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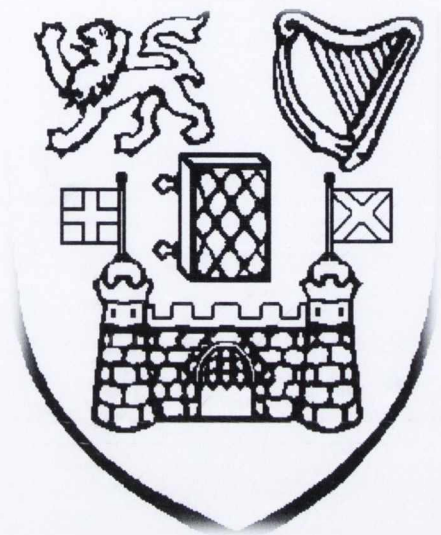
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A Study of the Glycosylphosphatidylinositol Phospholipase C in *Trypanosoma brucei*



By

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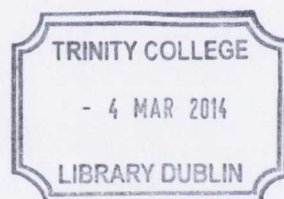
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for the degree of Doctor of Philosophy

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Summary

Trypanosoma brucei represents a clade of flagellated parasites that cause human and animal trypanosomiases in sub-Saharan Africa. The glycosylphosphatidylinositol phospholipase C (GPI-PLC) is an endogenous enzyme that has been shown to contribute to the virulence of the parasite *in vivo*. Although the physiological role of the GPI-PLC is currently unknown, the enzyme has been shown to hydrolyze the GPI-anchor of the trypanosome's protective variant surface glycoprotein (VSG) coat under a number of experimental conditions.

The GPI-PLC was recently found to localize along the flagellar attachment zone, on the outer bilayer of the flagellar membrane, as a linear, patchy array. However there is no recognized signal sequence in the enzyme's primary structure that can account for such a specific membrane localization. Furthermore, while the GPI-PLC primary structure contains no recognized Ca^{2+} binding domain, there has been strong evidence that implicates Ca^{2+} in the regulation of the enzyme. In this study, potential GPI-PLC partner proteins that could account for the specific localization of the GPI-PLC, and partner proteins that could be involved in the regulation of the GPI-PLC were sought.

A robust MALDI-ToF mass spectrometric procedure for the identification of unknown proteins was first established and validated. The standard proteins cytochrome c, ovalbumin, and bovine serum albumin, were all successfully identified by peptide mass fingerprinting following MALDI-ToF mass spectrometry of in-gel trypsin, and chymotrypsin, digests of the proteins. Immunoprecipitation of the GPI-PLC was successfully carried out in Triton[®] X-100, and n-octylglucoside based cell lysis buffers; the presence of the GPI-PLC in the immunoprecipitated material was confirmed by MALDI-ToF mass spectrometry. However, despite testing a range of non-ionic, ionic, and zwitterionic detergents, no specific co-immunoprecipitating candidate partner protein for the GPI-PLC was found.

The de-energization of bloodstream form trypanosomes was examined in an effort to determine how an intracellular calcium signal could be transduced to an activation signal for an enzyme located on the cell surface. In contrast to earlier

reports, here it was definitively demonstrated that the de-energization of trypanosomes leads to generalized cell rupture and it was further shown that this time course of cell rupture correlated with GPI-PLC-mediated release of the VSG. Cell rupture, with concurrent release of the VSG, was much more rapid in trypanosomes de-energized in the presence of Ca^{2+} and the calcium ionophore A-23187. Since the onset of cell rupture and GPI-PLC activation could not be distinguished in a number of different conditions, the hypothesis that the GPI-PLC might be specifically regulated *in situ* by a calcium binding protein should be reconsidered.

In this thesis, the *in vitro* growth characteristics of trypanosomes lacking the GPI-PLC were also investigated. Regular enumeration of trypanosomes maintained in different culturing conditions revealed that cells lacking the GPI-PLC had a longer doubling time than wild-type cells. It was additionally observed that GPI-PLC knockout cells could only reach approximately half the maximum cell density achievable by wild-type cells. While the true physiological role of the GPI-PLC has remained unclear since the enzyme's discovery, the findings of this study highlight a new, and previously unconsidered, importance of the GPI-PLC in trypanosomes.

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.... Almost there... How, *raises arms to the sky for dramatic effect*, how could I have survived my time in college without the support of the two lads back home, who have waited eagerly for me to finish. Cian and Fran have been there through it all. There is no doubt their friendship has helped keep me going. All the advice and encouragement offered over countless beverages (some hot and caffeinated, others cold and alcohol based) is deserving of the most sincere thanks! I guess Ger has also suffered my complaints over enough coffees and pints to warrant his inclusion too – he helped keep spirits up, so thanks Ger!

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Abbreviations

AAT	Alanine amino transferase
BES	Bloodstream expression site
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
α -CHCA	α -Cyano-4-hydroxycinnamic acid
CMC	Critical micellar concentration
CRD	Cross reacting determinant
CTAB	Cetyltrimethylammonium bromide
DEAE-52	Diethylaminoethyl cellulose
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
FBS	Foetal bovine serum
Gal	Galactose
GlcN	Glucosamine
GPR	General practice reagent
HMI-9	Hirumi modified Iscove's medium, formulation 9
HPLC	High performance liquid chromatography (grade)
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IgM	Immunoglobulin M
L_n	Natural logarithm
MALDI	Matrix assisted laser desorption ionisation
Man	Mannose
MBP	Maltose binding protein
mfVSG	Membrane-form variant surface glycoprotein
MITar	Molteno Institute Trypanozoon antigen repertoire
MITat	Molteno Institute Trypanozoon antigen type

MW	Molecular weight
N/A	Not applicable or not available
NEM	<i>N</i> -ethylmaleimide
NHS-(LC)	<i>N</i> -Hydroxysuccinimide (long chain spacer)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
pCMPS	<i>p</i> -Chloromercuriphenylsulfonic acid
(8-)pCPTcAMP	8-(4-Chlorophenylthio)adenosine 3',5'-cyclic monophosphate
(G)PI	(Glycosyl)Phosphatidylinositol
PLC	Phospholipase C
PMSF	Phenylmethanesulfonyl fluoride
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
sVSG	Soluble variant surface glycoprotein
TBS(T)	Tris buffered saline (with Tween)
TEMED	<i>N,N,N',N'</i> -Tetramethylethylenediamine
Tes	<i>N</i> -[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid
TFA	Trifluoroacetic acid
TLCK	<i>N</i> α-Tosyl-L-lysine chloromethyl ketone hydrochloride
ToF	Time of flight
TPCK	<i>N-p</i> -Tosyl-L-phenylalanine chloromethyl ketone
Tris	Tris(hydroxymethyl)aminomethane
TSB	Trypanosome separating buffer
VSG	Variant surface glycoprotein
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
YFP	Yellow fluorescent protein

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Chapter 1

Introduction

1.1. The African Trypanosome

1.1.1. Historical prologue

“...nagana – the pretty native name for an unknown something that made great stretches of South Africa into a desolate place, impossible to farm in, dangerous to hunt big game in, suicidal to travel in... Nagana steals into fine horses and makes their eyes stare and their hair fall out; while the fat of these horses melts away nagana grows watery pouches on their bellies and causes a thin rheum to drip from their noses; they droop, and at last die – every last horse touched by nagana dies. It was the same with cattle... Fat droves of cattle, sent away to far-off slaughter-houses, arrived there hairless, hidebound skeletons. There were strange belts of country through which it was death for animals to go. And the big game hunters!”

Paul de Kruif; extract from “Microbe Hunters”, 1926.

Although descriptions of diseases matching the vivid portrayal of Paul de Kruif were already known for some time, our story here begins with the assignment of Surgeon Major David Bruce to Africa in 1894. It was at the request of Sir Walter Hely-Hutchison, Governor of Natal, that Bruce was sent to South Africa to investigate an epidemic that was devastating the Zulu's cattle (Grogono, 1995a). At the time Bruce was already a well recognized pathologist, credited with the discovery of the Gram negative bacterium (later named *Brucella mellitensis*) responsible for Malta fever (Bruce, 1887).

Bruce, accompanied by his wife Mary Elizabeth Bruce, travelled to Ubombo – a small village North of Pietermaritzburg (the current capital city in the province of KwaZulu-Natal, in Eastern South Africa). After their arrival in Ubombo the Bruces were met with two theories concerning the cause of the fatal disease known locally as nagana. The first of these theories, supported by the natives, suggested that nagana was caused by wild game contaminating the water supply, while the second theory, put forward by the explorer David Livingston in the early eighteen fifties, proposed that the sickness was caused by the bite of the tsetse fly (Laveran and Mesnil, 1907; Steverding, 2008). At the time the Bruces were called for, the plains below Ubombo village were infested with flies, and it was in these fly-plagued regions that the Zulu's cattle were dying in such large numbers (Grogono, 1995a).

Bruce worked with a number of animals, and subjected them to the bites of the flies from regions where nagana was prevalent. Bruce also observed organisms swimming in the blood of animals suffering from the disease, and he eventually recognized the organisms as trypanosomes. It should be pointed out that by this stage an extensive literature had already developed on the trypanosome, and Bruce was undoubtedly aware of the earlier discovery by Griffith Evans that trypanosomes were the causative agent of surra in India – a disease of horses and camels that was similar to nagana (Evans 1880). Ultimately, Bruce elucidated the aetiology of nagana; big game animals acted as a reservoir for the nagana parasite, and the tsetse fly acted to transmit the parasite from these game animals to domestic cattle and horses during the fly's blood meal (Bruce, 1897).

In 1896, during his investigations in South Africa, Bruce had one of his infected dogs sent to England, and the parasites of this animal became the first pathogenic trypanosomes to be studied in laboratories across Europe. The researchers Plimmer and Bradford, who worked in London with the parasites sent by Bruce, named the organism *Trypanosoma brucei* (which was later corrected to *T. brucei* in accordance with standard nomenclature practice), (Plimmer and Bradford, 1899; Laveran and Mesnil, 1907). The name *Trypanosoma* (from τρύπανον, *trupanon*, a borer; and σῶμα, *soma*, body) had been already introduced by Gruby, who observed and accurately described a parasite, “smooth and twisted two or three times around its long axis like a wimble or a corkscrew”, in the blood of frogs (Gruby, 1843).

Until the beginning of the twentieth century Bruce's work had focused on the Trypanosome responsible for nagana. But at this time more investigations were also being pursued by other researches, and it was becoming clear that different species of trypanosomes were responsible for other afflictions. Of particular interest to this history, is the case of a patient of Robert Michael Forde in Banjul (the capital city of Gambia, which was called Bathurst at the time). Forde's patient was the captain of a steamer on the River Gambia who was suffering from presumed malarial fevers, or a condition that appears similar to descriptions of "trypanosome fever". With the aid of Joseph Everett Dutton, however, organisms in the captain's blood were identified as trypanosomes, which were called *T. gambiense* (Dutton, 1902).

About a year after Dutton's discovery, Aldo Castellani discovered trypanosomes in the cerebrospinal fluid of patients suffering from sleeping sickness in the town of Entebbe, on the Northern shores of Lake Victoria in Uganda. Sleeping sickness, was affecting native workers in the region and was characterized by various neurological effects before the victims ultimately fell into a fatal coma (Grogono, 1995b). When Castellani made his discovery, sleeping sickness was still considered a separate illness to trypanosome fever; the causative agent of trypanosome fever and the organism responsible for sleeping sickness were therefore also thought to be different species of trypanosome. Castellani named the trypanosome of sleeping sickness *T. ugandense* (Castellani, 1903a; Castellani, 1903b; Hoare, 1972), (some other researchers named the parasite *T. castellanii*). However it wasn't long before Bruce, who was also stationed in Entebbe, and other researchers established that *T. gambiense* and *T. ugandense* were in fact the same species of trypanosome (Bruce and Nabarro, 1903; Laverin and Mesnil, 1907) and it was further shown that trypanosome fever represented the early stage of sleeping sickness (Bruce, *et al.*, 1903; Gray and Tulloch, 1907).

This epidemic of sleeping sickness in Uganda, which broke out in the region of Busoga in 1896, ultimately claimed an estimated 500,000 lives across Uganda and Kenya, and a further estimated 300,000 lives in the Congo Basin (Steverding, 2008) before ending in 1906. Just two years later, however, another research commission would be established, under the direction of Bruce, to investigate another epidemic of sleeping sickness, this time in Nyasaland (present day Malawi), along the Eastern shores of Lake Nyasa (now Lake Malawi), (Grogono, 1995b). The research carried out by this later commission, between 1908 and 1915, was significant. The development of

trypanosomes in the tsetse vector was documented (e.g. Bruce *et al.*, 1914), and it was further established that this latest epidemic was caused by a trypanosome distinct to that responsible for the epidemic in Uganda; a subtly different trypanosome was found, in the blood of an infected individual, by Professor John Stephens, and named *T. rhodesiense* after its geographical distribution (Stephens carried out his work in Rhodesia, south of Lake Nyasa), (Stephens and Fantham, 1910).

Although *T. rhodesiense* was widely accepted as morphologically and pathologically distinct to *T. gambiense*, at the time the morphological distinctions between *T. rhodesiense* and *T. brucei* were much less obvious; many researches simply described *T. rhodesiense* as a human infective form of *T. brucei*. Other researchers, unable to distinguish between *T. brucei* and *T. rhodesiense* by microscopic examination, or in terms of the parasites' infectivity to laboratory animals, considered *T. rhodesiense* to be precisely the same species of trypanosome – the immunity of persons to the nagana parasite was, therefore, considered a variation in human susceptibility to *T. brucei*, and didn't necessarily prove that *T. rhodesiense* was a separate species of trypanosome (Wenyon, 1926). *T. rhodesiense* (referred to as *T. brucei* vel *rhodesiense* by Bruce) was also shown to be transmitted by the same arthropod vector as *T. brucei*, i.e. *G. Morsitans*, with wild game animals acting as a reservoir (Kinghorn and Yorke, 1912).

By Bruce's return to England in 1913, to report the findings of this commission on sleeping sickness, a comprehensive body of literature had documented the aetiology of trypanosomiasis (including animal and human forms of the diseases). Significantly, the first classification scheme for trypanosomes had also been developed (see section 1.2.2), although it would be some time still before the distinction between *T. brucei* and *T. rhodesiense* was confidently and widely accepted. Bruce would not return to the study of trypanosomes. Instead, after being appointed Commandant of the Royal Army Medical College at Netley, he was chosen to lead a commission investigating enteric fever and tetanus (Grogono, 1995b), a pursuit which is beyond the scope of this text. Undoubtedly, the research of Bruce, together with studies of the sleeping sickness commissions, provided a substantial foundation for the future study of trypanosomes, and indeed other tropical illnesses.

1.1.2. Classification of Trypanosomes

The third commission on sleeping sickness made the first distinction between different trypanosomes, and provided the first system for classifying the parasites. Originally there were three major groups of trypanosomes; trypanosomes belonging to the *brucei* group, the *pecorum* group or the *vivax* group where each defined by their morphology, their development, and their pathogenicity to animals. The classification scheme has changed significantly since, however, and has most recently been updated in 2012 by the International Society of Protistologists (Adl *et al.*, 2012).

The scheme put forward by the International Society of Protistologists represents a consolidation of the major changes established in the classification scheme of several years earlier (Adl *et al.*, 2005). This earlier scheme was the first system that abandoned the formal designations such as “class”, “sub-class”, and “phylum” *etc.*, in order to adopt instead a nameless, systematic ranking scheme. This development to the classification methods was made partly to avoid the cascade of changes that inevitably followed the repositioning of some organisms, or groups of organisms, in older systems. It has been recommended that when referring to a rank in this new ranking system, the rank (*e.g.* Trypanosoma) should be preceded by two or three higher ranks, placed in brackets, with the highest rank given first (Adl *et al.*, 2005).

Most eukaryotes are now divided into one of five monophyletic lineages, or “super-groups”, with Animalia and Fungi, for example, deriving from the Opisthokonta lineage, and the Plantae deriving from the Archaeplastida lineage. The Opisthokonta and Amoebozoa super-groups are considered members of the Amorphea clade (sometimes referred to as “Unikonta”), while the Sar and Archaeplastida super-groups, together with some other eukaryotes, are members of the Diaphoreticks clade. Trypanosomes, belong to the Excavata super-group.

Of particular relevance to this report is the Trypanosoma brucei clade [Excavata: Kinetoplastea: Trypanosomatida], of which the causative agent of nagana, and the organism used for this study (*T. b. brucei*), is a member. Other members of this clade include the human infective trypanosomes, *i.e.* the trypanosomes responsible for sleeping sickness, such as *T. b. gambiense* and *T. b. rhodesiense*. Interestingly, organization of the Trypanosoma clades, although largely based on genetic analysis of

18S rRNA, appears dependent on the natural host of the trypanosomes, with the *T. cruzi* and *T. brucei* clades containing the only trypanosomes pathogenic to humans (Stevens *et al.*, 1999). The revised classification system of Adl *et al.*, (2012) is presented in figure 1.1.

1.1.3. Trypanosome Morphology and Ultrastructure of the Flagellum

“...a curious little beast, a creature with a blunt rear-end and a long slim lashing whip with which he seemed to explore in front of him. A creature shaped like a panatela cigar, only it was flexible, almost tying itself in knots sometimes... What extraordinary creatures! They didn’t go stupidly along like common microbes – they acted like intelligent little dragons. Each one of them darted from one round red blood cell to another; he would worry at it, try to get inside it, tug at it and pull it, push it along ahead of him – and then suddenly off he would go in a straight line and bury himself under a mass of the blood cells...”

Paul de Kruif; extract from “Microbe Hunters”, 1926.

It is difficult to find a more vivid, and elegant, description of trypanosome morphology and behaviour than that of Paul de Kruif’s above. More conventionally however, the African trypanosome is described as a flagellated, protozoan parasite (or sometimes as a flagellated protozoon). The term protozoon (the singular form of protozoa) reflects the phylum Protozoa of traditional taxonomic classifications, the phylum to which trypanosomes belonged. Although the International Society of Protistologists has described ‘protozoa’ and ‘flagellum’ as “problematic” terms, the later in particular will be used throughout this thesis since its use is widespread in the trypanosomal literature for describing the parasite’s motility organelle, an organelle that is especially relevant to this treatise (the recommended term for the organelle is ‘cilium’; Adl *et al.*, 2005).

Trypanosomes are roughly cylindrical or worm-like in shape, with a bluntly tapered posterior end and slightly more finely tapered anterior end. The long slender bloodstream form *T. brucei* is approximately 2.8 μm in diameter, about 23 μm to 30 μm long, and is confined by a corset of sub-pellicular microtubules that underpin the well defined, undulating membrane. Short stumpy bloodstream form *T. brucei* (considered in section 1.1.6), which can be found in pleomorphic strains of the organism, are approximately 17 μm to 22 μm in length, and about 3.5 μm in diameter (Bruce, 1910; Hoare, 1972).

Like other members of the Euglenozoa, trypanosomes possess a single flagellum (most euglenoids however contain two or more flagella or cilia), that extends from the base of the flagellar pocket, an invagination of the cell membrane towards the posterior end of the trypanosome. In long slender bloodstream forms the flagellum extends beyond the anterior end of the cell for approximately 6 μm (this free flagellum is much shorter, or not present, in short stumpy forms; Hoare, 1972). The flagellum of *T. brucei* may be divided into four structural components, which will be briefly considered below: the axoneme, the paraflagellar rod (PFR; previously called the paraxial rod), flagellar (or flagellum) attachment zone (FAZ), and flagellar membrane (which is distinct from the membrane of the flagellar pocket, and from the plasma membrane). The main organization of the trypanosome flagellum is shown in figure 1.2.

Figure 1.1. Current Taxonomic Standing of *Trypanosoma brucei brucei*

Shown opposite is the current eukaryotic taxonomic classification scheme, as recommended by the International Society of Protistologists (Adl *et al.*, 2012). Representative species of the principal Trypanosoma clades are shown, although some species of trypanosome (*e.g.* *T. pestanai*) have not yet been assigned to particular clades, and more clades may still be found (Stevens *et al.*, 1998; Votýpka *et al.*, 2004; Simpson *et al.*; 2006; Rodrigues *et al.*, 2005). “*Incertae sedis*” identifies groups with an uncertain positioning in the scheme, but have been cautiously classified as shown. Information on such groups omitted from Eukarya, is best sourced from Adl *et al.* (2012). For clarity, two genera have been omitted from the Trypanosomatida rank: *Sergia*, and *Strigomonas*. Ranks shown in italics currently contain only a single identified species (*e.g.* *Tsukubamonas globosa* is, at present, the only identified species in the clade Tsukubamonas; Yabuki *et al.*, 2011). The five super-groups, Archaeplastida, Sar, Excavata, Amoebozoa and Opisthokonta, are shown.

