyte cytosolic proteins prevented efficient labeling of vacuolar proteins.

Biotin Accumulates in the Parasitophorous Vacuole in Permeabilized Infected Erythrocytes—Although the vacuolar membrane forms a barrier between the parasite surface and the host cell cytosol, it is most conceivable that the PVM allows the transport of nutrients essential for parasite survival. Using a patch-clamp technique, Desai *et al.* (4) measured high conductance channels that are permeable to organic and inorganic anions and cations. The channels are open 98% of the time, and they are highly abundant. In bilayers, this channel allows passage of molecules up to 1,400 Da (5), which is similar to the nonselective pores discovered in the PVM of the taxonomically related parasite *Toxoplasma* (25). The existence of these pores in *Plasmodium*-infected erythrocytes has not been verified biochemically, but if they exist, they should allow access of membrane-impermeable biotin derivatives that are ~600 Da in size.

To increase access of biotin to the interior of the infected erythrocyte, we utilized SLO to permeabilize the erythrocyte plasma membrane. In an initial assessment of our experimental strategy, infected erythrocytes were permeabilized and, after complete removal of the erythrocyte cytosol, incubated with the membrane-impermeable sulfo-NHS-LC-biotin, which was detected using a fluorescent streptavidin derivative (Fig. 2). The images show a distinct ring around the periphery of the intracellular parasite. The biotin co-localizes with the vacuolar marker protein SERP (Fig. 2*A*). This location is distinct from the location of the erythrocyte membrane protein band 3 and from the location of the parasite cytosolic protein aldolase (Fig. 2, *B* and *C*). In some parts, the streptavidin and the antibody to band 3 co-localize; this is due to the fact that proteins of the erythrocyte plasma membrane also react with the biotin. Little if any biotin was detectable in the erythrocyte cytosol and in the parasite cytosol, respectively. A ring-like staining around the periphery of the parasite can be attributable to a reaction of the biotin with membrane proteins of the PVM and with soluble vacuolar proteins. Because the resolution of fluorescence microscopy does not allow discrimination between a staining of the PVM and a lumenal staining, a more detailed biochemical analysis was performed to assess the biotinylation of soluble vacuolar proteins.

Biotin-labeled Parasite Proteins Bind to SAv-agarose—Because mammalian erythrocytes have lost the ability to synthesize proteins *de novo*, in infected erythrocytes, radiolabeled amino acids are incorporated exclusively into parasite proteins. The following experiment was designed to optimize the biotinylation conditions and the selective isolation of biotinylated proteins by SAv-agarose. Infected cells were cultivated for 30 min in the presence of L-[^{35}S]methionine, and subsequently, the erythrocyte plasma membrane was permeabilized with SLO to release the soluble contents of the erythrocyte cytosol. In all experiments, the integrity of the PVM and the parasite plasma membrane was assessed as described previously (10). Parasite-containing vacuoles were lysed by repeated freezing and thawing, and the fraction of soluble proteins was collected after centrifugation. One aliquot of the lysate was treated with sulfo-NHS-LC-biotin, and another aliquot remained untreated. Both samples were incubated with SAv-agarose beads, and bound proteins were eluted by boiling the beads in SDS sample buffer. The fractions of unbound and bound proteins were analyzed by SDS-PAGE and autoradiography (Fig. 3*A*). No radiolabeled proteins from the nonbiotinylated sample bound to SAv-agarose (Fig. 3*A, lane 2*), whereas after biotinylation, most radiolabeled proteins were found in the fraction of bound proteins (Fig. 3*A, lane 4*). It is noteworthy that the pattern of

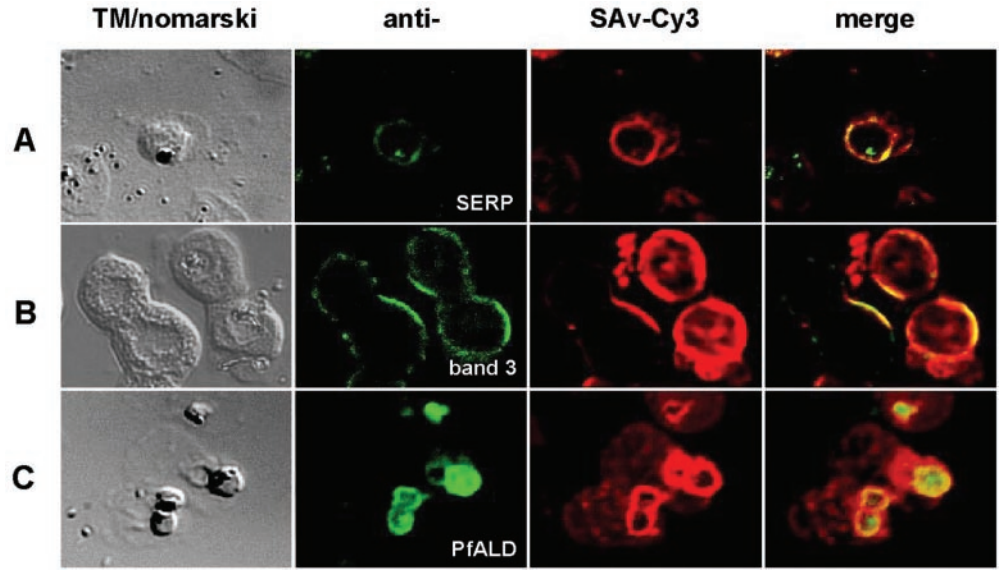

FIG. 2. **Biotin localizes to the parasitophorous vacuole.** Trophozoite-infected erythrocytes were permeabilized with SLO, biotin-labeled, and spread onto microscope slides. Cells were fixed and incubated with antibodies to SERP (*A*), a vacuolar resident protein. Conjugated secondary antibodies (*green color*) co-localize with SAv-Cy3 (*red color*). *B* and *C* show dual labeling of biotin and the erythrocyte membrane protein band 3 (*B*) or of biotin and the parasite cytosolic protein PfALD (*C*). Cells were imaged using a laser scanning confocal microscope. The *left panels* (*TM/nomarski*) show the transmission images of the respective fields, and the *right panels* (*merge*) show an overlay of fluorescence images for each of the antibodies used.

biotinylated proteins differed from that of the nonbiotinylated proteins and that the bands appeared less focused. This observation is attributable to the variant degrees of biotin labeling of individual polypeptide chains. To assess the effects of biotin incorporation on the electrophoretic motility of proteins, BSA was incubated with different molar ratios of sulfo-NHS-LC-biotin and analyzed by SDS-PAGE. As shown in Fig. 3*B,* the electrophoretic motility of BSA is altered depending on the degree of biotinylation.

The subsequent experiments were devised to demonstrate compartment-specific biotinylation of soluble parasite proteins in permeabilized IRBCs. The so-called GBP, the SERP, and parasite aldolase (PfALD) are soluble marker proteins that are located in different compartments of the infected erythrocyte. Aldolase is restricted to the parasite cytosol (19), SERP is restricted to the vacuole (13), and GBP is found both inside the vacuole and within the erythrocyte cytosol (10). In the first step, it was confirmed that these proteins can be biotinylated and that they bind to SAv-agarose, provided that they are accessible to biotin. This was assessed using lysates of permeabilized infected erythrocytes; infected erythrocytes were permeabilized with SLO, and after complete removal of the erythrocyte cytosol, a lysate containing soluble proteins of the parasite cytosol and of the PV was prepared. One aliquot of soluble proteins was treated with sulfo-NHS-LC-biotin as described above. Another aliquot remained untreated. The samples were incubated with SAv-agarose beads, and the bound and unbound proteins were analyzed separately by immunoblotting using antisera to the respective marker proteins (Fig. 4*A*). In the sample of nonlabeled proteins, the three marker proteins were recovered in the fraction of unbound proteins (Fig. 4*A,* compare *lanes 1* and *2*). After biotin treatment, GBP, SERP, and PfALD were recovered in the fraction of bound proteins (Fig. 4*A,* compare *lanes 3* and *4*). In the case of GBP and SERP, recovery was almost quantitative. Although recovery was not entirely complete in the case of PfALD, these results demonstrate that the marker proteins are biotinylated and can be isolated on SAv-agarose.