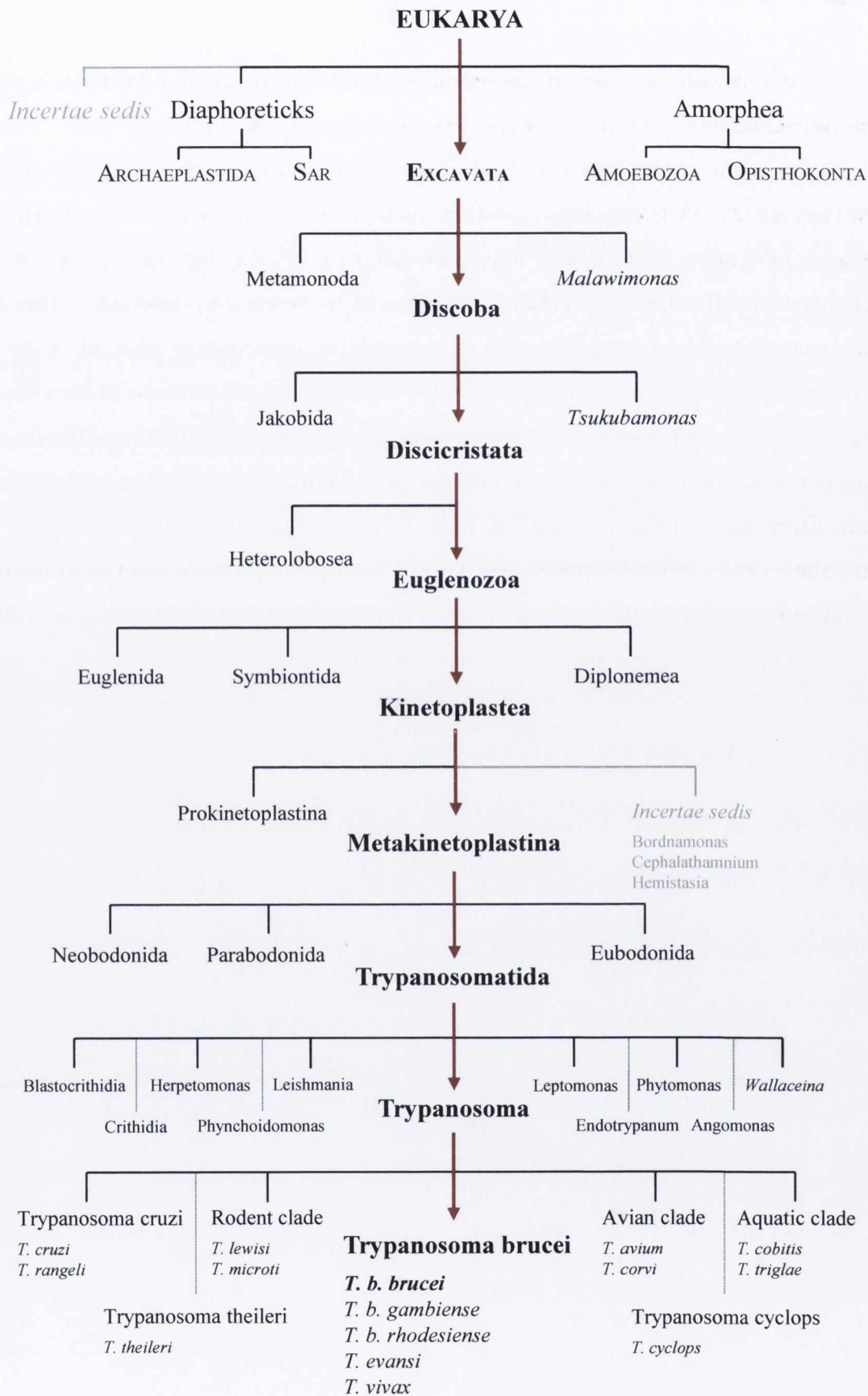
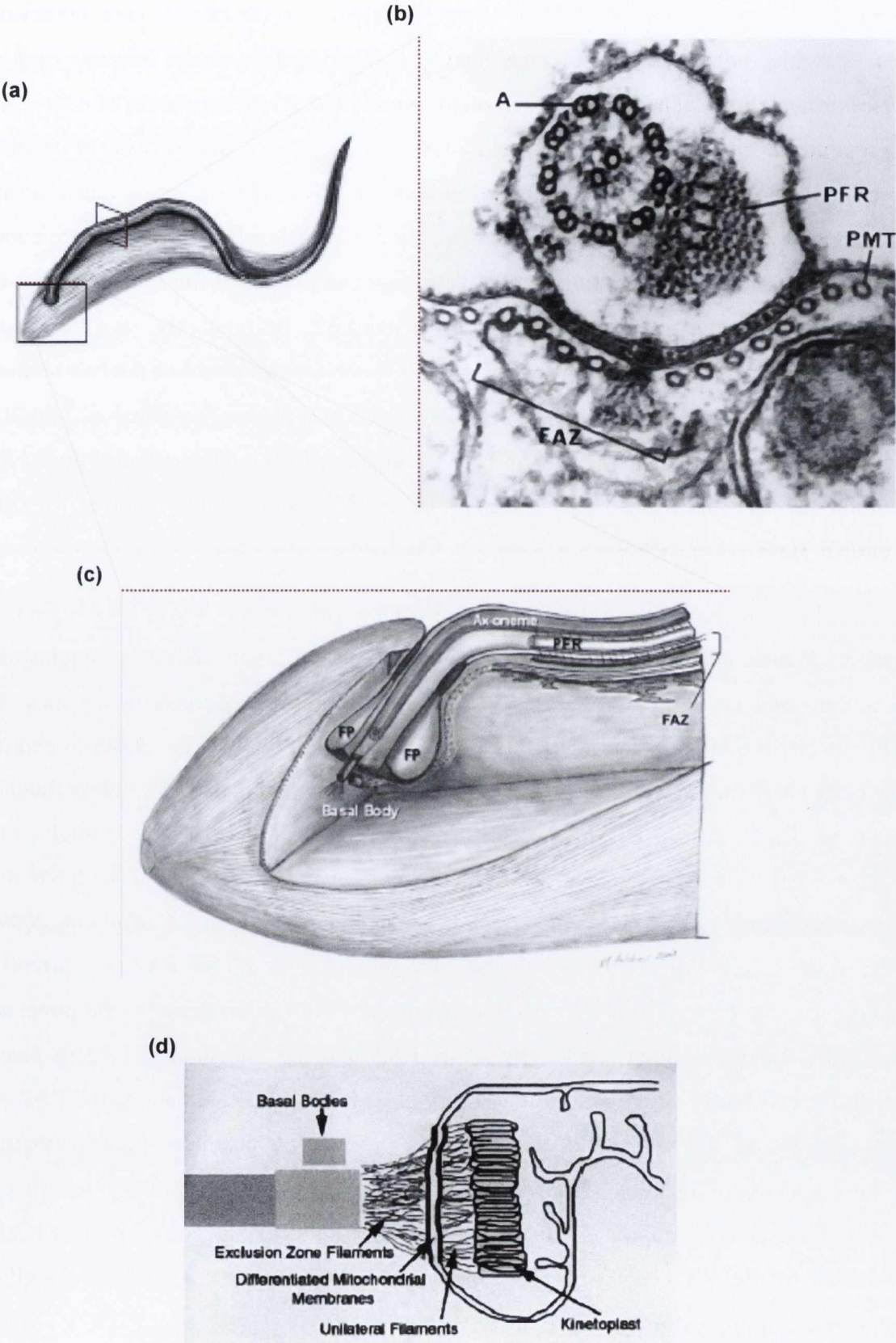


Figure 1.2. Ultrastructure of the African Trypanosome

A bloodstream form trypanosome is illustrated in (a) as a reference to show where the sectional images (b) and (c) are in relation to the entire cell body. The cut, highlighted planes in (a) are coloured red along one edge to match the equivalent edges in (b) and (c). A transmission electron micrograph of a transverse slice through the flagellum is shown in (b), with the paraflagellar rod (PFR), flagellar attachment zone (FAZ), subpellicular microtubules (PMT) and axoneme (A) labelled. The FAZ filament is seen as a region of high electron density immediately adjacent to the four specialized sub-pellicular microtubules; the four specialized microtubules are also seen to associate with a hook of endoplasmic reticulum. An enlarged view of the flagellar pocket is shown in (c), taken as an oblique section through the trypanosomal cell body; the, axoneme, flagellar pocket (FP), PFR, FAZ, and basal body are labelled. A final enlarged view of the kinetoplast and the fibrils connecting the kinetoplast to the basal body is presented in (d). Images (a) and (c) are taken from Oberholzer *et al.*, (2011), while images (b) and (d) are reproduced from Gull (1999).



The central shaft of the flagellum is composed of the axoneme and PFR, which run parallel to each other along most of the flagellum. Unlike the axoneme however, the PFR does not continue the entire length of the flagellum to the flagellar pocket (Vickerman, 1969). The axoneme is a highly ordered structure consisting of a “9 + 2” arrangement; nine doublets of microtubules that encircle a central pair of singlet microtubules. The outer doublet microtubules are connected by nexin links, while dynein motors attached to the doublets utilize ATP to fuel flagellar motion (Ralston and Hill, 2008). The pseudocrystalline PFR is a large component of the flagellum that is physically connected to the axoneme, and to the FAZ, by relatively long fibrils of currently unknown protein composition. The PFR itself is composed of the two related proteins PFR-A (a 69 kDa protein) and PFR-C (a 73 kDa protein) arranged as a highly organized lattice (Schlaeppli *et al.*, 1989; Deflorin *et al.*, 1994). Disruption of the *PFR-A* gene has revealed that correct formation of the PFR is absolutely required for cell motility (Bastin *et al.*, 1998).

There are a number of principle components within the *T. brucei* FAZ region: a group of four microtubules that are closely associated with smooth endoplasmic reticulum, and a multiply beaded structure that runs alongside the microtubular quartet. This specialized microtubule quartet extends from the basal body at the posterior end of the cell, where they then follow a right-handed helical path alongside the sub-pellicular microtubules to the anterior of the cell (Sherwin and Gull, 1989). Interestingly, the microtubular quartet of the FAZ, which are nucleated at the basal body, have the opposite polarity to the other sub-pellicular microtubules (Field and Carrington, 2009; Robinson *et al.*, 1995). Although the precise make-up of the FAZ is currently unknown, it is through direct connections from the PFR and axoneme to the punctate bead-like structures along the length of the FAZ that the flagellum is held in place along the cell body; the flagellum traces a right-handed helical path above the FAZ to the anterior of the cell, held in place by a series of punctate, transmembrane connections approximately 25 nm in diameter and spaced every 95 nm (Vickerman, 1969; Field and Carrington, 2009). The flagellar adhesion glycoprotein 1 (FLA1) localizes to the FAZ and has been shown to be crucial in establishing flagellar attachment in procyclic life cycle stages of the parasite (LaCount *et al.*, 2002). More recently, a heavily *N*-glycosylated 160 kDa protein, FLA3, which is located in the FAZ

region, has been shown to be crucial for maintaining flagellar attachment in bloodstream form trypanosomes (Woods *et al.*, 2013).

The lipid composition of the flagellar membrane is unclear, although the organelle's membrane has been reported to be enriched with sterols and other lipid raft constituents such as sphingolipids and glycolipids (Maric *et al.*, 2010; Landfear and Ignatushchenko, 2001). The unique lipid composition of the flagellar membrane, which is distinct to that of the plasma membrane, is thought to play some role in aiding the specific localization of some proteins to the organelle (Fridberg *et al.*, 2007). Calflagins, a family of calcium binding proteins for example, are hypothesized to associate specifically with the flagellar membrane through *N*-linked acylation; myristoylation and/or palmitoylation of the calflagins may allow their localization to the specialized flagellar lipid environment (Wu *et al.*, 1994; Fridberg *et al.*, 2007).

The flagellum is ultimately anchored to the cell at the basal body, which in turn is fixed to the kinetoplast, a large region of compacted, mitochondrial DNA common to all members of the Kinetoplastea (previously, order Kinetoplastida; Honigberg, 1963). Anchoring of the flagellum to the kinetoplast is achieved *via* the tripartite attachment complex, however little is known of this structure (Gull, 1999). Some out-dated terms for the kinetoplast give some indication of its characteristics, and earlier supposed function. Plimmer and Bradford (1989) for example, called the organelle the micronucleus, due to its proximity to the nucleus and because both organelles stained a similar crimson colour when trypanosomes were treated with a mixture of methylene-blue and erythrosine. Another term, “blepharoplast” (Wasielewsky and Senn, 1900), recognized the kinetoplast's close association with the base of the flagellum.

1.1.4. The Surface Coat and Antigenic Variation

Antigenic variation in trypanosomes was first observed at the beginning of the twentieth century when a number of researchers established that trypanosomes could change, in some immunological sense, in mammals suffering from sleeping sickness (Franke, 1905). The resistance of *T. brucei* to the host immune system was perhaps most classically shown to involve a periodic fluctuation in the numbers of parasites in the blood throughout the time-course of an infection (Ross and Thomson, 1910; Ross

and Thomson, 1911). Studies on this phenomenon in these early years was limited to testing the ability of various sera to agglutinate populations of trypanosomes, collected at various stages during a host infection, or at different stages of the parasite's life cycle. Nonetheless, it is from these experiments that much of our current understanding of antigenic variation is derived.

Description of antigenic variation is perhaps best illustrated by following the immunological changes that occur during infection of a mammal with African trypanosomes. Although each single strain of trypanosome is capable of producing a number of distinct antigens during an infection (Ritz, 1916) it has been shown that the antigenic characteristics of a number of trypanosome variants injected into a host by the tsetse vector are initially quite similar (Broom and Brown, 1940; Gray, 1965). This initial common antigen, termed the "basic antigen", was shown to persist until a new antigen was produced; the appearance of antigenically distinct variants, with some strains of *T. brucei* for example, occurred every two to four days in laboratory rodents (Gray, 1965). The destruction of the initial wave of parasitemia coincides with the production of variant specific antibody in the host, however some novel antigenic variants always survive the hosts initial defence and begin to proliferate in the bloodstream, characterizing the second wave of parasitemia. The result is an inevitably fatal cycle of destruction of each trypanosomal variant, and recolonization of the mammalian bloodstream by each new antigenic variant in turn.

A number of observations indicated that the main variable antigen of trypanosomes was related to the protein coat of the organism (Vickerman, 1969). For example, the basic antigen described above is also present in metacyclic form trypanosomes (Cunningham, 1966) but not in other stages of the trypanosomal life cycle within the insect vector. The reappearance of the basic antigen also corresponds to the acquisition of a dense layer of protein surrounding the trypanosome, which was also seen in bloodstream forms of the parasite (Vickerman, 1969).

Biochemical characterization of the major protein constituent of the bloodstream surface coat was first achieved by Cross (1975). There followed a large collection of studies on the protein, now called the variant surface glycoprotein (VSG), that was found to account for 10% of the total cellular protein, or approximately 10^7

molecules for each cell (Cross, 1975). The main biochemical features of the VSG will be considered in section 1.1.5.

As the understanding of the biochemical properties of the VSG has greatly improved, so too has the understanding of the genetic principles underlying antigenic variation. It has been found, for instance, that four alternative mechanisms allow *T. brucei* to change what VSG is expressed. Before these processes can be summarised it should first be noted that the multitude of VSG genes present in trypanosomes are spread across the eleven major chromosomes (Berriman *et al.*, 2005), and the many intermediate and mini chromosomes (Williams *et al.*, 1982; Melville *et al.*, 1998) found in the parasite. For the VSG to be expressed, the gene must first be located in an active bloodstream expression site (BES), a process which has been reviewed recently by Stockdale *et al.* (2008).

During the course of trypanosomiasis, the infected host will mount an immune response against the VSG that is expressed by the majority of the population of trypanosomes. However, a small number of the parasites have always switched their major surface antigen through any one of the processes “array VSG conversion”, “telomeric VSG conversion”, “segmental VSG conversion” or “transcriptional switch”. The major process in antigenic variation requires a recombination event during which a new VSG gene, located either in a VSG gene array (array VSG conversion) or at a chromosome telomere (telomeric gene conversion), replaces the currently expressed VSG gene in the active BES. Alternatively, portions of separate VSG genes located in VSG arrays can be recombined to yield a newly functional VSG, also to be expressed from the active BES (segmental VSG conversion). Finally, as trypanosomes contain up to twenty functional expression sites (Becker *et al.*, 2004), the parasite may simply change which of these expression sites is transcriptionally active (transcriptional switch), (Stockdale *et al.*, 2008). The frequency of VSG switching, *in vivo*, has been found to approximate 10^{-5} per generation (Myler *et al.*, 1985).

1.1.5. Some Biochemical Characteristics of the Variant Surface Glycoprotein

Sequencing of the trypanosome genome identified 806 VSG genes divided between each of the eleven megabase diploid chromosomes (Berriman *et al.*, 2005). Of

these genes only 57 (7%) were fully functional, however the final number of fully functional VSG genes may be much higher once sequence data for the intermediate chromosomes and minichromosomes becomes available. Despite the large number of VSGs, each variant analyzed to date can be grouped into one of a number of families based on the conservation of, in particular, potential *N*-linked glycosylation sites and groups of cysteine residues.

Analysis of the amino acid sequences of numerous VSGs has shown that there is a remarkable conservation of cysteine residues in the primary structure; cysteine conservations in the N-terminus allows the VSG to be classed as an N-terminal domain type A, B, or C, while cysteine conservations in the C-terminus allows VSG to be classed as a C-terminal domain type 1, 2, 3 or 4 (Carrington *et al.*, 1991; Cross 1984). The conservation of these cysteine residues is likely due to a requirement to conserve disulfide linkages important for correct folding of the VSG. Of particular interest to this thesis is the N-terminal domain type A, and C-terminal domain type 2, Molteno Institute Trypanozoon antigen type (MITat) 1.2 variant of the VSG, which is the variant used throughout this work. A number of MITat VSGs, all of which belong to the MITar (Trypanozoon antigen repertoire) serodeme, have been sequenced and classified according to their domain types.

The VSG is a heavily glycosylated protein, with carbohydrate accounting for 7-17% of the molecule by weight (Johnson and Cross, 1977). The two classes of carbohydrate attachments to the surface glycoprotein involve, firstly, a glycolipid anchor attaching the protein to the cell membrane, and an *N*-linked glycosylation comprising mannose and *N*-acetylglucosamine (Turner, 1985). Although the function of the glycolipid anchor is well established, the precise function and requirement of the later *N*-linked glycosylation is still uncertain, carbohydrate attachments of this later type may be required for providing a lateral stability in the VSG coat, spacing the VSG molecules appropriately across the surface of the trypanosome (Turner, 1985; Mehlert *et al.*, 2002). The *N*-linked glycosylation of the VSG variant MITat 1.5 has been shown to be necessary for the transport of the protein to the cell surface (Ferguson *et al.*, 1986) while in other variants the carbohydrate attachments seem to meet some structural requirement (Blum *et al.*, 1993). The carbohydrate backbone of the glycolipid anchor of the VSG (which is common to all VSGs) has the general form: ethanolamine-HPO₄-6Man α 1-2Man α 1-6Man α 1-4GlcN α 1-6PI (where Man represents mannose, GlcN is

glucosamine, and PI is phosphatidylinositol). However this glycolipid anchor can be further modified by addition of numerous galactose (Gal) moieties. Zitzmann *et al.*, (2000) have divided VSGs into two classes (class 1 and 2) based on combinations of carbohydrate modifications to the common glycolipid, and based on N-linked glycosylation of the glycoprotein. The different classes of VSG are summarized in figure 1.3.

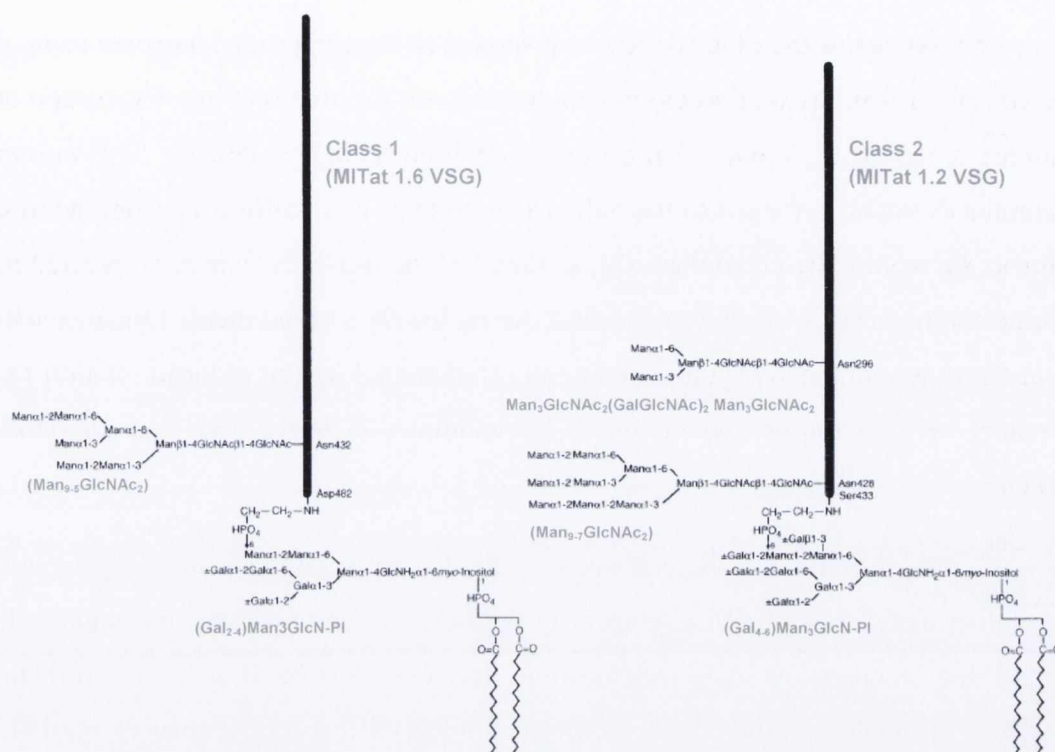
A single VSG monomer can be further considered as comprised of two domains, joined by a short unfolded segment that is particularly sensitive to proteolysis (Johnson and Cross, 1979, Gomes *et al.*, 1986). The three-dimensional crystal structures of both of these domains (the amino terminal domain and the carboxy terminal domain), for one surface glycoprotein variant (MITat 1.2), have been solved separately (Freyman *et al.*, 1990; Chattopadhyay *et al.*, 2005) and are presented in plate 1.1.

As a surface coat, approximately 5×10^6 VSG dimers form a dense, glycosylated, barrier 12-15 nm thick (Vickerman, 1969; Cross, 1975), preventing access of host antibody to invariant trypanosomal antigens. Although the infected host can successfully mount an immune response directed against the VSG coat, the ability of the parasite to switch to the expression of a new major surface protein with only limited sequence homology to that VSG previously expressed allows a small number of trypanosomes to escape the initial host response, and subsequent immune responses to each successive variant. It should finally be noted that despite the limited sequence homology between VSG variants (approximately 16%), the three dimensional structure of each variant remains conserved (Blum *et al.*, 1993; Freyman *et al.*, 1990), so the protective surface coat is consistently functional.