After confirmation that all marker proteins are biotinylatable, IRBCs were permeabilized with SLO, washed to remove

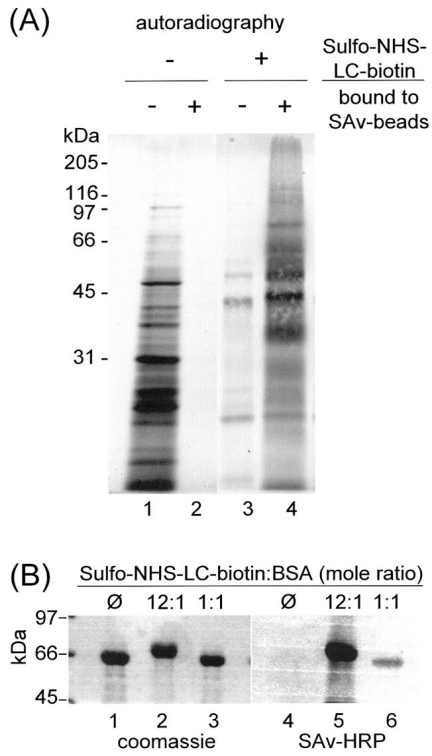

FIG. 3. *A,* biotin labeling of parasite proteins. Infected erythrocytes were metabolically labeled with L-[^{35}S]methionine. Erythrocytes were permeabilized with SLO, and the erythrocyte cytosol was extracted. The remaining fraction consisted of intact parasites, including an intact PVM and parasite plasma membrane. The PVM and the parasite plasma membrane were then lysed, and membrane proteins and soluble proteins were separated. One aliquot of soluble proteins, corresponding to 2×10^8 cells, was treated with sulfo-NHS-LC-biotin (*lanes 3* and *4*), and one aliquot remained untreated (*lanes 1* and *2*). Proteins that bound to SAv-agarose beads (*lanes 2* and *4*) and unbound proteins (*lanes 1* and *3*) were analyzed by SDS-PAGE and autoradiography. *B,* extensive biotin labeling increases the molecular size of proteins. BSA was treated with sulfo-NHS-LC-biotin at different molar ratios and analyzed by SDS-PAGE and Coomassie Blue staining (*lanes 1–3*) or by reaction with peroxidase-conjugated SAv on nitrocellulose filters (*lanes 4–6*).

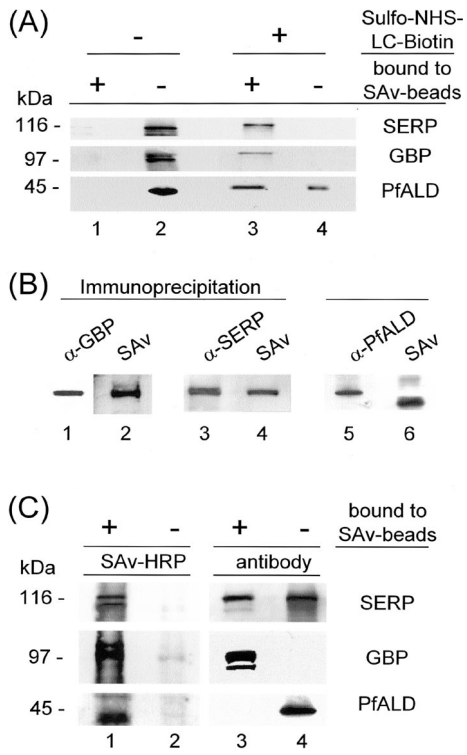

FIG. 4. **Selective biotin labeling of vacuolar marker proteins.** *A,* to confirm that the marker proteins can be biotinylated in solution, a soluble protein fraction was prepared and processed as described for Fig. 3*A*. The fraction of proteins bound to SAv-agarose beads (*lanes 1* and *3*) and the fraction of unbound proteins (*lanes 2* and *4*) were analyzed by immunoblotting for the presence of the marker proteins SERP, GBP, and PfALD. *B* and *C,* infected erythrocytes were permeabilized with SLO. After complete extraction of the erythrocyte cytosol, the cellular fraction consisting of intact parasites and the intact PV was incubated with sulfo-NHS-LC-biotin and lysed after removal of free biotin. *B,* proteins were immunoprecipitated using antisera against GBP and SERP, respectively, separated by SDS-PAGE, and transferred to nitrocellulose filters (*lanes 1–4*). The filters were first probed with antisera that were detected in a color reaction using alkaline phosphatase-conjugated secondary antibodies (*lanes 1* and *3*). The same filters were reprobed with peroxidase-conjugated SAv, which was subsequently detected by a chemiluminescence reaction (*lanes 2* and *4*). The incorporation of biotin into aldolase was analyzed in the same way (*lanes 5* and *6*), except that soluble proteins were separated electrophoretically without prior immunoprecipitation. *C,* the lysate was reacted with SAv-agarose beads, and the fractions of bound (*lanes 1* and *3*) and unbound (*lanes 2* and *4*) proteins were analyzed as described in *B* for the incorporation of biotin (*lanes 1* and *2*) and the presence of the respective marker proteins (*lanes 3* and *4*).

the contents of the erythrocyte cytosol, and incubated with sulfo-NHS-LC-biotin. Upon removal of free biotin and lysis of parasites, soluble proteins were immunoprecipitated using antibodies to SERP and GBP, respectively. Precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose filters. Initially, the filters were probed with the SERP and GBP antibodies, and their binding was detected using an alkaline phosphatase-conjugated secondary antibody (Fig. 4*B, lanes 1* and *3*). Subsequently, the filters were reprobed with peroxidase-conjugated SAv and analyzed by chemiluminescence (Fig. 4*B, lanes 2* and *4*). The same analysis was carried out for PfALD, but because the antiserum against aldolase does not immunoprecipitate the protein, the total lysate was separated by SDS-PAGE and analyzed (Fig. 4*B, lanes 5* and *6*). The antisera specifically recognized the marker proteins. The bands that were detectable with SAv were of identical sizes as GBP and SERP, indicating that both proteins were biotin-labeled. No biotin-labeled band corresponding in size to aldolase was detectable; a protein band of smaller size reacted with SAv, but not with the aldolase-specific antibody.

Affinity isolation of biotin-labeled proteins on SAv-agarose beads and subsequent analysis by immunoblotting confirmed the compartment-specific labeling of the respective proteins (Fig. 4*C*). In permeabilized cells, PfALD was not labeled, and, consequently, it was not found in the fraction of proteins bound to SAv, whereas GBP was quantitatively labeled with biotin and recovered. Apparently, in permeabilized cells, the accessibility of the biotin to SERP is restricted; all of the biotin-labeled SERP protein is bound to SAv-agarose beads (Fig. 4*C, lanes 1* and *2*); however, a substantial proportion of the SERP molecules remained unbound, obviously because these molecules have not been labeled with biotin (Fig. 4*C,* compare *lanes 3* and *4*). A 5-fold increase of biotin in the biotinylation reaction did not result in more efficient labeling of SERP (data not shown). It is noteworthy that in lysates, *i.e.* when SERP is released, the protein is labeled and binds to SAv quantitatively (Fig. 4*A*). Although it is possible that the unlabeled protein in Fig. 4*C* represents an intraparasite pool of SERP en route to the parasite plasma membrane, we consider this possibility unlikely. In previous experiments, we were unable to detect levels of SERP within the parasite, unless secretion was actively inhibited by the drug brefeldin A (19, 10). It is more likely that, within the vacuole, at least a proportion of the molecule assumes a tight conformation that severely compromises the access of biotin. In no experiments did we detect labeled or unlabeled hemoglobin, the most abundant soluble protein in trophozoite-infected erythrocytes, indicating that the soluble erythrocyte contents had been removed efficiently from permeabilized cells.

The Chaperone PfBiP Associates with Biotin-labeled Proteins—Although in none of our experiments using SLO-permeabilized erythrocytes were we able to detect biotin-labeled aldolase, we analyzed the fraction of SAv-agarose-bound proteins for the presence of several other intraparasite proteins. In the fraction of proteins that were isolated by affinity chromatography on SAv-agarose beads, we consistently detected PfBiP (26), the plasmodial homologue of BiP, a molecular chaperone and endoplasmic reticulum resident protein in eukaryotic cells (Fig. 5*A*). Because it is possible that some of this protein may not be retained within the parasite's secretory pathway and hence may be transported into the vacuole, we investigated whether PfBiP was biotin-labeled. Permeabilized cells were treated with sulfo-NHS-LC-biotin and subsequently lysed, and PfBiP was immunoprecipitated. The precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose filters. In the first reaction, binding of the anti-PfBiP antiserum was determined using an alkaline phosphatase-conjugated secondary antibody (Fig. 5*B, lane 1*). In a second reaction, biotin was detected by chemiluminescence using peroxidase-conjugated SAv (Fig. 5*B, lane 2*). Under these conditions, biotin was undetectable, suggesting that recovery of PfBiP in the fraction of proteins that bound to SAv-agarose was due to an interaction of the chaperone with other biotin-labeled proteins. PfBiP did not bind to SAv-agarose beads in the absence of the biotin (Fig. 5*A*), ruling out the possibility that the protein directly interacted with SAv or the resin. Most likely, when cell lysates are prepared, intraparasite chaperones such as PfBiP associate with biotinylated proteins bound to SAv-agarose beads and are co-isolated. In the subsequent experiments, the protocol was revised to reduce the possibility of mistaking intraparasite chaperones as putative vacuolar resident proteins. Chaperones are released from their substrates in a process that requires the hydrolysis of ATP. Therefore, SAv-agarose beads were washed in the presence of 5 mM ATP before electrophoretic analysis. This step eliminated the co-isolation of PfBiP almost entirely,