Figure 1.3. Biochemical Properties of the VSG

Differences in the glycosylation between VSG class 1 and 2 are shown in (a), with MITat 1.6 VSG used as an example of class 1 VSG and MITat 1.2 VSG used as an example of class 2 VSG. Carbohydrate moieties in parenthesis are optional attachments. VSGs can also be grouped by their conserved C-terminal domain cysteine residues, shown in (b). Gaps in the sequences represent the place of cleavage to generate the mature C-terminus. All cysteine residues have been coloured red, while one of the C-terminal glycosylation sites in MITat 1.2 VSG sequence (*i.e.* Asn426) has been coloured blue (together with aligning asparagine residues). VSG structures in (a) were taken from Zitzmann *et al.*, (2000). Sequences and alignments for (b) were taken from Carrington *et al.*, (1991), with the sequence of MITat 1.3 VSG (accession number AAX36025) additionally aligned.

(a)



(b)

C-Terminal domain type 1

	terminal domain type 1	***	*	*	*	***	***	***	***	*	*	*	*	****	***
MITat 1.3	NTETKK C SDKKTEG DCK --DGCKWDGKECKD									SSILATKKFALTTVVSAAFVALLF					
MITat 1.4	EATTDKCKGKL-EDT C KKESNCKWENNACKD									SSILVTKKFALTTVVSAAFVALLF					
MITat 1.6	EATPECKKGDAK-TCQT T QGCKWEGETCKD									SSILVTKKFALTTVVSAAFVALLF					
ILTat 1.24	DTPAECKKGKEKD- C -KSPDK C WGE T CKD									SSILANKQFALSVSAAAFVALLF					
ILTat 1.3	QTTDDKCKDKKK-DD C -KSPDK C WGE T CKD									SSFILNKQFALSVSAAFAALLF					
MVAT 4	DKKEEKCKGKLE-PE C TKAPECKWEGETCKD									SSILVKNQFTLSMISAAFM----					
ANTAT 1.1	ETPAECKTGKKK-DD C -K-DGCKWEA E TCKD									SSILVTKKFALSLVSAAFASLLF					
ANTAT 1.10e	EATTDKCKDKTK-DE C -KSPNCKWEGETCKD									SSILVTKKFALSLVSAAFASLLF					
ILTat 1.25	EA---KKCSDDKKEE C -KSPNCKWDGKECKD									SSILANKQFALSVSAAAFVALLF					

C-Terminal domain type 2

[illegible]

C-Terminal domain type 3

MITat 1.5 KENTPCQSAVCGFRKKGKDGESDPEKDKEKCRN GSFLTskQFAFSVSVSAAFMALLF

C-Terminal domain type 4

MVAT7 IONOTECEGVKGTPPTGKAKVCGWIEGKCOD SSFLLSKOFALSVVSAFAALLF

1.1.6. A Brief Summary of the Cell Cycle in *Trypanosoma brucei*

The *Trypanosoma* rank (which corresponds to the genus *Trypanosoma* using the previous classification) was previously organised into the two sections Stercoraria and Salivaria, based on the mode of transmission of representative species. *Trypanosoma brucei*, for example, belonged to the Salivaria section as it is transmitted from the insect vector to the mammalian host through the saliva of the tsetse fly, which is injected into the mammalian host by the anterior station during the fly's blood meal. Organisms that belonged to Stercoraria were contaminatively transmitted by the posterior station from the insect vector's faeces and included, for example, *T. lewisi* and *T. cruzi* (Hoare, 1964).

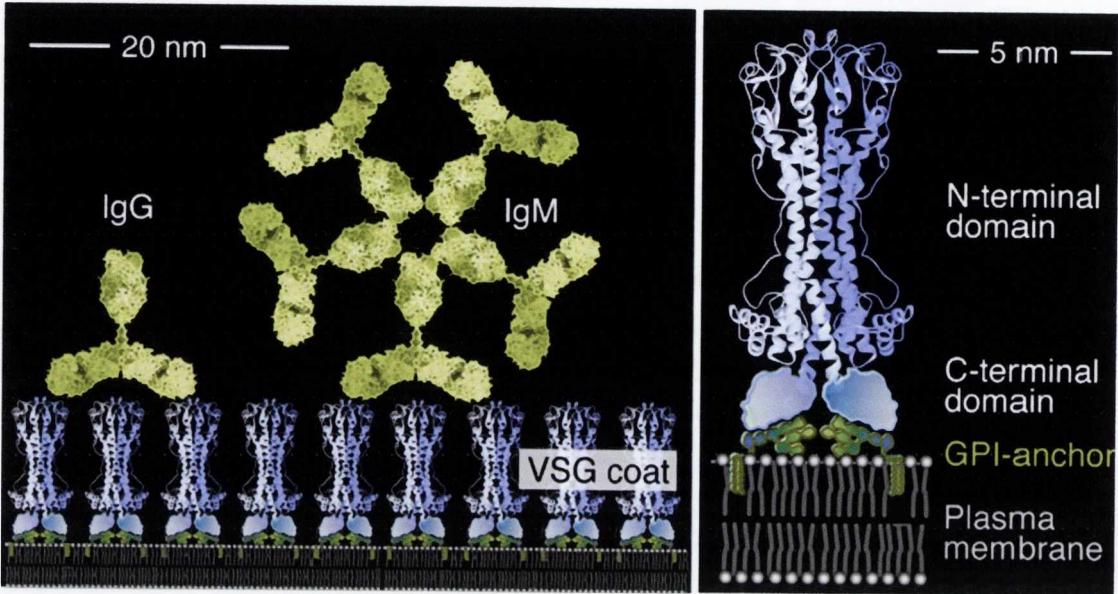
Shown in figure 1.4, the cell cycle is divided between those stages spent in the mammalian bloodstream and those spent in the tsetse midgut and salivary glands. The pleomorphic bloodstream stage trypanosome may exist as one of at least two distinct cell types, originally characterized by morphological differences (Bruce *et al.*, 1912): the long slender form, or the short stumpy form. Only the long slender bloodstream form is proliferative in the mammal and increasing numbers of these forms appear to induce a density-dependent differentiation into the non-proliferative stumpy form (Vassella *et al.*, 1997). The ability of the cell-permeable cyclic adenosine monophosphate (cAMP) analogue, 8-4-chlorophenylthio-cAMP (8-pCPT-cAMP), to induce differentiation to stumpy forms has implicated the cAMP pathway in this process; a zinc finger kinase (ZFK) and MAP kinase (TbMAPK5) have also been implicated (Vassella *et al.*, 2001; Domenicali Pfister *et al.*, 2006).

Differentiation of long slender bloodstream forms to the growth-arrested short stumpy forms, as well as being important in limiting parasitemia, helps the transmission of the parasite to the tsetse fly, as only these later stumpy forms are preadapted to the environment of the tsetse fly midgut (Matthews and Gull, 1994). Interestingly, and for unknown reasons, some laboratory strains of trypanosomes have been shown to lose their pleomorphism when maintained by serial passage in animals, and instead only long slender forms of the resulting monomorphophic trypanosomes are seen in the bloodstream (Hoare, 1972). However, if a population of the MITar serodeme, which is monomorphic in mice and rats, is transferred to a rabbit, then the trypanosomes rapidly develop a pleomorphic phenotype (Voorheis, personal communication).

Plate 1.1. Molecular Structure of the Variant Surface Glycoprotein

Shown in (a) is a molecular model of the VSG coat, with IgG and IgM shown as a comparison (first panel), and a single VSG dimer anchored to the plasma membrane (second panel), reproduced from Engstler *et al.* (2007). The most complete molecular structure currently available of the VSG N-terminal domain and C-terminal domains are shown in (b), reproduced from Chattopadhyay *et al.* (2005), (the structure is for the MITat 1.2 variant). Structural data for the region linking the C-terminal and N-terminal domains (dashed white line) are currently unavailable, as are the data for sixteen residues between the C-terminal domain and the ethanolamine bridge of the GPI anchor.

(a)



(b)



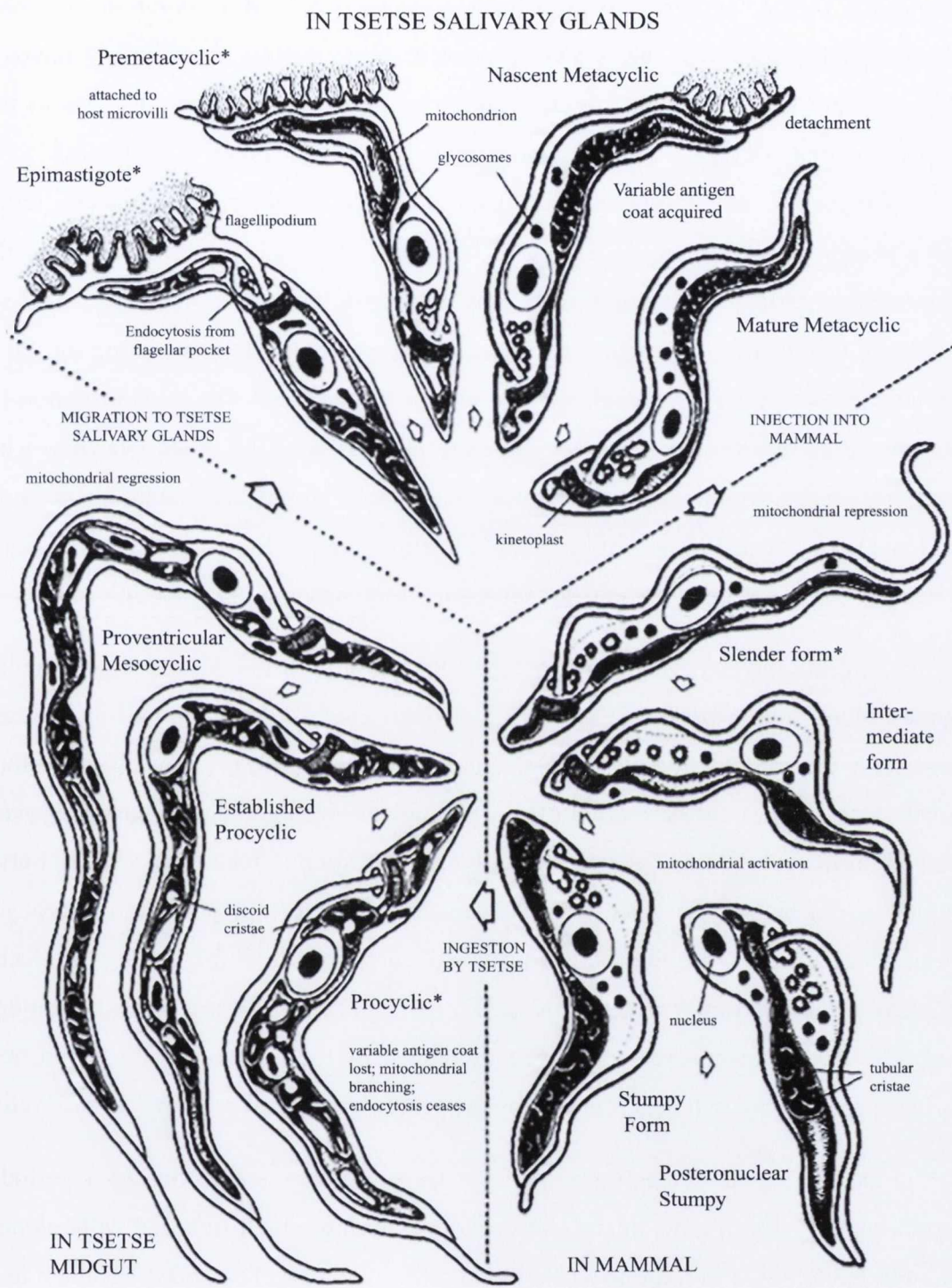


Figure 1.4. The Life Cycle of *Trypanosoma brucei*

Reproduced from Vickerman, 1985.

Numerous biochemical changes in the trypanosome are required for survival in the insect vector, with replacement of the VSG coat and elaboration of energy metabolism being perhaps the best characterized. Long slender form trypanosomes, for example, rely entirely on glucose circulating in the mammalian bloodstream as their carbon source (Riley, 1956). The glycolytic conversion of glucose to 3-phosphoglycerate and phosphoenolpyruvate occurs in a specialized organelle termed the glycosome (Visser *et al.*, 1981). These last glycolytic intermediates are then converted to pyruvate in the cytosol. This glycolytic pathway is the organisms' sole source of adenosine triphosphate (ATP); each glucose molecule responsible for the net production of two ATP molecules under aerobic conditions. The transformation into stumpy forms results in an enlargement and elaboration of the mitochondrion, which develops cristae; expression of enzymes involved in amino acid metabolism is also evident (Vickerman, 1985). Differentiation to short stumpy forms prepares the parasite for an amino-acid dependent metabolism required for survival in the tsetse vector.

Ingestion of trypanosomes by tsetse flies results in the destruction of the long slender bloodstream forms of the parasite through glucose depletion and by proteases present in the midgut of the fly (Fenn and Matthews, 2007). Short stumpy forms however undergo a further series of morphological and biochemical changes over a forty eight to seventy two hour period following ingestion (Vickerman, 1985). Further mitochondrial elaboration occurs as the parasites develop into proliferative procyclic forms that utilize L-proline as the principal carbon source for energy production (Vickerman, 1985; van Weelden *et al.*, 2003; Brinkaud *et al.*, 2006). Transformation to procyclic form trypanosomes is also accompanied by the removal of the bloodstream surface protein coat, which is replaced by the protease-resistant protein coat procyclin.

Development of the procyclic form trypanosomes occurs during a period of approximately eleven days in the tsetse midgut, during which time the cells elongate and ultimately begin to migrate to the tsetse salivary gland. The parasites attach to the epithelial cells of the salivary gland *via* the flagellum (Vickerman, 1985). A small proportion of these epimastigote cells have been reported to undergo an exchange of genetic material in the salivary glands, or ducts, prior to attachment to the epithelial microvilli (Gibson *et al.*, 2008; Vickerman, 1985), although the precise mechanism behind this trypanosomal mating is still unknown.

Transformation to metacyclic forms occurs as the trypanosomes begin to pre-adapt to the mammalian bloodstream. The precise metabolic development of the organism during the metacyclic stages is still poorly understood, although mature metacyclic form trypanosomes, which exist in the salivary gland unattached to the lumen, have a mitochondrion and glycosomes that are morphologically similar to those of long slender bloodstream form trypanosomes (Vickerman, 1985). The mature metacyclic forms, also possess a protein coat similar to the bloodstream VSG coat, in preparation for their transmission to the mammalian host during the insect vector's blood meal.

1.1.7. The Development of Conditions for Culturing Bloodstream Form *T. brucei*

A general summary of African trypanosomes could not be completed without at least briefly considering the enormous efforts, of very many researchers, that have culminated in our ability to culture the bloodstream form of *T. brucei* used extensively throughout this present work. Although even the best culturing conditions still cannot match the vigour of trypanosomal growth *in vivo*, there can be no doubt that the development of *in vitro* culturing conditions has greatly aided the study of the parasite.

Undoubtedly, the first culture medium that supported trypanosomal growth was that of McNeal and Novy (McNeal and Novy, 1904). This medium, which was used to grow both *T. lewisi* and *T. brucei*, consisted of a nutrient agar mixed with defibrinated rabbit blood. In this medium, *T. brucei* required a significantly higher blood content than *T. lewisi* to grow; *T. lewisi*, for example, was capable of growing in mixtures consisting of 10 parts nutrient agar to 1 part blood, while *T. brucei* required 1 part nutrient agar to 2 or 3 parts blood. However, while *T. brucei* could be maintained (McNeal and Novy speculated indefinitely) in this medium, it was found that the infectivity of the parasite to animals fell over a relatively short time, particularly when the cultures were maintained at temperatures above 25 °C.

It was some seventy three years after McNeal and Novy's *in vitro* growth of bloodstream form *T. brucei* before infective forms of the organism could be maintained indefinitely in culture. Hirumi *et al.* (1977a; 1977b) succeeded in showing that animal infective forms of *T. brucei* could be cultivated, in the presence of bovine fibroblast-

like cells, in Roswell Park Memorial Institute (RPMI) 1640 medium (Moore, *et al.*, 1967) buffered with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and supplemented with 20% heat-inactivated foetal bovine serum (FBS). While the development of techniques for axenically culturing animal infective forms of *T. brucei* was proving very challenging, this same year saw the publication of an excellent medium suitable for axenic culturing of procyclic forms of *T. brucei*. This semi-defined medium published in 1977 (SDM-77; Brun and Jenni, 1977) was the latest medium suitable for growing procyclic *T. brucei* and was soon refined to SDM-79, a medium which still remains very popular for culturing the tsetse midgut stage of *T. brucei* and is currently in use by our laboratory (Brun and Schönenberger, 1979).

The first axenic culture of bloodstream form trypanosomes that maintained their infectivity to animals was established by Baltz *et al.* (1985), who showed that the bloodstream form of the parasite could be cultured in a fibroblast-free system after an initial short adaptation to culture in the presence of a macrophage feeder layer of cells. Baltz's medium was based on Eagle's minimal essential medium (MEM; Eagle, 1959), with supplements including heat-inactivated rabbit serum and 2-mercaptoethanol (amongst additional ingredients). However in a second report, published in the same year as Baltz's findings, Duszenko *et al.* (1985) also succeeded in axenically culturing infective, bloodstream form *T. brucei*. This later study achieved greater culturing densities of trypanosomes compared to Baltz *et al.*, and further elucidated the requirement for the mammalian fibroblast-like cells in Hirumi's earlier system. Using MEM lacking the cystine normally present, and containing some additional supplements, Duszenko and his colleagues showed that cysteine (the reduced, monomeric form of cystine) was absolutely necessary for trypanosomal growth as the bloodstream form. The fibroblast cells present in earlier systems were required for the conversion of the cystine present in RPMI 1640 medium, and MEM, to cysteine, which the trypanosomes could then take up and utilize for protein synthesis. As neither RPMI 1640 medium nor MEM contain any cysteine necessary for trypanosomal growth, Duszenko's report suggested that the inclusion of 2-mercaptoethanol in Baltz's recipe allowed for reduction of the available cystine to the necessary cysteine.

Duszenko's medium, however, required precise addition of the correct amounts of L-cysteine twice daily. The addition of too much cysteine (*i.e.* a final concentration greater than 0.2 mM) was actually toxic to the trypanosomes due to the autoxidation of

cysteine generating hydrogen peroxide, to which the trypanosomes were acutely sensitive (Duszenko *et al.*, 1985). Baltz had speculated that the inclusion of 2-mercaptoethanol (in their medium) was actually partly required for reducing glutathione, which could then function as a scavenger of toxic free radicals. However, Duszenko's culturing medium was soon simplified by Hirumi and Hirumi (1989), who developed the medium currently in use for bloodstream form *T. brucei*. A ninth formulation of Hirumi's modified Iscove's medium (HMI-9) avoided the requirement for continuously adding L-cysteine to the cultures (Iscove's medium is Iscove's modified Dulbecco's medium, IMDM, which in turn is Dulbecco's modification of MEM, DMEM; Iscove and Melchers, 1978; Dulbecco and Freeman, 1959). Key to the HMI-9 recipe is the inclusion of the metal chelator bathocuproine sulfonate, which acts to prevent the copper-catalyzed autoxidation of cysteine to cystine (*e.g.* Harman *et al.*, 1984). The complete standard HMI-9 recipe is given in table 1.1.

Table 1.1. Complete HMI-9 Recipe

Component	Molecular Weight	Concentration (mg·l ⁻¹)	Concentration (mM)
Basic IMDM formulation (Iscoe and Melchers, 1978)*			
Glycine	75	30	0.4
L-Alanine	89	25	0.281
L-Arginine hydrochloride	211	84	0.398
L-Asparagine (free base)	150	25	0.167
L-Aspartic Acid	133	30	0.226
L-Cystine (2HCl)	240	91.4	0.381
L-Glutamic Acid	147	75	0.51
L-Glutamine	146	584	4
L-Histidine hydrochloride-H ₂ O	210	42	0.2
L-Isoleucine	131	105	0.802
L-Leucine	131	105	0.802
L-Lysine hydrochloride	183	146	0.798
L-Methionine	149	30	0.201
L-Phenylalanine	165	66	0.4
L-Proline	115	40	0.348
L-Serine	105	42	0.4
L-Threonine	119	95	0.798
L-Tryptophan	204	16	0.0784
L-Tyrosine, disodium salt	225	104	0.462
L-Valine	117	94	0.803
Biotin	244	0.013	0.0000533
Choline chloride	140	4	0.0286
D-Calcium pantothenate	477	4	0.00839
Folic Acid	441	4	0.00907
Niacinamide	122	4	0.0196
Pyridoxal hydrochloride	204	4	0.0196
Riboflavin	376	0.4	0.00106
Thiamine hydrochloride	337	4	0.0119
Vitamin B12	1355	0.013	0.0000096
i-Inositol	180	7.2	0.04
Calcium chloride (CaCl ₂)	111	165	1.49
Magnesium sulphate (MgSO ₄)	120	97.67	0.814
Potassium chloride (KCl)	75	330	4.4
Potassium nitrate (KNO ₃)	101	0.076	0.000752
Sodium bicarbonate (NaHCO ₃)	84	3024	36

Table 1.1. Complete HMI-9 Recipe (Continued)

Component	Molecular Weight	Concentration (mg·l ⁻¹)	Concentration (mM)
Sodium chloride (NaCl)	58	4500	77.59
Sodium phosphate (NaH ₂ PO ₄ ·H ₂ O)	138	125	0.906
Sodium selenite (Na ₂ SeO ₃ ·5H ₂ O)	263	0.0173	0.0000658
D-Glucose	180	4500	25
HEPES	238	5958	25.03
Phenol Red	376	15	0.0399
Sodium pyruvate	110	110	1

Additions by Hirumi and Hirumi (1989)

Bathocuproine disulfonate	565	28	0.05
L-Cysteine	121	182	1.5
Hypoxanthine	136	136	1
2-Mercaptoethanol	78	15.6	0.2
Sodium pyruvate	110	110 (220)	1 (2)†
Thymidine	242	38.7	0.16
FBS		20 % (v/v)‡	

Other components routinely added in this laboratory

Adenosine	267	255	0.955
Guanosine	283	200	0.707
pH		7.4	

* The formulation presented here can be found on the Gibco (Invitrogen) website (<http://products.invitrogen.com/ivgn/product/12440046>).

† Additional sodium pyruvate (1 mM) was added to the basic IMDM recipe by Hirumi and Hirumi, which brought the final sodium pyruvate concentration in HMI-9 to 2 mM (shown in parenthesis).