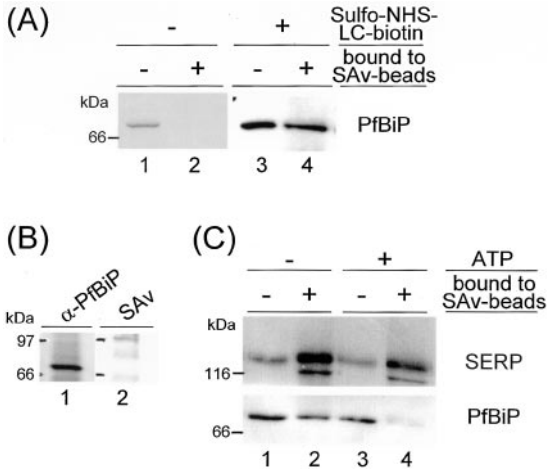

FIG. 5. **Co-isolation of PfBiP with biotinylated proteins.** *A,* infected erythrocytes were permeabilized, and one aliquot of the cells was treated with sulfo-NHS-LC-biotin. Lysates of cells were prepared and incubated with SAv-agarose beads. The fraction of unbound proteins (*lanes 1* and *3*) and the fraction of bound proteins (*lanes 2* and *4*) were analyzed for the presence of PfBiP using a specific antiserum. *Lanes 1* and *2,* untreated sample; *lanes 3* and *4,* biotinylated proteins. *B,* PfBip was immunoprecipitated from a sample of biotin-treated cells, and the precipitate was analyzed by immunoblotting using either the specific antiserum (*lane 1*) or peroxidase-conjugated SAv (*lane 2*). *C,* biotinylated proteins were bound to SAv beads, and the beads were washed with either HEPES buffer (*lanes 1* and *2*) or HEPES buffer containing 5 mM ATP (*lanes 3* and *4*). Proteins that remained bound to the beads (*lanes 2* and *4*) and unbound proteins (*lanes 1* and *3*) were analyzed for the presence of SERP and PfBiP.

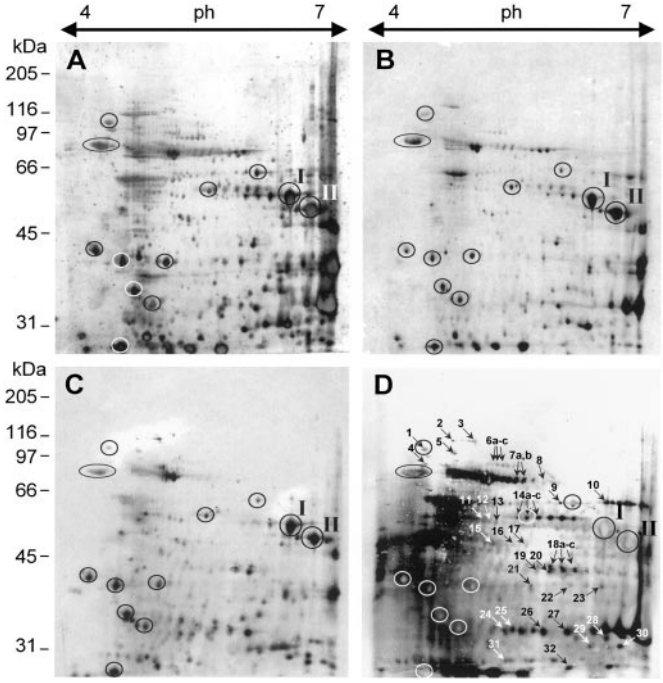

FIG. 6. **Protein pattern of putative vacuolar proteins.** Proteins in intact infected erythrocytes were metabolically labeled, and cells were permeabilized and treated with biotin. The fraction of soluble proteins, each corresponding to 2×10^9 infected erythrocytes, was subjected to two-dimensional gel electrophoresis using a pH gradient of 4–7 in the first dimension and 12% SDS-PAGE in the second dimension. *A,* silver-stained gel. *B,* Coomassie Blue-stained gel. *C* and *D,* proteins from a third two-dimensional gel were transferred to a nitrocellulose filter. The filter was first exposed to x-ray film to visualize metabolically labeled parasite proteins (*C*) and subsequently reacted with alkaline phosphatase-conjugated SAv to visualize biotinylated proteins (*D*). The most prominent spots that were identified as parasite proteins are marked with *circles*. Proteins that are both biotinylated and radiolabeled are indicated by *Arabic numerals*; spots that may represent identical polypeptides at various degrees of biotinylation are marked with *lowercase letters*. *I, P. falciparum* enolase; *II, P. falciparum* ornithine aminotransferase.

but it had no effect on the isolation of SERP (Fig. 5*C*) or of other biotin-labeled proteins.

Two-dimensional Gel Electrophoretic Analysis of Biotin-labeled Parasite Proteins—As a prerequisite for the identification of novel vacuolar proteins, IRBCs were labeled metabolically with L-[^{35}S]methionine, permeabilized with SLO, and treated with sulfo-NHS-LC-biotin. After cell lysis, soluble proteins were separated in triplicate on different two-dimensional gels using a pH gradient of 4–7 in the first dimension. To estimate the relative abundance of proteins in permeabilized erythrocytes, one gel was silver-stained (Fig. 6*A*), and one gel was stained with Coomassie Blue (Fig. 6*B*). As expected, silver staining revealed the most complex protein pattern. The most abundant proteins were also detectable using the less sensitive Coomassie Blue stain. To compare biotinylated proteins *versus* total parasite proteins, proteins of a third gel were transferred to a nitrocellulose filter. The filter was exposed to x-ray films to analyze the pattern of radiolabeled proteins (Fig. 6*C*). Subsequently, biotin-labeled proteins were detected using alkaline phosphatase-conjugated SAv (Fig. 6*D*). A comparison of the biotin-labeled proteins and the radiolabeled proteins clearly demonstrates that the pattern of biotin-labeled proteins is less complex and that it does not simply reflect the pattern of the most abundant radiolabeled proteins. In contrast, the most intensely radiolabeled proteins appear not to be biotin-labeled. Considering that cytosolic housekeeping proteins of the parasite, such as enzymes of the glycolytic pathway, are likely to be highly abundant, this result underscores the notion that the incorporation of the biotin is selective. In fact, when a matrix-assisted laser desorption ionization time-of-flight analysis was carried out using the two most prominent spots (I and II), they were identified unequivocally as *P. falciparum* enolase and as *P. falciparum* ornithine aminotransferase, respectively (data not shown). Biotin-labeled proteins form characteristic strings of individual spots. We attribute this pattern to various degrees of biotin incorporation into the same polypeptide chain. Because each sulfo-NHS-LC-biotin adds a negative charge, molecules with a high incorporation of the derivative are expected to migrate toward the acidic end of the gradient. The shift to the acidic end of the gradient also correlates with a slight increase in molecular size. A detailed comparison reveals 12 prominent spots, *circled* in Fig. 6, *A–D,* which represent highly abundant parasite proteins that are detectable by Coomassie Blue staining. Nine of these spots appear negatively stained in Fig. 6*D* because of the high local concentration of nonbiotinylated protein. Thirty-nine protein spots were identified that were both biotinylated and radiolabeled and that therefore fulfill the criteria expected for vacuolar proteins. Some of these spots may represent identical polypeptides that are biotinylated to various degrees. In conclusion, the accessibility of nonpermeant biotin derivatives to the vacuolar lumen in permeabilized IRBCs has allowed the identification of candidate vacuolar proteins. These proteins are restricted in number, and they segregate into a distinct two-dimensional pattern. The sequencing of the parasite's genome has almost been completed. This will allow the rapid identification of the genes encoding vacuolar proteins, as a prerequisite for a molecular understanding of the cell biological functions of this unusual compartment in the infected erythrocyte.

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