‡ Hirumi and Hirumi originally added 10% (v/v) FBS and 10 % (v/v) Serum Plus™ (a serum containing a low level of serum proteins, while containing some other added proteins, from Hazleton Biologics). For this work, only 10% (v/v) FBS is used in HMI-9.

1.2. The GPI-Specific Phospholipase C of *Trypanosoma brucei*

1.2.1. Discovery and Early Characterization of the GPI-PLC

The early eighties saw a number of publications investigating the characteristics of VSG release from the surface of *T. brucei*. Release of the surface coat could first be experimentally initiated by calcium ions, in the presence of the calcium ionophore A-23187, and could also be initiated by incubating cells in the presence of benzyl alcohol. The process of VSG release was shown to be temperature dependent, and found to be inhibited by EGTA and also by zinc cations (Bowles & Voorheis, 1982; Voorheis *et al.*, 1982). It was speculated by these authors that release of the trypanosome surface coat was enzyme dependent, and that activation of this enzyme was triggered by release of calcium from intracellular stores, an idea that was supported by showing that inhibition of the energy metabolism of trypanosomes, which would in turn prevent the energy-dependent sequestration of calcium in intercellular stores, also caused surface coat release (Voorheis *et al.*, 1982).

A critical observation by Cardoso de Almeida and Turner was that membrane bound VSG was biochemically, and immunochemically, distinct from the released form of VSG. In a publication in 1983, it was shown that a single VSG variant could exist in two forms: a soluble, released form (sVSG), and an insoluble, membrane-bound form (mfVSG), (Cardoso de Almeida and Turner, 1983; Jackson, 1983). The observed difference in mobility's of these VSGs, on a denaturing gel, was thought to be due to a covalent modification of the released or membrane-bound form, which the authors first postulated as being a result of a difference in phosphorylation states between mfVSG and sVSG. Although the authors succeeded in showing that mfVSG incorporated radiolabeled phosphate, they were unable to show any difference in the extent of radiolabeled phosphate present in VSG before and after its release (Cardoso de Almeida and Turner, 1983).

The difference in electrophoretic mobility's observed between mfVSG and sVSG, on denaturing polyacrylamide gels, was soon shown to be dependent on the cleavage of a phosphate ester, which linked VSG to the cell surface (Jackson and Voorheis, 1985). In agreement with the earlier observation by Cardoso de Almeida,

cleavage of the phosphodiester bond securing the VSG to the membrane by an enzyme, in a manner shown to mimic *Bacillus cereus* phospholipase C activity (Jackson and Voorheis, 1985), results in the VSG retaining phosphate after its release. The involvement of a phospholipase C was also suggested by Ferguson and his co-workers, who established that membrane-bound VSG was covalently linked to *sn*-1,2-dimyristylglycerol (Ferguson *et al.*, 1985a), and who later revealed that VSG was anchored to the cell membrane through a covalent linkage to glycosyl-*sn*-1,2-dimyristylphosphatidylinositol (Ferguson *et al.*, 1985b).

Purification of the enzyme responsible for VSG release was achieved simultaneously, and independently, by a number of groups; three papers describing purification and characterization of the enzyme were all received by The Journal of Biological Chemistry within the space of one month. Although the enzyme was separately referred to as the membrane-form variant surface glycoprotein hydrolase (Bulow and Overath, 1986), the glycan-phosphatidylinositol-specific phospholipase C (Fox *et al.*, 1986), and the variant surface glycoprotein hydrolase (Hereld *et al.*, 1986), by the three groups, the name glycosylphosphatidylinositol-specific phospholipase C (GPI-PLC, EC 3.1.4.47) has become the more commonly adopted nomenclature and is therefore used throughout this work.

Although the laboratories of Englund, Cross, and Overath each reported similar properties of the GPI-PLC, a number of differences between their observed characteristics makes interpreting the results of their publications something less than straightforward. Undoubtedly, the precise conditions used for the purification of the GPI-PLC, by each of the groups, may explain some of the observed differences in the determined enzyme properties. Both Overath's and Cross's groups did agree that purified GPI-PLC ran as a band of approximately 39,000 Da on polyacrylamide gels, in the presence of SDS (Fox *et al.*, 1986; Bulow and Overath, 1986). Englund's group, however, found the slightly different molecular mass of 36,000 Da, also by SDS-PAGE, (Hereld *et al.*, 1986). With regards to the number of GPI-PLC molecules present, per trypanosome, Overath's and Englund's groups reported closely agreeing figures of 27,000 and 30,000 molecules respectively; Fox and her colleagues, however, concluded that there were a more generous 100,000 copies of the GPI-PLC in each cell. Nonetheless, in the present literature, a copy number of 30,000 has become the accepted value.

In each of the studies of the GPI-PLC, the effects of a number of reagents on enzyme activity were monitored. Perhaps most interesting was the finding that the addition of CaCl_2 to purified enzyme slightly decreased its activity, while addition of EGTA or EDTA stimulated phospholipase activity (Hereld *et al.*, 1986; Fox *et al.*, 1986), although no such effect was seen by Bülow and Overath. Dithiothreitol (DTT) was found to also stimulate activity, approximately two fold, while mercury or zinc ions had a considerably inhibitory effect. The sulfhydryl reagents *N*-ethylmaleimide (NEM), and *p*-chloromercuriphenylsulfonic acid (pCMPS) were also shown to have an inhibitory effect on purified GPI-PLC (Hereld *et al.*, 1986). The effect of hydrogen ion concentration differed between the three reports; in one instance activity maxima were seen at pH values of 7.0 and 10.0 (Fox *et al.*, 1986), in contrast to maximum activity being observed at pH 8.5 by Bülow and Overath. This later result was in agreement with Hereld and Englund who observed that the GPI-PLC was optimally active between pH 7.5 and 8.5.

Despite no region of significant hydrophobicity in the primary structure of the GPI-PLC, the enzyme behaves as an integral membrane protein during purification. Detergent is required to solubilise enzyme activity, and critically the GPI-PLC is predominantly found in the detergent phase following phase separation with Triton[®] X-114 (Fox *et al.*, 1986). The GPI-PLC remains active in non-ionic detergents such as Triton[®] X-100, and *n*-octylglucoside (which Fox and colleagues found to be the most effective), but is inhibited by cationic detergents, anionic detergents, and also by zwitterionic detergents such as 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), (Fox *et al.*, 1986; Bülow and Overath, 1986).

Substrate specificity of the GPI-PLC is well established; although the enzyme can act on phosphatidylinositol, the hydrolysis of the GPI anchor of VSG occurs approximately one hundred and seventy times faster (Bulow and Overath, 1986). The enzyme has also been shown to act as a lysophospholipase C since it has the ability to act on a monoacyl derivative of mfVSG (Fox *et al.*, 1986). The phospholipids phosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine cannot be cleaved by the GPI-PLC (Bulow and Overath, 1986).

1.2.2. The Cross-Reacting Determinant

Prior to the discovery of the GPI-PLC, or indeed prior to the characterization of the VSG GPI anchor, it had been shown that all VSG variants possess an immunogenically identical domain (Barbet and McGuire, 1978). Antibodies raised against purified variant specific *Trypanosoma brucei*, and *Trypanosoma congolense*, VSG would only recognize, in immunofluorescent experiments, the trypanosome variant against which the antibodies were raised. Critically, purified variant specific VSG could be precipitated by antibody raised against any other variants (Barbet and McGuire, 1978). Although characterization of this cross-reactivity would not be achieved for another decade, Barbet and McGuire correctly postulated that there was a region of the VSG common to all variants, and that this region was only exposed when the VSG they purified from the cell surface. This conclusion supported the observation that purified variant-specific antibody would not cross react with other variants during microscopic studies of intact cells, likely because the domain was inaccessible to the antibody.

Characterization of the membrane anchor of the VSG (Jackson and Voorheis, 1985; Ferguson *et al.*, 1985a; Ferguson *et al.*, 1985b) finally allowed the nature of the immunologically conserved region in VSG to be understood. The GPI-PLC of *T. brucei* catalyzes the conversion of mfVSG to sVSG and dimyristoylglycerol (Ferguson *et al.*, 1985b), by cleavage of the phosphodiester bond between the inositol ring and the dimyristoylglycerol. The determination of the complete structure of the VSG membrane anchor, shown in figure 1.5, established VSG as the first GPI-anchored protein to be discovered.

Several features of the released form of the VSG contribute to the binding of antibodies raised against other variant specific VSGs. The majority of cross-reacting antibodies (70%) require the presence of the cyclic phosphate on the inositol ring (Zamze *et al.*, 1988). Other determinants of the sVSG which allow for binding include an unmodified 2-deoxy-2-*N*-acetylamino-D-galactose (GalNac) residue and the first galactose residue of the galactose branch, while for 30% of the cross-reacting antibodies, the ethanolamine-phosphate bridge was required together with the first galactose residue (although in this later case the cyclic phosphate was not required), (Zamze *et al.*, 1988).

Figure 1.5. Structure of the Variant Surface Glycoprotein GPI Anchor

The structure of the VSG GPI anchor core is shown (a), and is common to most characterized GPI anchors. Substitutions onto this core structure can be made at each position labelled 'R', with some possibilities listed below. Bonds that can be cleaved by various chemical agents or enzymes are shown (with HONO being nitrous acid, HF being hydrogen fluoride and GPI-PLD being GPI-specific phospholipase D). Hydrolysis of the VSG GPI anchor by the GPI-PLC in *T. brucei*, producing sVSG and dimyristylglycerol, is shown in (b). A legend for the carbohydrate moieties is given below. This figure is adapted from Essentials of Glycobiology (Ferguson, *et al.*, 2009; Chapter 11, Glycosylphosphatidylinositol Anchors).

Some variable substituents in GPI anchors:

R₁: fatty acid or OH (myristate, *i.e.* CH₃(CH₂)₁₂COO, in *T. brucei* VSG)

R₂: fatty acid or alkyl or alkenyl chain (myristate in *T. brucei* VSG)

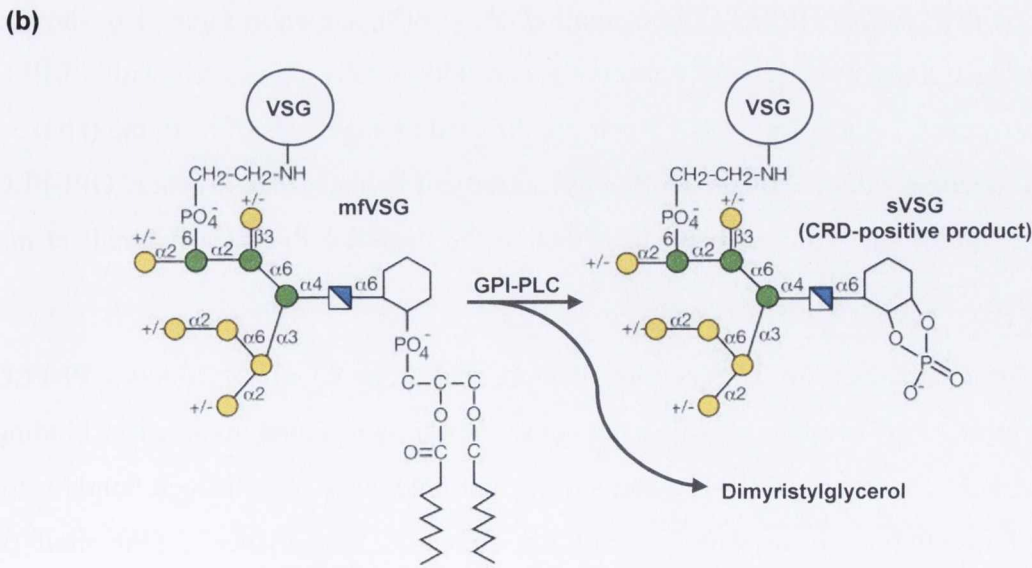
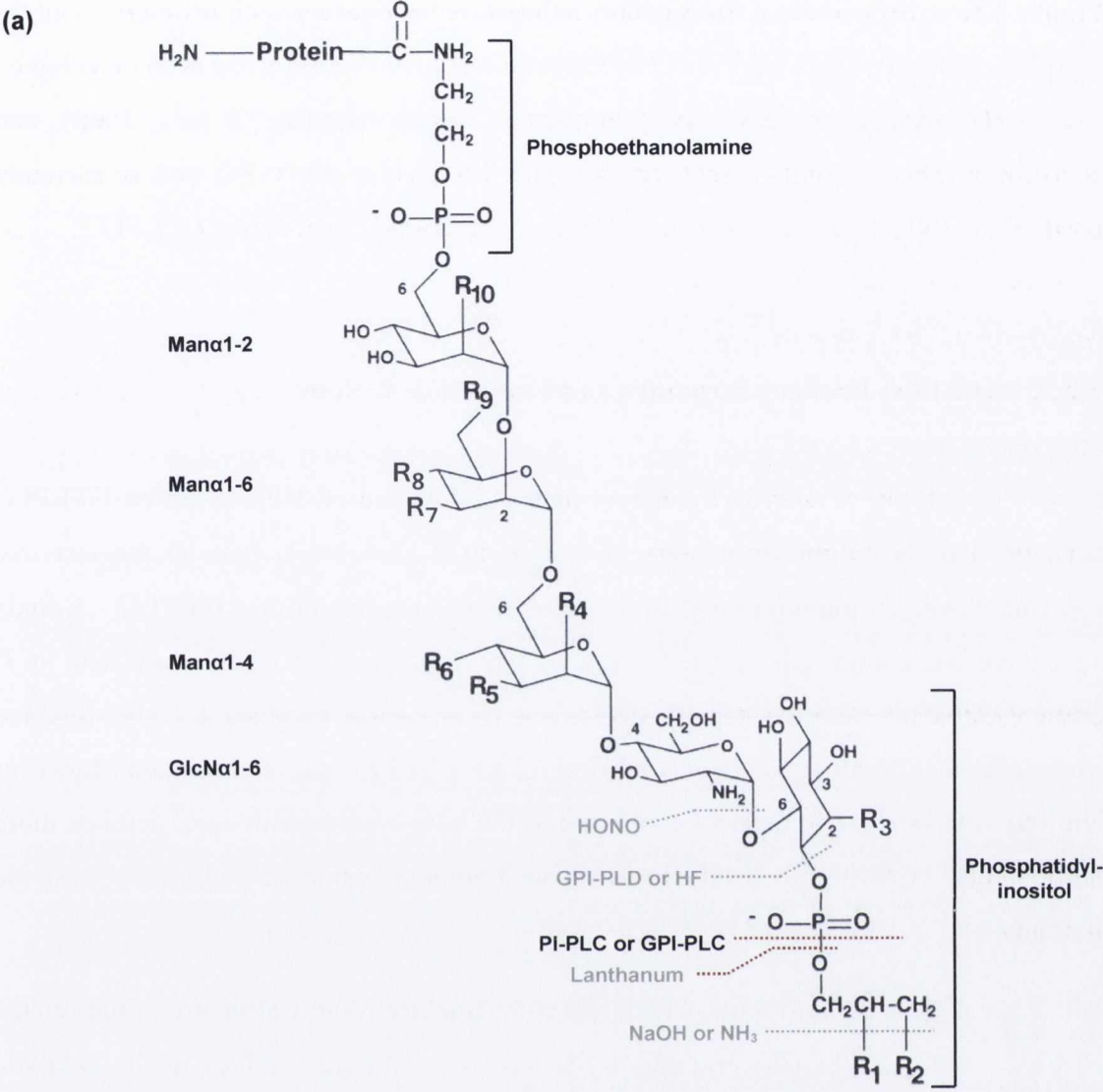
R₃: fatty acid or OH (may be positioned at C2 or C3 of the inositol ring)

R₄, R₉: ethanolamine phosphate or OH

R₅, R₆, R₇, R₈, R₁₀: carbohydrate substituents or OH (combinations of galactose moieties in *T. brucei* VSG)

Carbohydrate symbol legend for (b):

- Galactose (Gal)
- Glucosamine (GlcN)
- Mannose (Man)



The cross reacting determinant has therefore become a principle determinant for the identification of GPI anchors. The CRD epitope, for example, has been detected on the Gp63 surface protease of *Leishmania major* (Bordier *et al.*, 1986) and acetylcholinesterase from human erythrocytes (Stieger *et al.*, 1986) and is currently used as a marker of released *T. brucei* VSG, and as a marker for active GPI-PLC.

1.2.3. GPI-PLC Primary Structure and Enzymatic Activity

The ability of sulfhydryl reagents such as NEM and pCMPS to inhibit GPI-PLC activity, and the slight stimulatory action of DTT, led researchers to suspect that cysteine played an important role in the catalytic mechanism of the GPI-PLC. A study was therefore conducted in which each of the eight cysteine residues present in *T. brucei* GPI-PLC were systematically mutated to serine residues by site directed mutagenesis (Carnall *et al.*, 1997). None of the GPI-PLC cysteine mutants however showed any decrease in activity compared to the wild-type enzyme and, perhaps more surprisingly, pCMPS still exhibited identical inhibitory action in each of the cysteine mutants.

A slightly earlier report, which had elucidated the atomic structure of the related PI-PLC from *Bacillus (B.) cereus*, implicated two histidine residues in the catalytic mechanism of bacterial PI-PLCs (Heinz *et al.*, 1995). Sequence alignment of *B. cereus* PI-PLC with *T. brucei* GPI-PLC by Carnall *et al.*, (1997), shown in figure 1.6, showed that this histidines were largely conserved, and additionally conserved with PI-PLC from *Listeria (L.) monocytogenes*. Since site directed mutagenesis of histidine (His) 34 (to a glutamine residue) abolished GPI-PLC activity, it is likely that *T. brucei* GPI-PLC shares a similar catalytic mechanism to that in the bacterial PI-PLCs (Carnall *et al.*, 1997).

Structural data from *B. cereus* PI-PLC, and from *L. monocytogenes* PI-PLC (Moser *et al.*, 1997) further revealed the conservation of residues involved in binding *myo*-inositol. Heinz *et al.*, (1995), had shown that a network of hydrogen bonds were involved in binding *myo*-inositol within the enzyme's active site; a first shell of hydrogen bonds that interacted directly with the *myo*-inositol, and a second shell of amino acids that held the first shell in place, also through hydrogen bonding. As

revealed by the sequence alignment of Carnall and her co-workers, amino acids involved in this hydrogen bonding network are largely conserved between the three PLCs.

Despite the positive evidence implicating His34 in the catalytic mechanism of the GPI-PLC in *T. brucei*, a later report suggested that glutamine (Gln) 81 was also critically involved in GPI-PLC activity. A Gln81Glu mutant displayed only 0.0018 times the activity of the WT GPI-PLC; a Gln81Asn mutation resulted in a 125-fold drop in enzyme activity (Rashid *et al.*, 1999). Interestingly, although this glutamine residue is not required for either of the active site hydrogen bonding shells in *B. cereus* PI-PLC or *L. monocytogenes* PI-PLC, the residue is conserved between these PI-PLCs and with the GPI-PLC of *T. brucei* (Gln81 in *T. brucei* GPI-PLC corresponds to Gln51 in *B. cereus* PI-PLC), shown in figure 1.6. Rashid *et al.*, (1997) also suggested that cysteine (Cys) residue 80 in *T. brucei* GPI-PLC was resident in the enzyme's active site, and that this residue was the target for pCMPS-mediated inactivation of the enzyme. It was speculated that the formation of a bulky adduct between Cys80 and pCMPS occluded the active site. In support of this hypothesis, it was shown that Cys80Ala and Cys80Thr mutants that maintain GPI-PLC activity (in agreement with the findings of Carnall's Cys80Ser mutant) gained resistance to pCMPS. Furthermore, site directed mutagenesis of Cys80 to the more bulky phenylalanine (Phe) residue inhibited the enzyme. However, Carnall's Cys80Ser mutant did not gain any resistance to pCMPS-mediated inactivation (Carnall *et al.*, 1997), as would have been expected by Rashid's hypothesis, so interpreting the role of Cys80 in the active site of *T. brucei* GPI-PLC is difficult.

Figure 1.6. GPI-PLC Sequence Notes

The amino acid sequences of *T. brucei* GPI-PLC (Accession number: P09194), *B. cereus* PI-PLC mature protein (Accession number: P14262), and *L. Monocytogenes* PI-PLC mature protein (Accession number: P34024), were aligned using the online Clustal Omega package (available at www.ebi.ac.uk/Tools/msa/clustalo/). Identical amino acids are highlighted with a '*'. Amino acids involved in the first shell of hydrogen bonding with *myo*-inositol in *B. cereus* PI-PLC are highlighted in red font, underlined, and are residues His32, Arg69, Lys115, Arg163, Trp178, Asp198 and Tyr200 (Heinz *et al.*, 1995). First shell residues in *L. Monocytogenes* PI-PLC (His45, Arg84, Arg124, Lys126, Glu177, Asp204, and Tyr 206) are similarly highlighted. Residues involved in a second shell of hydrogen bonds in bacterial PI-PLCs are coloured red (but not underlined). Amino acids in *T. brucei* GPI-PLC that align with the residues involved in the bacterial PI-PLC hydrogen bonding network are also coloured red. Histidines involved in the catalytic mechanism of *B. cereus* PI-PLC (His32 and His82) are highlighted red and boxed, with corresponding residues from *L. monocytogenes* PI-PLC (His44 and His96) and *T. brucei* GPI-PLC (His34 and His114) also highlighted. A majority of the residues involved in the hydrogen bonding network, and the proposed catalytic histidines, are conserved across each of these phospholipases. All of the trypanosomal GPI-PLC cysteine residues are coloured blue, and labelled. Cys80 and Gln81 (also blue) have been speculated to be part of the active site in *T. brucei* GPI-PLC (Rashid *et al.*, 1999), while Cys269, Cys270, and Cys273 can be acylated in *T. brucei* GPI-PLC (Paturiaux-Hanocq, *et al.*, 2000). This figure is adapted from Carnall *et al.*, (1997).

The results of Carnall *et al.*, have showed that none of the cysteine residues in *T. brucei* GPI-PLC are required for enzyme activity. However, it has been shown that the cluster of three cysteine residues between positions 269 and 273 (*i.e.* Cys269, Cys270, and Cys273) may be important in regulating the GPI-PLC. After identifying two forms of the GPI-PLC, differing in electrophoretic mobility by an approximate molecular weight of 3 kDa, it was speculated that one of the forms (the 39 kDa form) represented an acylated form of the protein (Paturiaux-Hanocq, *et al.*, 2000). After showing that the addition of palmitic acid or myristic acid to the GPI-PLC represented a thioacylation, it was ultimately shown that this thioacylation occurred normally on any two of the three residues Cys269, Cys270 or Cys273. Loss of the acyl chains by mild alkaline treatment results in a conversion of the 39 kDa form of the GPI-PLC to a 42 kDa form (Paturiaux-Hanocq, *et al.*, 2000).

However, since non-acylated and acylated forms of the GPI-PLC are able to convert mfVSG to sVSG, it remains unclear as to what the precise purpose of the thioacylation of the protein is. Both forms of the GPI-PLC appear detectable, in equivalent amounts, by Western blot analysis of cell lysates. An attractive hypothesis by Paturiaux-Hanocq *et al.* (2000), was that complete acylation of the GPI-PLC affected the localization of the enzyme under certain circumstances, however this idea has not yet been further investigated since the initial observations.

1.2.4. A Physiological Role for the GPI-PLC?

Despite the large amounts of data collected it has still remained unclear as to what the physiological role of the GPI-PLC is. Although as early as the mid-nineteen eighties the enzyme had been successfully purified, characterized, and shown to cause a release of VSG *in vitro*, it had not been definitively shown what the *in vivo* requirement of the enzyme was; while a number of theories had been put forward, only circumstantial evidence had suggested that the function of the GPI-PLC, *in vivo*, was to release the VSG coat. For instance, expression of both the GPI-PLC and the VSG are limited to the bloodstream, and metacyclic, stages of the organism (Lamont *et al.*, 1987; Ross, 1987; Grab, 1987), with synthesis of both proteins being undetectable during growth of procyclic cells (Bulow and Overath, 1985). What was certainly clear,

however, was that there were at least three circumstances, *in vivo*, in which release of the VSG coat was required.

Firstly, upon initial assault of trypanosomes into their mammalian host, following a tsetse blood meal, the trypanosomes must replace their metacyclic VSG coat for a bloodstream VSG coat (Esser and Schoenbechler, 1985). Secondly, when cells are undergoing antigenic variation, a procedure is required for replacing the old VSG coat with a new, antigenically distinct, VSG coat (Webb *et al.*, 1997). Although in both these cases a rapid process for removing the VSG could be considered advantageous, it is the final scenario that most likely demands a rapid release of the surface coat. This final scenario is during the differentiation of the bloodstream stage trypanosome to the procyclic stage trypanosome; after trypanosomes are ingested by the tsetse vector, they must promptly exchange the VSG coat for the new protease-resistant coat, procyclin (Roditi *et al.*, 1989) that offers sufficient protection in the entirely different environment of the insect gut. Classically, the GPI-PLC was considered to be involved in one or more of these roles.

In an attempt to elucidate the *in vivo* requirement of the GPI-PLC, a null mutant cell line (GPI-PLC^{-/-}) was generated (Webb *et al.*, 1997). After confirming that incubating GPI-PLC^{-/-} trypanosomes under hypotonic conditions did not result in the exposure of the CRD, and after additionally showing that the CRD was not exposed after incubating GPI-PLC^{-/-} cells in the presence of detergent, it seemed that the GPI-PLC was indeed responsible for conversion of mfVSG to sVSG, as previously thought. But, the null mutant cell line could still transform to procyclic form trypanosomes, and successfully replaced VSG with procyclin (Webb *et al.*, 1997). It should also be noted that the null mutants were also capable of undergoing antigenic variation after establishing infections in mice. A few important differences were observed, however, that suggested that the GPI-PLC somehow influences the virulence of the bloodstream stage of the parasite.

Under normal cases of infection, *i.e.* the infection of laboratory mice with wild-type *T. brucei*, the mean survival time of each mouse was found to be twenty eight days, a stark contrast to the mean survival time of seventy four to eighty one days observed in GPI-PLC^{-/-} infections (some mice even survived through to the completion of the experiment after some ninety nine days of infection). Critically, the much

reduced virulence of the GPI-PLC^{-/-} cell line was partly reversed in trypanosomes expressing low levels of GPI-PLC activity (approximately one sixteenth of wild-type activity); mice infected with trypanosomes expressing these low levels of GPI-PLC showed a mean survival time of fifty seven days (Webb *et al.*, 1997). GPI-PLC^{-/-} cells also showed a much reduced first peak of parasitemia in infected animals (approximately one third that of wild-type infections), and similarly showed a noticeably reduced parasitemia throughout the course of infection. Again, these differences were partially reversed when animals were infected with trypanosomes expressing low levels of GPI-PLC activity.

But, if the classic defence ascribed to trypanosomes is their ability to undergo antigenic variation, why then do GPI-PLC^{-/-} trypanosomes display reduced virulence despite their ability to still undergo VSG switching? The ability of the GPI-PLC^{-/-} trypanosomes to undergo a complete life cycle, and their ability to establish a persistent infection in laboratory animals, raises some questions over our understanding of this enzyme. Webb and her colleagues suggested that the reduced virulence of the GPI-PLC^{-/-} cell line could be due to a reduced growth rate of the parasite, or alternatively due to the infected animals somehow clearing the knock-out cell line with greater ease. They also suggested that repeating animal infections of GPI-PLC^{-/-} trypanosomes in the organisms' natural host, rather than laboratory animals, may offer a more significant phenotype that could more clearly point to the physiological role of the GPI-PLC.

To add to our confusion, the GPI-PLC is not just able to hydrolyze GPI anchors, but it can also act on phosphatidylinositol (PI) under some detergent conditions (Bütikofer *et al.*, 1996). In the presence of 0.02% Triton[®] X-100, GPI-PLC was shown to be active against PI and GPI over a similar pH range (with a pH optimum of approximately 8). Under these detergent conditions, the GPI-PLC was found to have a K_m for the GPI-anchor of acetylcholinesterase (from bovine erythrocytes) of 785 nM, and a K_m for PI of 37 μ M. Furthermore, a biosynthetic precursor of VSG, lipid A, can also act as a substrate for the GPI-PLC (Hereld *et al.*, 1986). Other authors have suggested that the GPI-PLC may indeed be involved in regulating the levels of intracellular inositol phosphates (Ochatt, *et al.*, 1999). Despite these additional candidates for physiological substrate of the GPI-PLC, the conversion of mfVSG to sVSG has remained the favoured physiological role of the GPI-PLC in the literature.

However, the physiological circumstance during which the GPI-PLC mediates the hydrolysis of the GPI anchor remains unknown.

1.2.5. Localization of the GPI-PLC

The localization of the GPI-PLC has been the subject of intense study for a number of years. The most recent work has provided evidence that localizes the GPI-PLC to the outside of the flagellar membrane and, more specifically, along the flagellar attachment zone (Hanrahan *et al.*, 2009). This localization was shown by immunofluorescent microscopy, and confirmed after transfecting bloodstream form trypanosomes with a yellow fluorescent protein (YFP)-GPI-PLC recombinant protein; YFP-GPI-PLC was also visualized as a linear array along the flagellum. A key aspect of this latest study was the proposition that some trypanosomal surface proteins (such as the GPI-PLC) could not be detected by immunofluorescent microscopy under normal fixing conditions. When trypanosomes were fixed with paraformaldehyde at temperatures greater than room temperature, it was proposed, the fixed VSG effectively blocked access by anti-GPI-PLC antibody to the GPI-PLC. Detection of the GPI-PLC on the surface of the cell could only be achieved if the cells were instead fixed at 0 °C, a condition under which the VSG, with less thermal energy available for flexing over other invariant surface proteins, did not hinder antibody binding to the GPI-PLC (Hanrahan *et al.*, 2009).

A similar phenomenon to that observed by Hanrahan *et al.*, (2009) was observed by Bülow *et al.*, (1989). In their study, the GPI-PLC could only be detected by immunofluorescence in trypanosomes chemically fixed with 2% formaldehyde for ten minutes. Fixing trypanosomes for several hours was found to completely abolish their ability to detect the GPI-PLC, which they thought was due to an inability of the antibody to gain access to intracellular protein after more a drastic fixation procedure (note that in their report immunofluorescent imaging localized the GPI-PLC to intracellular vesicles). It is likely that the ability to access proteins below the protective VSG coat in *T. brucei*, with antibody, is dependent on a combination of temperature, concentration of fixative, and during of fixation.

The report of the phospholipase localization at the flagellar membrane described by Hanrahan *et al.*, (2009) is convincing, and agrees with at least some of the results published earlier by other authors. However earlier reports showing different results should also be considered. Using a fractionation approach for example, it has been shown that GPI-PLC activity is recovered in membrane fractions, particularly the membranes of the flagellum and flagellar pocket (Grab *et al.*, 1987). It should be mentioned though that these workers also found the phospholipase associated with the glycosomal membranes (Grab *et al.*, 1987), a localization that was not found by Hanrahan *et al.*, but was supported by other researchers (Subramanya and Mensa-Wilmot, 2006). Subramanya and Mensa-Wilmot, however, have found no evidence linking the GPI-PLC to either the membrane of the flagellum or flagellar pocket.

The differences observed between Hanrahan *et al.* (2009), Subramanya and Mensa-Wilmot (2006) and Grab *et al.* (1987) are not unique. The approach adopted by Grab *et al.* (1987) to localize the GPI-PLC involved making preparations of trypanosomal organelles, testing these purified organelles for marker enzyme activity and then testing the specific GPI-PLC activity in each separated organelle. By these criteria, the GPI-PLC was concluded as normally resident in the flagellar membrane and flagellar pocket, and some phospholipase activity was also found associated with the Golgi complex. However, after adopting a different fractionation approach based on isopycnic sucrose-gradient centrifugation, GPI-PLC activity could not be found in fraction densities correlating to flagellar membrane or plasma membrane fractions (Bülow *et al.*, 1989). Instead, GPI-PLC activity was detected in those fractions with a density of 1.25 g.cm^{-3} and displaying hexokinase activity, suggesting the phospholipase was localized to the glycosomes. Additionally, Bülow *et al.* could detect phospholipase activity in fractions correlating to lysosomal membranes, mitochondrial membranes and the membrane of the flagellar pocket.

The orientation of the GPI-PLC should also be considered. Two opposing theories have valid evidence. It has been shown that while cells remain intact during trypsin treatment, for approximately twenty minutes, the GPI-PLC activity also remains normal. After twenty minutes of trypsin treatment however, GPI-PLC activity begins to fall, and this fall in activity correlates with cell lyses (Bülow *et al.*, 1989). This evidence suggests that trypsin does not have access to the phospholipase until the cell membrane becomes permeable to either of the enzymes, and therefore implicates that

the phospholipase is located on the cytosol-facing side of intracellular membranes. However, considering the ratio of VSG molecules to GPI-PLC molecules (a ratio of 10^7 to 3×10^3 , or approximately 1:3000), it is perhaps unsurprising that proteolysis of the GPI-PLC begins some time after the proteolysis of the VSG has started. Paradoxically, it has also been shown that the GPI-PLC can be detected by immunofluorescence in the absence of any membrane-permeabilizing conditions (Hanrahan *et al.*, 2009). Hanrahan and her co-workers have shown that immunofluorescent detection of intracellular proteins, such as tubulin, requires a small amount of detergent to be present; no detergent is required for the detection of the GPI-PLC by the same means. Finally, the GPI-PLC could also be detected by non-penetrative surface labelling with ^{125}I , an observation that convincingly shows the GPI-PLC to be at least partially orientated on the extracellular side of the flagellar membrane (Hanrahan *et al.*, 2009).

The orientation of the GPI-PLC on the outside of the flagellar membrane is an attractive idea, particularly since it solves a previous topological problem regarding the access of the GPI-PLC to its substrate. However until the localizations, and orientations, reported by all of the studies can be collectively rationalized, the localization of the phospholipase will remain contentious.

1.2.6. A Final Note: the Involvement of a Zinc Metalloprotease

More recently it has become apparent that a second enzyme is involved in VSG release from trypanosomes. The major surface protease B from *T. brucei* (TbMSP-B), is a zinc metalloprotease located on the surface of trypanosomes (Grandgenett *et al.*, 2007) and related to the major surface zinc metalloprotease (MSP, or GP63) of *Leishmania* spp. (LaCount *et al.*, 2003). Unlike the GPI-PLC, TbMSP-B protein is not detectable, by Western blot analyses, in bloodstream form trypanosomes despite the presence of its mRNA; the protein does, however, become detectable within 24 hrs of the induction of differentiation to procyclic cells (Grandgenett *et al.*, 2007). Under the differentiation conditions employed by Grandgenett and his co-workers, it has been shown that the TbMSP-B is responsible for the appearance of a 37 kDa fragment of VSG in the culture medium 48 hrs after initiation of differentiation.

After establishing a cell line lacking both alleles of the *TbMSP-B* gene (*TbMSP-B*^{-/-} cells) it was shown that VSG release during differentiation of bloodstream trypanosomes to procyclic forms is slower when *TbMSP-B* is absent. This slower release of VSG is also observed in *GPI-PLC*^{-/-} cells (Grandgenett *et al.*, 2007). However, when mutant trypanosomes in which both the alleles of the *GPI-PLC* and the *TbMSP-B* have been deleted, VSG release during differentiation cannot be completed and only in this later circumstance do the cells fail to differentiate (Grandgenett *et al.*, 2007). Critically, the effect of the *TbMSP-B* on VSG was only monitored under these culture conditions, and the significance of the protease has not been established in trypanosomes differentiating *in vivo*.

Although the presence of a second enzyme capable of releasing the VSG from the surface of trypanosomes aids our understanding as to how the *GPI-PLC*^{-/-} cells constructed by Webb *et al.*, (1997) were capable of undergoing a complete life cycle, *in vivo*, the precise timing of events, *i.e.* the induction of *TbMSP-B* translation and VSG release prior to, and subsequent to, this induction, remains poorly understood.

1.3. Aims

Ultimately, this thesis endeavours to identify any protein that may interact with the *GPI-PLC* in bloodstream form *T. brucei*. In particular, this thesis aims:

- 1) To establish and validate a robust procedure for confidently identifying unknown proteins. A MALDI-ToF mass spectrometric approach will be adopted and validated for identifying unknown proteins, particularly unknown protein bands present on Coomassie stained polyacrylamide gels.
- 2) To investigate if *GPI-PLC* partner proteins can be specifically co-immunoprecipitated with the *GPI-PLC* using non-ionic, ionic, and zwitterionic detergent conditions.
- 3) To investigate the mechanism of *GPI-PLC* activation during the de-energization of bloodstream form trypanosomes. The role of Ca^{2+} in the de-energization

process, and in the stimulation of GPI-PLC-mediated VSG release from the cell surface, will be studied to determine if Ca^{2+} regulates the GPI-PLC *in situ*.

- 4) To rigorously characterize the *in vitro* growth characteristics of GPI-PLC^{-/-} cells in order to determine if the *in vitro* growth of GPI-PLC^{-/-} cells is consistent with *in vivo* findings. This characterization will aid in developing a general understanding of the GPI-PLC and could help uncover a physiological role for the enzyme.

Chapter 2

Materials and Methods

2.1. General Methods

2.1.1. Notes on Standard Equipment used

An Oertling analytical balance, or an AnD HR-120 analytical balance, was used to weigh reagents between 1 mg and 100 mg. Greater masses were measured using an Ohaus Adventurer. Gilson Pipetteman micro-pipettes were used appropriately to measure volumes between 1 μ l and 1 ml. An Amagross glass electrode, connected to an EDT RE357 microprocessor pH meter, was used to measure the hydrogen ion concentration of solutions. The pH meter was calibrated daily using standards solutions of pH 4.0, 7.0, and 10.0, from Sigma. Absorbance values of solutions were measured using a Cary 50 UV spectrophotometer. For centrifugation of samples, a refrigerated Sorvall RC5C centrifuge, a refrigerated Rotanta 460R centrifuge, and numerous bench-top microcentrifuges (at room temperature or at 4°C) were used as appropriate.

2.1.2. Standard Buffer Preparation and Recipes

All buffers and solutions were prepared with distilled, deionised water. Unless otherwise specified, all buffers and solutions were titrated at room temperature using 12 M HCl or 5 M NaOH when necessary. The base recipes of standard buffers used throughout this work are given in table 2.1.

Table 2.1. Standard Buffer Compositions

	TSB	5X TSB	PBS	10X PBS	Tes
NaCl	44 mM	220 mM	136 mM	1.36 M	140 mM
KCl	4 mM	20 mM	3 mM	30 mM	4 mM
Na ₂ HPO ₄	57 mM	285 mM	16 mM	160 mM	---
NaH ₂ PO ₄	3 mM	15 mM	---	---	---
KH ₂ PO ₄	---	---	3 mM	30 mM	---
Tes	---	---	---	---	30 mM
Sucrose*	70 mM	---	40 mM	400 mM	60 mM
Glucose*	10 mM	---	10 mM	100 mM	10 mM
Adenosine*	0.1 mM	---	0.1 mM	1 mM	0.1 mM
NaN ₃ †	0.002% (w/v)	---	0.02% (w/v)	---	---
pH	8.0	8.0	7.4	7.4	7.4

* Component omitted from trypanosome separation buffer (TSB) for storage of diethylaminoethyl cellulose (DEAE) resin.

† NaN₃ was only added for long-term storage of buffers that were not required for work with live cells.

2.1.3. Use and Storage of Chemicals

Unless otherwise specified, all chemicals and working stock solutions were prepared and stored as recommended by the relevant manufacturer (listed in section 2.9.1).

2.1.4. Preparation and use of Protease Inhibitors

Where noted that protease inhibitors were used, these protease inhibitors (unless otherwise specified) are leupeptin, pepstatin A, phenylmethylsulfonyl fluoride (PMSF) and N_α-Tosyl-L-lysine chloromethyl ketone (TLCK). Each protease inhibitor was prepared as recommended by the manufacturer; in particular, PMSF and TLCK were

always prepared immediately prior to use. The concentrations of each of the protease inhibitors used, and the targets of each of the protease inhibitors, are given in table 2.2.

Table 2.2. Protease Inhibitors

Protease Inhibitor	Working Concentration*	Target†
Leupeptin	10 µg·ml ⁻¹ (21 µM)	Serine proteases and thiol proteases
Pepstatin A	2 µg·ml ⁻¹ (3 µM)	Acid proteases
PMSF	52 µg·ml ⁻¹ (300 µM)	Serine proteases
TLCK	37 µg·ml ⁻¹ (100 µM)	Trypsin-like serine proteases

* Stock solutions of protease inhibitors were prepared as recommended by the manufacturers.

† Information on the targets of each protease inhibitors was obtained from the Sigma-Aldrich website, at www.sigmaaldrich.com.

2.2. Cell Culture and Purification of Trypanosomes from Infected Rats

2.2.1. Source of Trypanosomes

Bloodstream form *Trypanosoma brucei brucei* used for this study were originally sourced, in 1960, from the blood of an infected sheep in Uganda (Cunningham & Vickerman, 1962). This original strain was classed as ‘strain 427’, and clones of these trypanosomes were prepared in 1975, yielding cultures expressing homologous VSGs (Cross, 1975). Organisms used here consisted solely of long slender, bloodstream forms of the Molteno Institute Trypanozoon antigen type (MITat) 1.2 prepared by Cross and previously termed, in the literature, as clone 221. Both wild-type and null GPI-PLC mutant (GPI-PLC^{-/-}), (Hanrahan *et al.*, 2009), MITat 1.2 clones were used.

2.2.2. *In Vitro* Culturing Conditions

Bloodstream form trypanosomes were maintained by culturing in sterile HMI-9 (Hirumis’ modified Iscove’s medium, formulation 9) growth medium, originally prepared by Hirumi and Hirumi (1989) and supplemented additionally as shown in table 2.3. Cultures were incubated at 37°C, 5% CO₂ and normally kept between a density of 1 x 10⁵ cells·ml⁻¹ to 2 x 10⁶ cells·ml⁻¹. Cells were enumerated using an Improved Neubauer haemocytometer, with a silvered stage, and an inverted Olympus phase contrast microscope.

Table 2.3. Composition of Culture Medium

	Concentration
HMI-9 powder	9.07 g·l ⁻¹
Sodium bicarbonate	2 g·l ⁻¹
Guanosine	200 mg·l ⁻¹
Adenosine	255 mg·l ⁻¹
2-Mercaptoethanol	14 µl·l ⁻¹
FBS*	10% (v/v)
pH	7.4

* Foetal bovine serum (FBS) was heat-inactivated by heating at 60 °C for forty five minutes before use.

2.2.3. Preparation of Glycerol Stocks for *in Vitro* Culturing

Glycerol stocks of cell lines, for storage in liquid nitrogen, were prepared by mixing an equal volume of cell cultures (typically 500 µl) with growth medium supplemented as previous, but additionally containing 30% (v/v) glycerol. These samples were slowly cooled to -80 °C and left overnight before transferring to liquid nitrogen for long term storage.

2.2.4. Infection of Rats

Stabilates, taken from liquid nitrogen immediately prior to use, were settled in cool water before flushing the trypanosome suspension from the capillary with approximately 200 μ l to 300 μ l of trypanosome separating buffer (TSB, table 2.1). Male Wistar rats, typically three months old, or older, were infected with the trypanosome suspension by intraperitoneal injection. Rats were housed under appropriate conditions until the parasitemia reached between 5×10^8 and 1×10^9 cells per millilitre of blood. Blood used for counting the level of parasitemia during an infection was collected from the animal's tail vein.

2.2.5. Collection of Infected Rat Blood

Infected rats were anaesthetized with a lethal dose of sodium pentobarbitone (approximately 0.5 ml, of a 200 $\text{mg} \cdot \text{ml}^{-1}$ solution, per rat) once the appropriate level of parasitemia was reached (approximately seventy two hours after infection). The rats were exsanguinated by aortic puncture, and blood withdrawn through a 21-gauge needle attached to a 20 ml syringe containing 2 ml of EGTA (100 mM, pH 7.4). Once collected, the blood was kept on ice.

2.2.6. Purification of Trypanosomes from Infected Blood

Trypanosomes were separated from blood using the method originally developed by Lanham (1968), and subsequently modified by later authors (Owen and Voorheis, 1976, Lonsdale-Eccles and Grab, 1987). Briefly, the infected blood was centrifuged at 650 g , in a Rotanta 460 A centrifuge (swing-out rotor) for ten minutes at 4°C. After aspirating the upper layer of pale-yellow plasma, an equal volume of TSB was used to resuspend the second (white) layer, which was composed of the trypanosomes, with minimal disturbance of the bottom (red) layer of erythrocytes. The resuspended layer of trypanosomes was then applied to a 15 ml (approximately 1.2 cm diameter) DEAE-52 column equilibrated with TSB. The column was washed with TSB, at 4°C, until healthy trypanosomes were eluted; erythrocytes, non-viable

trypanosomes, and other cells present in the crude suspension remain bound to the column. Collected trypanosomes were finally washed twice with buffer (as described in the relevant sections) before counting and use in experiments.

2.2.7. Preparation of Glycerol Stocks for Infecting Rats

Infected rat blood, after collection, was brought to a final glycerol content of 10% (w/v) and thoroughly mixed. Blood was drawn into a heparinized, glass micro-haematocrit tube (75 mm length x 1.15 mm internal diameter, from Fisher Scientific) until the tube was approximately two thirds full. Both ends of the haematocrit tube were then sealed in a Bunsen flame before gently cooling the tube to -80 °C overnight. The prepared glycerol stocks were then stored in liquid nitrogen until use.

2.2.8. Establishing *in Vitro* Cultures from non-Sterile Stocks

In some circumstances it was necessary to use the non-sterile glycerol stocks used for infecting rats to establish *in vitro* cultures. In such circumstances, the appropriate stabilate was warmed as before, but flushed with HMI-9 medium containing kanamycin (50 $\mu\text{g}\cdot\text{ml}^{-1}$) and ampicillin (100 $\mu\text{g}\cdot\text{ml}^{-1}$). The resulting cultures were maintained in the presence of these antibiotics for at least one week before sub-culturing into fresh media containing no antibiotics.

2.2.9. Ethics Statement

All work with live animals performed during this project was first approved by the relevant local and national bodies. Animals were always handled in accordance with the guidelines of these bodies.

2.3. Gel Electrophoresis and Western Blotting

2.3.1. Preparation of Denaturing Gels

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was carried out using a modification of the method developed by Laemmli (1970). For this work, 10%, 12.5%, and 15%, polyacrylamide gels were used, as outlined in the relevant results sections. The components of these gels are given in table 2.4, with the volumes presented being sufficient for four gels. Resolving gels were prepared, and poured between two glass plates (from Atto Corp.) separated by a 1mm spacer before covering the top of the gel with a thin layer of ethanol. Once the resolving gels had set, after approximately twenty minutes, the ethanol was poured off, and the region above the resolving gel, between the glass plates, was washed with distilled, deionised water. This region was quickly wiped dry before pouring the 5% stacking gel and inserting the comb. Typically, the stacking gel set after twenty or thirty minutes, after which time the combs were removed and a fine spatula was used to clear the wells of fragments of polyacrylamide. The gels were either used immediately after preparation, or stored for up to two weeks at 4°C until use.

2.3.2. Sample Preparation for SDS-PAGE

Prior to loading samples on a denaturing gel, the sample was mixed with 5X sample buffer (recipe given in table 2.5) to yield a final ratio of sample to sample buffer of 4:1, *i.e.* the 5X sample buffer was diluted one in five times in the sample. The sample was then heated at 70 °C for five minutes prior to loading

DALTON MARK VII-L Standard Mixture, from Sigma, and Unstained Thermo Scientific Protein Molecular Weight Marker Mixture, were used as a source of molecular weight standards for denaturing gel electrophoresis and prepared essentially as instructed by the manufacturer were necessary. The standards were stored at -20 °C until use, and heated at 70 °C for one minute prior to loading. The protein contents of the standard mixtures are given in table 2.6.

Table 2.4. Compositions of Denaturing Gels

Percent Gel:	10% (Resolving Gel)	12.5% (Resolving Gel)	15% (Resolving Gel)	5% (Stacking Gel)
30% w/v Acrylamide	10 ml	12.5 ml	15 ml	3.4 ml
1% w/v Bisacrylamide	3.9 ml	3.1 ml	2.6 ml	2.6 ml
1.5 M Tris (pH 8.7)	7.5 ml	7.5 ml	7.5 ml	---
1.0 M Tris (pH 6.8)	---	---	---	2.5 ml
20% SDS	150 µl	150 µl	150 µl	100 µl
Water	8.0 ml	6.4 ml	4.4 ml	8.8 ml
10% (w/v) Ammonium persulphate*	100 µl	100 µl	100 µl	100 µl
TEMED	10 µl	10 µl	10 µl	15 µl

* Component was prepared freshly, immediately prior to use.

Table 2.5. Sample Buffer Composition

5X Sample Buffer	
Tris	160 mM
SDS	6.25% (w/v)
Glycerol	25% (v/v)
1% (w/v) Bromophenol blue *	1 ml
2-Mercaptoethanol†	4% (v/v)

* Bromophenol blue was initially dissolved in ethanol.

† Component only added immediately prior to use.

Table 2.6. Components of Protein Standard Mixtures

Protein	Approximate Molecular Weight (Da)	Sigma- Aldrich	Thermo- Scientific
β-galactosidase	116,000		/
Albumin, bovine	66,000	/	/
Albumin, egg	45,000	/	/
Glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle	36,000	/	
Lactate dehydrogenase	35,000		/
Carbonic anhydrase, bovine	29,000	/	
REase Bsp981	25,000		/
Tyrpsinogen, bovine pancreas	24,000	/	
Trypsin inhibitor, soybean	20,100	/	
β-Lactoglobulin	18,400		/
Lysozyme	14,400		/
α-Lactalbumin, bovine milk	14,200	/	

2.3.3. Electrophoresis Conditions

Polyacrylamide gels were run using a constant current of 25 mA, supplied by a Bio-Rad Power Pac 3000, per gel. During electrophoresis the gels were docked in a dual slab chamber, from Atto Corp., filled with running buffer (0.3 M glycine, 0.08 M Tris, 0.1% w/v SDS). Gels were run until the dye front reached within a few millimetres of the end of the resolving gel (typically taking about ninety minutes). The gels were removed from the chamber and stained for protein, or transferred to a nitrocellulose membrane, as outlined below.

2.3.4. Gel Staining and De-staining

After electrophoresis, gels were soaked in Coomassie Brilliant Blue R-250 (0.25% w/v in 10% v/v methanol, 7% v/v acetic acid) for twenty minutes before washing with de-staining solution (10% v/v methanol, 7% v/v acetic acid). Gels were washed with a number of changes of de-staining solution, while rocked gently, until background staining was completely removed.

2.3.5. Electrophoretic Transfer of Protein to Nitrocellulose Membranes

Protein was transferred from polyacrylamide gels by a modification of the procedure described originally by Towbin, (Towbin *et al.*, 1979). Nitrocellulose membrane and filter paper (both from Whatman) were soaked in transfer buffer (table 2.7) for half an hour prior to use. Proteins from polyacrylamide gels were transferred by placing the gel on top of the wetted nitrocellulose sheet, before sandwiching both gel and nitrocellulose between eight sheets of filter paper (four pieces on either side). The assembly was then placed into a semi-dry blotting chamber, with the nitrocellulose sheet on the anode facing, and the gel on the cathode facing. A constant current of 90 mA was applied, for each gel in the blotting chamber, and protein was transferred for one hour.

2.3.6. Ponceau Staining of Molecular Weight Standards

Following transfer of protein to the nitrocellulose membrane, the molecular standards were stained with Ponceau S (1% w/v Ponceau S in 5% v/v acetic acid) and marked with pencil. To de-stain the Ponceau S, the blot was washed with TBST (see table 2.7). Approximately four or five changes of TBST were required for complete destaining of the Ponceau S.

2.3.7. Immunological Detection of Alanine Aminotransferase

Prior to probing nitrocellulose sheets with antibody, non-specific binding sites on the sheet were blocked by incubating the blot for one hour, at room temperature, in TBST containing 5% (w/v) marvel. The blot was then washed with TBST over half an hour, with six changes of buffer, before applying rabbit anti-alanine aminotransferase immune serum, diluted 1 in 2,000 in TBST containing 1% (w/v) marvel. The blot was incubated with immune serum overnight, at 4°C, and with gentle agitation, after which time the blot was washed again, over half an hour with six changes of TBST. Bound antibody was detected as described in section 2.3.9 below.

A control blot probed with pre-immune serum confirmed that the anti-alanine aminotransferase immune serum reacted specifically with alanine aminotransferase under ideal loading conditions (figure 2.1).

2.3.8. Immunological Detection of the GPI-PLC

The GPI-PLC was detected by Western blotting in precisely the same manner as alanine aminotransferase, but using a 1 in 1,000 dilution of anti-GPI-PLC IgG. Although the anti-GPI-PLC was found to react predominantly with a protein of molecular weight 36 kDa in WT and not in GPI-PLC^{-/-} cells, there was some weak cross-reactivity with a small number of additional proteins (figure 2.2). Purification of anti-GPI-PLC IgG from rabbit serum is detailed in section 2.8.1, and the concentration of the purified IgG (1.2 mg·ml⁻¹) was determined using the Pierce 660 nm protein assay reagent (see section 2.8.2).

2.3.9. Detection of Rabbit Immunoglobulin G

Primary rabbit IgGs were detected using a peroxidase-conjugated goat anti-rabbit IgG as a secondary antibody. This secondary antibody was diluted 1 in 10,000 (yielding a final antibody concentration of 80 ng·ml⁻¹) in TBST containing 1% (w/v) marvel, and allowed to react with the blot for one hour, at room temperature, with gentle agitation. Secondary antibody solution was then poured off before washing the blot with six changes of TBST, over half an hour, and finally detecting the peroxidase conjugate as outlined in section 2.3.10.

2.3.10. Enhanced Chemiluminescence and Visualization of Peroxidase Conjugates

After the final washes in TBST, blots were immersed in substrate solution (table 2.7) for one minute before blotting the edges of the nitrocellulose membrane dry and placing between two thin plastic sheets in an X-ray cassette. Light sensitive film (Kodak X-Omat LS), was exposed to the membrane for a recorded amount of time (typically five to thirty seconds) and developed using a Fuji-Colenta Mediphot 937.

Table 2.7. Buffers used for Western Blotting

	Transfer Buffer	TBST	Substrate Solution*
NaCl	---	154 mM	---
Tris	25 mM	50 mM	100 mM
Glycine	150 mM	---	---
Methanol	10% (v/v)	---	---
Tween 20	---	0.1% (w/v)	---
Iodophenol (9 mg·ml ⁻¹ stock, in DMSO)	---	---	1 ml
Luminol	---	---	25 mg
30% (w/w) H ₂ O ₂	---	---	31 µl
pH	8.3	7.4	8.5

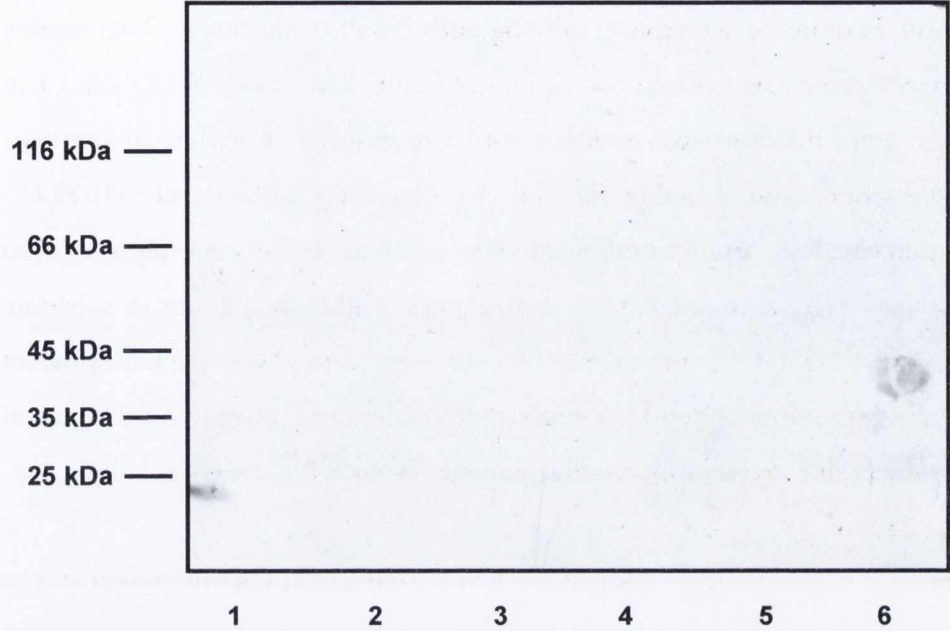
* Values presented for substrate solution are correct for a 100 ml solution.

Figure 2.1. Rabbit anti-Alanine Amino Transferase (AAT) IgG binds Specifically with AAT on a Western Blot

Increasing amounts of whole cell WT MITat 1.2 SDS-lysates were loaded onto a 10% polyacrylamide gel for blotting with anti-AAT antibody. Two replica gels were run and transferred to nitrocellulose membranes as described. For the control blot, shown in (a), the nitrocellulose membrane was incubated with a 1 in 2,000 dilution of pre-immune rabbit serum as the primary ‘antibody’. For the anti-AAT blot, shown in (b), the membrane was incubated with an identical dilution of immune serum containing anti-AAT IgG. Both membranes were exposed to the same piece of film for fifteen seconds, following a ten minute resting period, after incubating in substrate solution. Anti-AAT IgG reacts specifically with a band of the expected apparent molecular weight (approximately 55 kDa, highlighted with a red arrow) under the ideal loading condition (5×10^5 cells). The loading scheme for each blot is given below:

Blots (a) and (b):	Lane 1:	molecular weight standards (Thermo Scientific)
	Lane 2:	1.25×10^5 cells
	Lane 3:	2.5×10^5 cells
	Lane 4:	5×10^5 cells
	Lane 5:	1×10^6 cells
	Lane 6:	2×10^6 cells

(a)



(b)

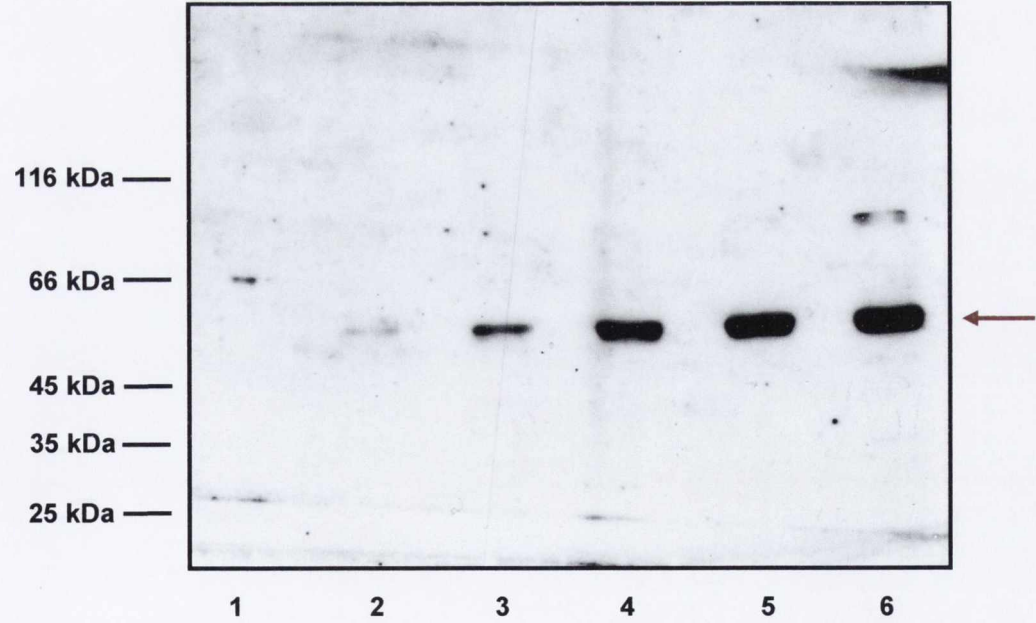
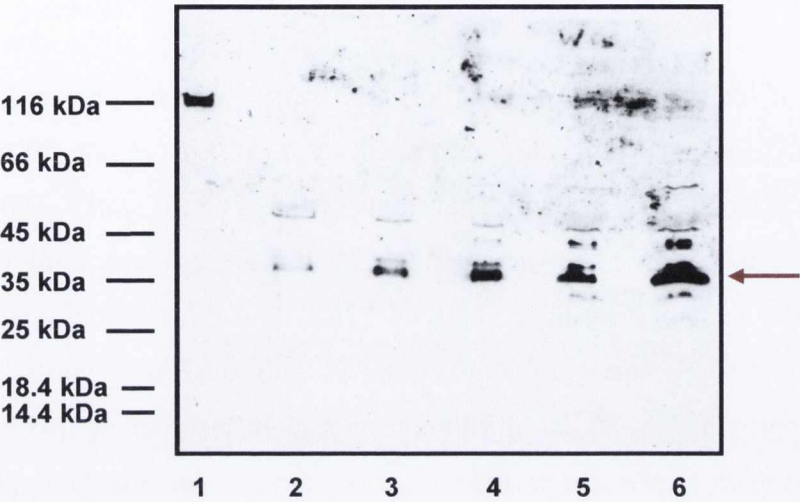


Figure 2.2. Rabbit anti-GPI-PLC IgG Specificity on a Western Blot

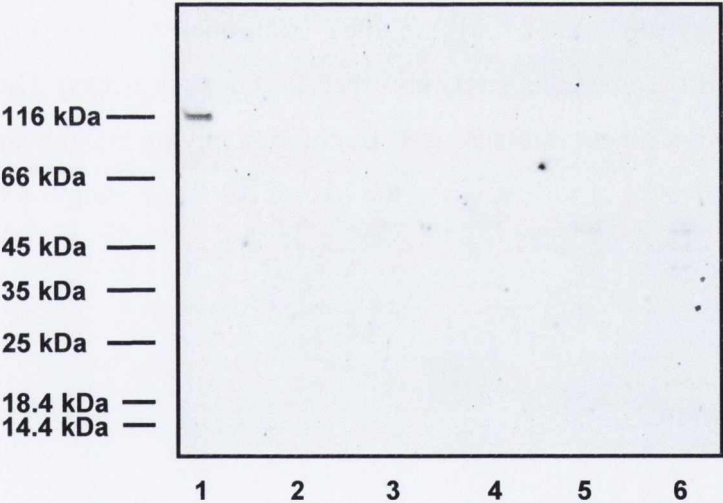
Increasing amounts of bloodstream form trypanosomal SDS-lysates were loaded onto a 15% polyacrylamide gel for blotting with anti-GPI-PLC antibody. Two replica gels were run, transferred to nitrocellulose membranes, blocked, and probed with a 1 in 1,000 dilution of anti-GPI-PLC IgG as described. For blot (a) increasing amounts of WT trypanosomes were loaded, while for blot (b) increasing amounts of GPI-PLC^{-/-} trypanosomes were loaded. Both membranes were exposed to the same piece of film for fifteen seconds following a ten minute resting period after incubating in substrate solution. Anti-GPI-PLC IgG reacts primarily with a band of the expected apparent molecular weight (approximately 36 kDa, highlighted with a red arrow) under the ideal loading condition (5×10^6 cells). The loading scheme for each blot is given below:

Blot (a) and (b):	Lane 1:	molecular weight standards (Thermo Scientific)
	Lane 2:	1.25×10^6 cells
	Lane 3:	2.5×10^6 cells
	Lane 4:	5×10^6 cells
	Lane 5:	1×10^7 cells
	Lane 6:	2×10^7 cells

(a)



(b)



2.4. MALDI-ToF Mass Spectrometry and Peptide Mass Fingerprinting

2.4.1. Gel De-staining and In-Gel Protein Digests

Proteins of interest were cut from polyacrylamide gels and prepared for mass spectral analysis using the procedure described by Lin (Lin *et al.*, 2008), based originally on the protocol developed by Shevchenko (Shevchenko *et al.*, 1996). Bands of interest were cut from the polyacrylamide gel, transferred to 1.5 ml tubes and incubated in 1 ml de-stain overnight at 4 °C. This de-stain solution was removed before incubating the gel pieces, for thirty minutes with occasional vortexing, in an equal volume (approximately 50 µl) of 50 mM ammonium bicarbonate (pH $\approx$ 8.0) and acetonitrile. This wash step was repeated until either the gel pieces had completely de-stained, or until no further destaining was achievable. Gel pieces were then dehydrated by incubating at room temperature in 500 µl of acetonitrile, until the gel piece became white and smaller in size.

After this dehydration step, the gel pieces were rehydrated with 2.5 µl of 10 µg.ml⁻¹ TPCK treated trypsin. After thirty minutes, additional protease was added if the previous aliquot had become completely absorbed into the gel pieces. The samples were incubated for another ninety minutes, at 4 °C, before applying enough ammonium bicarbonate (25 mM, pH $\approx$ 8.0) to just cover the gel pieces. The samples were then incubated overnight at 37 °C.

2.4.2. Peptide Extraction

De-stained, protease-treated, gel pieces were incubated in 25 µl of 50% (v/v) acetonitrile containing 5% (v/v) trifluoroacetic acid for thirty minutes, with agitation. The supernatant was then transferred to a fresh tube before repeating the extraction step. Both supernatants were combined and subsequently dried using an Integrated SpeedVac System ISS100 set to a low heat. The resulting powder was stored at -80 °C until use. Immediately prior to sample spotting (see below), the powdered protein

sample was dissolved in 5 μl of 5% (v/v) acetonitrile (diluted in 25 mM ammonium bicarbonate) containing 0.1% (v/v) trifluoroacetic acid.

2.4.3. Sample Spotting

Samples to be analyzed by mass spectrometry were spotted onto the target plate using the dried-droplet method (Karas & Hillenkamp, 1988). For this procedure, 1 μl of sample solution was mixed with 1 μl of the matrix solution α -cyano-4-hydroxycinnamic acid (α -CHCA), (10 $\text{mg}\cdot\text{ml}^{-1}$, in 60% v/v acetonitrile, 0.1% v/v trifluoroacetic acid). This solution was then pipetted directly onto the target plate and allowed to dry.

2.4.4. External Calibration of the Mass Spectrometer

Half a micro litre of a calibrant mixture, a one to one solution of Applied Biosystems Calibrant Mixture 1 and Calibrant Mixture 2, was mixed with an equal volume of α -CHCA and spotted next to each sample spot, on the target plate, using the dried droplet method as described above. The components of the calibrant mixtures are given in table 2.8. Once all sample, and calibrant, spots had fully crystallized the target plate was placed inside the mass spectrometer, a Voyager-DE PRO MALDI TOF system, from Applied Biosystems.

The machine was allowed to “warm-up” for approximately twenty minutes after activating the high voltage, after which time the calibrant spot was irradiated with the nitrogen laser set to a manual laser intensity of 800. Mass data was collected in reflector positive mode, using an accelerating voltage of 25,000 V, grid and guide wire voltages of 76.1% and 0.002% respectively, and a delay time of 125 ns. The mass range was limited to between 800 Da and 4,000 Da, with the low mass gate adjusted to prevent masses below 750 Da reaching the detector. Each spectrum was collected from two hundred laser shots from each of five areas of the calibrant spot; the final mass spectrum was, therefore, compiled from the acquisition of one thousand spectra.

Calibrant spectra were analyzed using version 4 of Data Explorer, from Applied Biosystems, and processed as recommended by the manufacturers. Briefly, the mass spectrum was first subjected to the standard baseline correction feature of the software, before de-isotoping the calibrant peaks. The peaks were then calibrated, with no peak weighting factor applied, by matching the masses of the detected peaks to the known masses of the calibrant mixture. Before applying the calibration, it was manually ensured that all the detected masses of the modified spectrum were within 0.05 Da of the known mono-isotopic masses, shown in table 2.8.

An external calibration was used for each sample to be analyzed. An example of the mass spectrum of the calibrant mixture is shown in figure 2.3. Using the method of calibration described here, it was found to be unnecessary to employ internal calibration procedures.

Table 2.8. Components of Calibration Mixtures 1 and 2

Calibration Mixture	Component	Charge	Mass (average)	Mass (mono-isotopic)
Mixture 1	des-Arg1-Bradykinin	+1	905.05	904.4681
	Angiotensin I	+1	1297.51	1296.6853
	Glu1-Fibrinopeptide B	+1	1571.61	1570.6774
	Neurotensin	+1	1673.96	1672.9175
Mixture 2	Angiotensin I	+1	1297.51	1296.6853
	ACTH (clip 1-17)	+1	2094.46	2093.0867
	ACTH (clip 18-39)	+1	2466.72	2465.1989
	ACTH (clip 7-38)	+1	3660.19	3657.9294
	Insulin (bovine)	+1	5734.59	5730.6087
		+2	2867.80	2865.8083

2.4.5. Acquisition of Mass Spectra

Spectra were obtained by irradiating sample spots with a manual laser intensity set between 600 and 1500. A complete spectrum of each sample was obtained by combining a total of one thousand spectra, collected from five spots on the sample. Spectra were collected in reflector positive mode, with accelerating voltage, grid and guide wire voltages, identical to those used for acquiring calibrant spectra. The mass range analyzed was between 800 Da and 4,000 Da, with the low mass gate set to prevent masses below 750 Da reaching the detector, as during calibration.

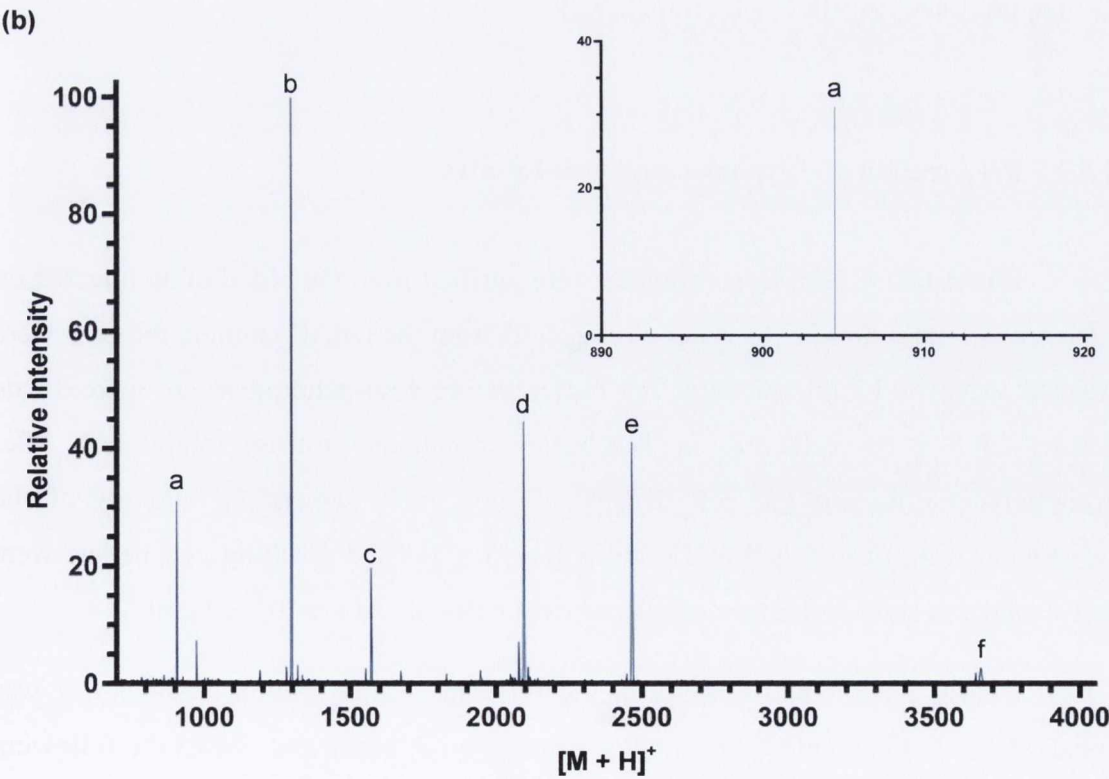
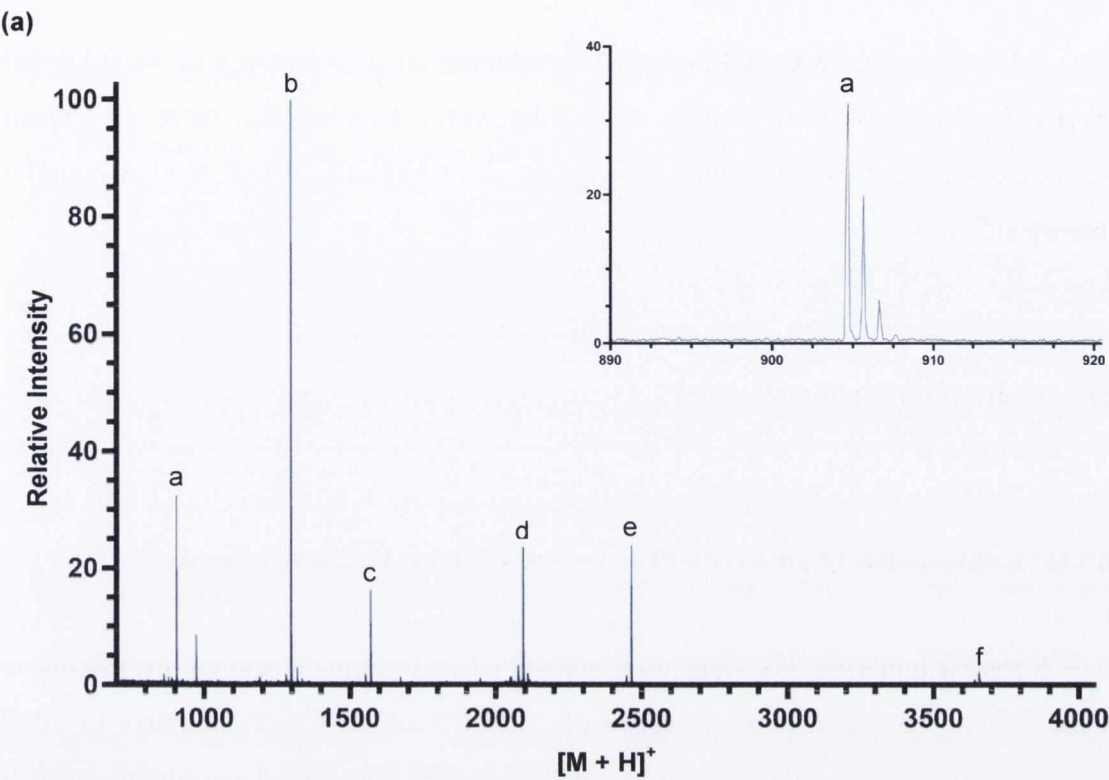
Once collected, the spectra were processed as follows. Initially, a default noise filtering was performed, prior to setting the peak detection limits low enough to ensure detection of monoisotopic peaks; the “%max peak area” was set to zero. The peaks were subsequently de-isotoped using the software’s de-isotoping function (see figure 2.3). The resulting data were analyzed as described below.

2.4.6. Peptide Mass Fingerprinting

Once raw mass spectra had been processed, as described in section 2.7.5., the mono-isotopic peak masses were entered into the MS-Fit database searching program on the ProteinProspector (versions 5.2.2 to 5.10.2 were used) web package (<http://prospector.ucsf.edu/>). Typically, only peak masses that had a relative intensity of 5% were entered, although when appropriate, and where mentioned in the relevant results section, masses of at least 2% or 10% relative intensity were entered. Usually, no constant or variable modifications of the peptides were selected (sometimes it was necessary to allow the mass of oxidized methionine residues to be included in the searching algorithm), and the “max missed cleavages” was set between two and three. In the first instance of searching, the search was not limited to any particular taxonomy, although in some cases searching had to be limited to the appropriate species, as described in results. The number of peptides required to match the predicted trypsin digests of proteins in the SwissProt database, within a mass tolerance of 0.1 to 0.2 Da, was dependent on the mass spectrum obtained.

Figure 2.3. Typical Calibrant Spectrum for MALDI-ToF MS

A mass spectrum of the calibrant mixture was obtained by combining the spectra from each of 1,000 laser shots, as described in section 2.4.4. The spectrum prior to any processing is shown in (a), with the peaks labelled a, b, c, d, e and f, corresponding to des-Arg1-Bradykinin, Angiotensin I, Glu-1-Fibrinopeptide B, ACTH (clip 1-17), ACTH (clip 18-39) and ACTH (clip 7-38) respectively. The spectrum after the described processing is shown in (b). The inset spectrum in each panel is focused on the des-Arg1-Bradykinin fragment, to show more clearly the effect of peak processing on the spectrum; in particular, peaks have been reduced to a single, mono-isotopic species. A mono-isotopic peak corresponds to the first peak mass in a family of peaks, and is usually the most abundant mass present in that family (*i.e.* the intensity of this mass detected is greatest). Each mass peak in a family of peaks is different in mass by a single neutron (*i.e.* one atomic mass unit).



2.4.7. Validation of MALDI-ToF Protocol

The protocol presented here for the identification of proteins by MALDI-ToF mass spectrometry was verified by testing the protocol on samples of in-gel trypsin-digested cytochrome c, ovalbumin, and bovine serum albumin (BSA), and is covered in chapter 3.

2.5. Immunoprecipitations

2.5.1. Conjugation of anti-GPI-PLC IgG to Protein-A Agarose Beads

Rabbit anti-GPI-PLC IgG was conjugated to Protein-A agarose by mixing an equal volume of antibody solution (50 μ l) with a Protein-A agarose slurry (a 50:50 suspension of Protein-A agarose beads in PBS, which was mixed thoroughly prior to use). The mixture was incubated overnight at 4 °C, with gentle agitation. Unbound antibody was washed free of the Protein-A beads by washing the beads twice with 500 μ l of appropriate lysis buffer (section 2.5.2).

2.5.2. Preparation of Trypanosomal Cell Lysates

Bloodstream form trypanosomes were purified from the blood of an infected rat as described in section 2.2.6. After eluting cells from the DEAE column, the cells were washed twice in 15 ml standard Tes buffer before resuspending to an approximate density of 5×10^9 cells·ml⁻¹ in Tes buffer containing protease inhibitors. After accurately determining the cell density of this stock suspension, aliquots of the suspension were centrifuged at 18,000 *g* for 45 s and the resulting cell pellets were resuspended in lysis buffer (see below) to yield a density of 1×10^9 cells·ml⁻¹.

Unless specified otherwise, in the relevant results section, lysis buffer was standard Tes buffer (table 2.1) containing protease inhibitors and one of the following detergents: n-octyl- β -D-glucopyranoside (n-octylglucoside; 20 mM or 30 mM), n-

dodecyl-β-D-maltoside (0.1 mM or 0.8 mM), Triton® X-100 (approximately 0.2 mM or 1.2 mM), 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; 6 mM, or 12 mM), SDS (7 mM or 12 mM), or cetyltrimethylammonium bromide (CTAB; 1 mM or 1.5 mM). Trypanosomes were lysed for twenty minutes, on ice, before centrifuging at 15,000 g, at 4 °C, and keeping the supernatant for immunoprecipitation experiments. Samples of these supernatants were also taken for SDS-PAGE analysis. Relevant information of each of these detergents is shown in table 2.9.

Trypanosome lysates to be used for immunoprecipitations were always prepared fresh, immediately prior to use, and were never frozen. Whichever lysis buffer was used to lyse cells was used to wash Protein-A agarose beads free of unbound anti-GPI-PLC IgG.

Table 2.9. Detergents used for GPI-PLC Immunoprecipitation

Detergent	MW	CMC (mM)	Concentrations Used	Concentrations Used (% w/v)	Class
n-Octylglucoside	292.4	20 – 25	20 mM, 30 mM	0.59%, 0.88%	Non-ionic
n-Dodecyl-β-D-maltoside	510.62	0.1 – 0.6	0.1 mM, 0.8 mM	0.005%, 0.04%	Non-ionic
Triton® X-100	650*	0.2 – 0.9	0.2 mM, 1.2 mM*	0.01%, 0.08%	Non-ionic
CHAPS	614.9	6.0 – 10	6 mM, 12 mM	0.37%, 0.74%	Zwitterionic
SDS	288.4	7.0 – 10.0	7 mM, 12 mM	0.20%, 0.35%	Anionic
CTAB	364.5	1.0	1 mM, 1.5 mM	0.04%, 0.06%	Cationic

* An average molecular weight of 650 was used for calculating the molarities of Triton® X-100 solutions used.

2.5.3. Immunoprecipitation of the GPI-PLC

The GPI-PLC was immunoprecipitated from freshly prepared cell lysates by incubating 1 x 10⁹ cell equivalents with 25 µl of anti-GPI-PLC conjugated Protein-A

agarose, overnight, at 4 °C and with gentle agitation. The samples were then centrifuged at 5,000 *g* for thirty seconds and the resulting pellet was washed twice in 500 µl of the same lysis buffer that was used to prepare the cell lysate. After the second wash, the agarose beads were gently resuspended in 20 µl of distilled, de-ionised, water and 5 µl of 5X sample buffer was added before heating the mixture at 70 °C for five minutes. Immunoprecipitated material was stored at -20 °C until required. Before samples were loaded on polyacrylamide gels, it was necessary to add a few crystals of sucrose to the immunoprecipitate in order to ensure the solution was dense enough to sink in the running buffer.

2.6. Experimental Release of the Variable Surface Glycoprotein

2.6.1. VSG Release Induced by Osmotic Shock

Bloodstream form cells were washed twice in standard Tes buffer (table 2.1.1). After the second wash in buffer, cells were centrifuged at 18,000 *g* for thirty seconds and the resulting pellet was resuspended, to a density of 2.5×10^8 cells.ml⁻¹, in water that contained protease inhibitors (table 2.1.2) and 30 mM Tes. The cell suspension was incubated for twenty minutes, at room temperature, before centrifuging at 18,000 *g* for thirty seconds and resuspending the resulting pellet in Tes buffer to achieve a density of 2.5×10^8 cell equivalents per millilitre. The resuspended pellet and the saved supernatant were prepared for SDS-PAGE as described in section 2.3.2 before storing at -20 °C until ready for gel electrophoresis.

2.6.2. VSG Release Induced by De-energization

De-energization of cells was carried out with slight modifications of the procedures described by Voorheis *et al.* (1982) and Hanrahan *et al.*, (2009). Bloodstream form trypanosomes were washed twice in hyper-osmotic Tes buffer (table 2.6.1) before re-suspending in hyper-osmotic Tes buffer, containing protease inhibitors,

to a density of 5×10^9 cells.ml⁻¹. Cells were then diluted 1 in 5 into the appropriate incubation buffer (see table 2.6.1) and maintained at 37 °C with gentle stirring.

Trypanosomes were incubated in buffer (de-energizing Tes buffer, or energizing Tes buffer) for two hours, with 250 µl samples taken at appropriate time points and cold-quenched in 1.25 ml of ice-cold hyper-osmotic Tes buffer. These samples were immediately centrifuged at 18,000 *g* for forty five seconds and the pellet kept and prepared for SDS-PAGE as described earlier. The supernatant was further centrifuged at 15,000 *g* for fifteen minutes to minimize contamination by insoluble cellular material. Aliquots of the resulting supernatant were prepared for SDS-PAGE, as described, with the remaining supernatant stored at -20 °C for later measurement of protein content.

Table 2.6.1. De-energization Buffer Recipes

	Hyper-osmotic Tes Buffer	De-energizing Tes Buffer	(Hyper-osmotic) Energizing Tes Buffer
NaCl	140 mM	140 mM	140 mM
KCl	4 mM	4 mM	4 mM
Tes	30 mM	30 mM	30 mM
Sucrose	150 mM	150 mM	150 mM
Glucose	---	---	10 mM
2-Deoxy- D -glucose*	---	25 mM	---
EGTA*	---	5 mM	---
Calcium acetate*	---	0.1 mM	---
A-23187*†	---	5 µg.ml ⁻¹	---
pH	7.5	7.5	7.5

* 2-deoxy-**D**-glucose, EGTA, calcium acetate, and the calcium ionophore A-23187, were added only when described in the relevant results section.

† A-23187 was initially prepared as a stock 5 mg·ml⁻¹ solution in DMSO.

2.7. Microscopic Techniques

2.7.1. Preparation of Cover-Slips

Prior to use, cover-slips were bathed in 0.01% (w/v) poly-L-lysine solution for ten minutes at room temperature. The cover-slips were then dried in an oven before painting the edges of the cover-slips with a thin layer of nail varnish, diluted appropriately with ethyl acetate, to maintain enough space between the cover slip and the slide to prevent damage to the cells.

2.7.2. Preparation of Paraformaldehyde Solution

Paraformaldehyde (6%, w/v) solution was prepared by adding the required mass of paraformaldehyde to five volumes of water. This mixture was shaken vigorously while adding, in a drop-wise manner, 5 M NaOH, until the paraformaldehyde had completely dissolved. The required volume of 10X phosphate buffered saline (PBS, see table 2.1.1.) was then added and the pH adjusted back to 7.4 using 12 M HCl. Finally, the solution was brought to the required volume before finally checking the pH and titrating slightly if appropriate. Paraformaldehyde solution was always prepared freshly on the day of use.

2.7.3. Cell Fixation

Cells were harvested from culture and washed twice by successive centrifugation (1,500 *g*, five minutes, 4 °C) and resuspension of the pellet in ice-cold PBS. Cell suspensions were adjusted to 1×10^8 cells. ml^{-1} before adding an equal volume of ice-cold 6% (w/v) paraformaldehyde solution and incubating on ice for ten minutes. Fixation was quenched by adding an equal volume of PBS containing 0.002% (w/v) NaN_3 and additionally containing 0.2 M glycine. Fixed cells were centrifuged at 18,000 *g* for thirty seconds, resuspended in PBS containing 0.02% (w/v) NaN_3 and brought to a density of approximately 5×10^7 cells. ml^{-1} before storing at 4 °C.

2.7.4. Nuclear Staining and Mounting Cover-Slips

Fixed trypanosomes were pipetted (25 μ l) onto the centre of a poly-L-lysine-coated cover-slip and allowed to adhere for ten to fifteen minutes. The cover-slips were then gently washed with PBS; excess PBS was blotted dry after washing. Approximately 2 - 3 μ l of anti-fade solution was applied onto the centre of the cover-slip before immediately inverting the cover-slip onto a glass slide and sealing the edges with nail varnish. Anti-fade solution was 50% (w/v) glycerol in PBS containing 4% (w/v) propyl gallate, 0.02% (w/v) NaN_3 and 1 $\mu\text{g}\cdot\text{ml}^{-1}$ Hoechst 33352. Completed cover-slips were stored at 4 °C in the dark until viewing.

2.7.5. Image Acquisition

Slides were viewed manually using an inverted fluorescent Axiovert 100TV Zeiss microscope over a 63X oil immersion objective. Phase contrast images were captured using an AxioCam MR camera (from Zeiss) and processed with AxioVision (version 3.1) software. Excitation wavelength (350 nm) for viewing Hoechst 33342 staining was provided by a multi-line Argon laser. All images were collected using the default software settings.

2.8. Miscellaneous Techniques

2.8.1. Purification of Rabbit anti-GPI-PLC IgG from Immune Serum

Rabbit serum containing anti-GPI-PLC IgG was already available in the laboratory, and had been prepared as described (Hanrahan *et al.*, 2009). Approximately 1.0 ml of this serum was mixed with an equal volume of column buffer (0.1 M Tris, 0.15 M NaCl, pH 8.0) and then applied to a Protein-A agarose column that had been previously equilibrated with column buffer. After the serum had entered the column, the column was washed thoroughly with column buffer before eluting bound antibody with 0.1 M glycine (pH 3.0). Eluted material was collected in an equal volume of column

buffer and the absorbance, at 280 nm, of each collected fraction was monitored (figure 2.4). Fractions containing suitable quantities of protein were combined. The final sample was aliquoted appropriately before storing at -20 °C. Once frozen, no aliquot of anti-GPI-PLC antibody was thawed for use more than twice.

2.8.2. Protein Concentration Assay

Protein concentration was measured using the Pierce 660 nm protein assay reagent. The reagent was used as directed by the manufacturer. Briefly, in a 96 well plate, 10 µl of sample was mixed with 150 µl of Pierce 660 nm protein assay reagent and the sample was incubated for thirty minutes at room temperature. The absorbance of the resulting solution was then measured at 660 nm. Absorbance measurements were compared to a BSA standard curve for determining protein concentration in the case of most protein mixtures, but a trypsin inhibitor standard curve was used for determining IgG concentration (as recommended by the manufacturer). Sample standard curves are shown in figure 2.5.

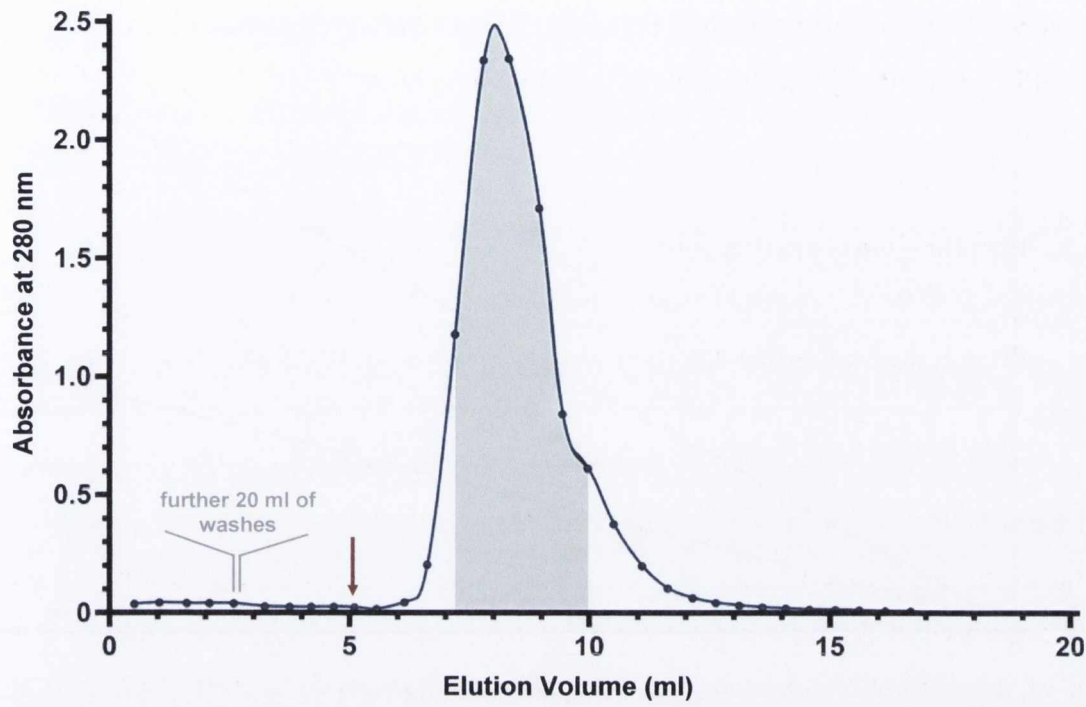


Figure 2.4. Elution of anti-GPI-PLC IgG from Protein-A Agarose

Anti-GPI-PLG IgG was purified from rabbit serum by passage through a DEAE column as described in section 2.8.1. After washing the Protein-A resin with 50 ml of column buffer the absorbance of subsequent fractions was monitored at 280 nm (shown above). Bound immunoglobulin was eluted with 0.3 M glycine (pH 3.0), (application is highlighted with a red arrow). Eluted fractions containing sufficient amounts of protein (shaded green above) were pooled before aliquoting and storing at -20 °C.

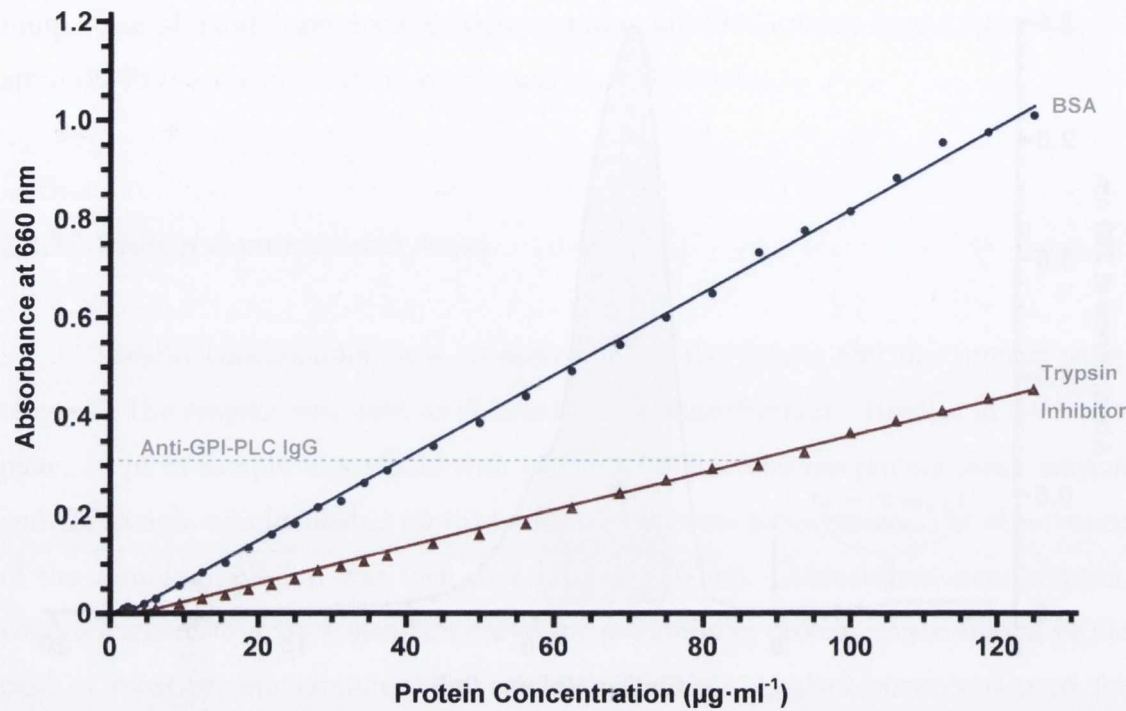


Figure 2.5. Standard Curves for the Pierce 660 nm Protein Assay Reagent

Dilutions of BSA and Trypsin Inhibitor were prepared from 2 mg·ml⁻¹ stock solutions (made in water), covering the range 20 µg·ml⁻¹ to 2 mg·ml⁻¹. 10 µl of each dilution was then added to 150 µl of the Pierce 660 nm protein assay reagent, mixed for thirty minutes and the absorbance was then read at 660 nm. A BSA standard curve was used for determining trypanosomal protein concentration during VSG release experiments (chapter 4). The trypsin inhibitor standard curve was used to determine the concentration of purified anti-GPI-PLC. The mean absorbance of three separate measurements of anti-GPI-PLC antibody (mixed with the Pierce protein assay reagent) gave an absorbance at 660 nm of 0.3112. This absorbance value corresponded to a protein concentration of 86.73 µg·ml⁻¹ present in the assay wells and therefore reflects a concentration of the stock anti-GPI-PLC IgG of approximately 1.4 mg·ml⁻¹.

2.8.3. Protein Labelling with NHS-LC-Biotin and sulfo-NHS-LC-Biotin

Labelling trypanosomal protein with NHS-LC-biotin and sulfo-NHS-LC-biotin was carried out essentially as recommended by the manufacturer. As described in the relevant results sections, bloodstream form trypanosomes were washed twice in appropriate buffer (PBS, TSB, or Tes buffer; Tris buffer was never used) and adjusted to cell densities between 2.5×10^7 cells·ml⁻¹ and 1×10^9 cells·ml⁻¹ (as described). Stock solutions (200 mM) of NHS-LC-biotin and sulfo-NHS-LC-biotin were prepared immediately prior to use in DMSO and buffer respectively (note, 200 mM labelling reagent corresponds approximately to 3 mg in 33 µl DMSO for NHS-LC-biotin, and 4 mg in 36 µl buffer for sulfo-NHS-LC-biotin). Stock solutions of labelling reagent were then diluted 1 in 100 into the cell suspensions, which were then incubated for twenty minutes on ice, or at 37 °C as described in the relevant results sections.

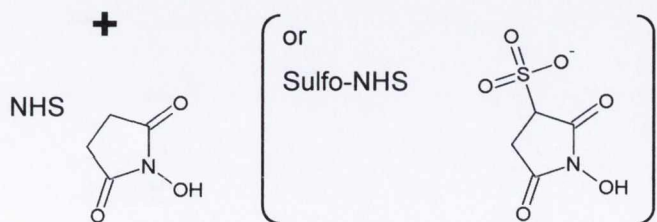
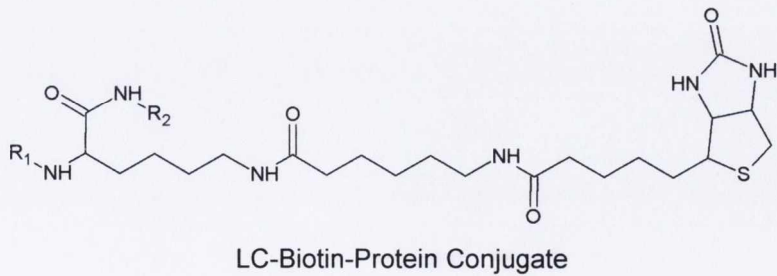
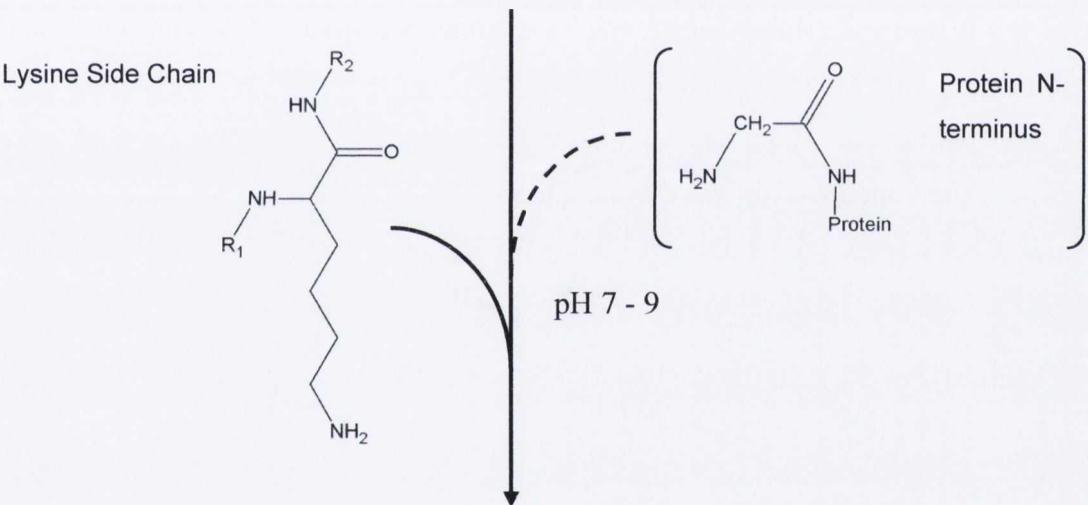
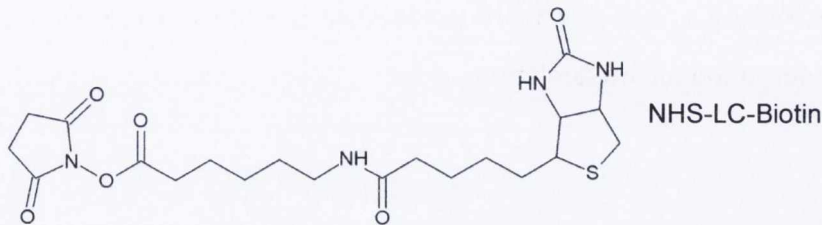
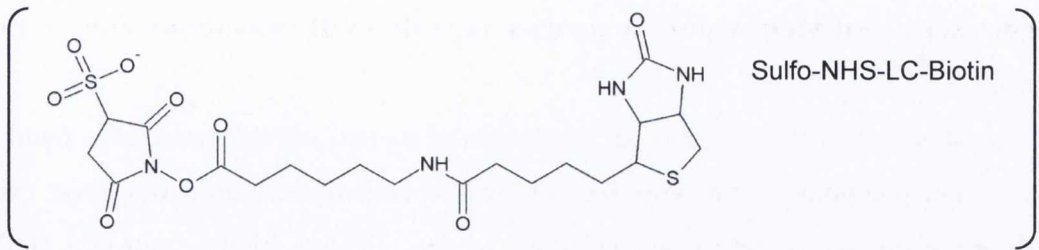
The labelling reaction (which is illustrated in figure 2.6) was quenched by the addition of an equal volume of buffer containing 100 mM glycine. Labelled cells were then washed twice in buffer containing 100 mM glycine and adjusted to a density of 1×10^8 cells·ml⁻¹ before boiling in the presence of 5X sample buffer (in the usual fashion), sonicating, and finally storing at -20 °C until use.

2.8.4. ECL Detection of Biotin-Labelled Proteins

Samples of labelled trypanosomes were electrophoresed on polyacrylamide gels, transferred to nitrocellulose membranes, stained with Ponceau S and then briefly washed, precisely as outlined for Western blotting in section 2.3. After blocking non-specific binding sites using 5% (w/v) marvel in the usual fashion, biotin-labelled proteins were detected using peroxidase-conjugated streptavidin. Peroxidase-conjugated streptavidin was diluted 1 in 10,000 (yielding a final concentration of 100 ng·ml⁻¹) in TBST containing 1% (w/v) marvel, and allowed to react with the blot for one hour, at room temperature, with gentle agitation. The peroxidase-conjugated streptavidin solution was then poured off before washing the nitrocellulose membrane with six changes of TBST, over half an hour, and finally detecting the peroxidase conjugate as outlined in section 2.3.10.

Figure 2.6. Structures and Reactions of Sulfo-NHS-LC-Biotin and NHS-LC-Biotin

The reactions of sulfo-NHS-LC-biotin and NHS-LC-biotin with protein primary amines are shown. Lysine side chains and the protein N-terminus react with the NHS ester to yield a stable LC-biotin-protein conjugate *via* the formation of an amide bond and the release of NHS (or sulfo-NHS). The reaction occurs from physiological to slightly basic pH, however there are marked increases in the rate of hydrolysis of the NHS ester as the pH increases.



2.8.5. Statistical Methods

Where necessary experimental data was plotted as the mean of a number of repeated experiments, with error bars showing either the standard deviation (SD) or standard error of the mean as described in the relevant results section. Both the standard deviation and the standard error of the mean (SE) were calculated as described (Cumming *et al.*, 2007), and shown below.

For the standard deviation:

$$SD = \sqrt{\frac{\sum(X - M)^2}{n - 1}}$$

Where n is the number of repeat experiments carried out, X represents each data point value, and M is the mean of the data set.

The standard error was calculated as:

$$SE = \frac{SD}{\sqrt{n}}$$

2.9. Materials

2.9.1. Sources of General Materials and Chemicals

The sources of each of the chemicals and materials used throughout this work are listed alphabetically below. Where appropriate, the grade and/or purity of the material is also shown.

<u>Name of Material</u>	<u>Grade and/or Purity</u>	<u>Supplier</u>
A-23187	≥ 98%	Sigma-Aldrich
Acetic acid	100%	Riedel-de Haën*
Acetonitrile	HPLC	Lab Scan
Acrylamide	99.9%	Melford
bis-Acrylamide	> 99%	Melford
Activated charcoal	100%	Duchefa Biochemie
Adenosine	> 98%	Duchefa Biochemie
Ammonium bicarbonate	≥ 99%	Melford
Ammonium persulphate	> 98%	Melford
Benzyl alcohol	> 99%	Sigma-Aldrich
Bovine serum albumin	≥ 97%	Sigma-Aldrich
Bromophenol blue	‘Electran’	BDH†
Butanol	GPR	HMF‡
Calcium acetate	N/A	N/A
CHAPS	> 98%	Melford
CTAB	≥ 98%	Merck
α-CHCA	N/A	Applied Biosystems
p-CMPS		Sigma-Aldrich
Coomassie brilliant blue R	≈ 50%	Sigma-Aldrich
Cytochrome c	> 99%	Sigma-Aldrich
DE-52 (DEAE cellulose)	N/A	Whatman§
2-Deoxy-D-glucose	≥ 98%	Carbosynth
DMF	> 99%	Fluka*
DMSO (anhydrous)	≥ 99.9%	Sigma-Aldrich

n-Dodecyl- β -D-maltoside	> 99%	Melford
EGTA	N/A	Melford
Ethanol	GPR	HMF‡
Foetal bovine serum	N/A	Biosera
Glucose	$\geq 99.5\%$	Sigma-Aldrich
Glycerol	$\geq 99\%$	Sigma-Aldrich
Glycine	> 98.5%	Duchefa Biochemie
Guanosine	N/A	Carbosynth
HMI-9 medium	N/A	Gibco§
Hoechst 33342	N/A	Molecular Probes
Hydrochloric acid	37%	Sigma-Aldrich
Hydrogen peroxide solution	30% (w/w)	Sigma-Aldrich
Hypoxanthine	$\geq 97\%$	Melford
Iodophenol	99%	Sigma-Aldrich
Kanamycin	> 750 IU.mg ⁻¹	Duchefa Biochemie
Leupeptin hydrochloride	$\geq 90\%$	Sigma-Aldrich
Luminol sodium salt	$\geq 98\%$	Sigma-Aldrich
Magnesium chloride	> 98%	Duchefa Biochemie
Marvel (powdered milk)	N/A	Purchased locally
Methanol	GPR	HMF‡
2-Mercaptoethanol	99%	Riedel-de Haën*
NHS-LC-Biotin	N/A	Thermo Scientific§
Nitrocellulose membrane	Protran (BA83)	Whatman§
n-Octylglucoside		Melford
Ovalbumin	$\approx 99\%$	Sigma-Aldrich
Paraformaldehyde	97%	BDH†
Pentobarbitone (Euthatal)	N/A	BioresourcesI
Pepstatin A	$\geq 90\%$	Sigma-Aldrich
Peptide standards	N/A	Applied Biosystems
Peroxidase-conjugated streptavidin	N/A	Merck
Pierce 660 nm protein assay reagent	N/A	Thermo Scientific§
PMSF	> 99%	Merck
Poly-L-lysine solution	0.1% (w/v)	Sigma-Aldrich
Ponceau S	Practical Grade	Sigma-Aldrich

Potassium chloride	> 99%	Duchefa Biochemie
Propyl galate	≥ 98%	Fluka*
Protein-A agarose	N/A	Merck
Protein standards	N/A	Sigma-Aldrich and Thermo Scientific§
SDM-79	N/A	Gibco§
SDS	> 99%	Melford
Sodium azide	≥ 95.5%	Sigma-Aldrich
Sodium bicarbonate	> 99.7%	Melford
Sodium chloride	> 99%	Duchefa Biochemie
Sodium hydroxide	98%	Sigma-Aldrich
Sodium phosphate dibasic	> 98%	Duchefa Biochemie
Sodium phosphate monobasic	99%	Riedel de-Haën*
Sorbitol	≥ 98%	Sigma-Aldrich
Sucrose	> 99.7%	Duchefa Biochemie
Sulfo-NHS-LC-biotin	N/A	Thermo Scientific§
TEMED	> 99%	Melford
TLCK	≥ 99%	Sigma-Aldrich
Tes	> 99%	Melford
Tris	> 99.9%	Duchefa Biochemie
Triton® X-100	‘Laboratory Grade’	Sigma-Aldrich
Trypsin (TPCK-treated)	13.8 kU.mg ⁻¹	Sigma-Aldrich
Trypsin Inhibitor		Sigma-Aldrich
Tween 20	‘General Use’	Sigma-Aldrich

* Riedel-de Haën and Fluka are now part of the Sigma-Aldrich group; the products listed here from Riedel-de Haën and Fluka are therefore best sourced from Sigma-Aldrich.

† BDH (British Drugs Houses) is now part of The Merck Group (Merck).

‡ Butanol, ethanol and methanol were supplied by the on-campus hazardous materials facility (HMF) service.

§ Products from Whatman and Thermo Scientific were sourced via the distributors Van Waters & Rogers (VWR), Medical Supply Company (MSC) or from Fisher Scientific. Products from Gibco were sourced through Invitrogen.

|| Bioresources is an on-campus facility which provides services and products relevant to work with animals.

2.9.2. Sources of Antibodies

Rabbit IgG against *T. brucei* AAT was the kind gift of Dr. D. Nolan (School of Biochemistry and Immunology, Trinity College Dublin).

Rabbit immune serum against *T. brucei* GPI-PLC was generously provided by Dr. Mark Carrington (Department of Biochemistry, University of Cambridge).

Peroxidase-conjugated goat IgG raised against the Fc fragment of rabbit IgG was purchased from Jackson ImmunoResearch.

2.9.3. Sources of Tissue Culture Consumables

All sterile pipettes, and culture flasks, were sourced from Starstedt. Stericup filters (0.22 μ m pore filter units with a polyethersulfone membrane) for sterilization of media were purchased from Millipore (part of The Merck Group).

2.9.4. Contact Information for Suppliers

The contact information for each material supply company is listed below.

Applied Biosystems		Biosera	
Website:	www.appliedbiosystems.com	Address:	Biosera Ltd – Head Office
Email:	uk.orders@eur.appliedbiosystems.com		1 Acorn House
			The Broyle
Phone:	0800 5150840		Ringmer
			East Sussex, UK
		Website:	www.biosera.com
		Email:	info@biosera.com
		Phone:	+44 1273 814466

Carbosynth

Address: Carbosynth Limited
8 & 9 Old Station Business Park
Comptom
Berkshire
RG20 6NE
UK

Website: www.carbosynth.com
Email: sales@carbosynth.com
Phone: +44 (0)1635 578444

Duchefa Biochemie

Website: www.duchefa.com
Email: info@duchefa.nl
Phone: +31(0) 23 531 9093

Fisher Scientific

Address: Fisher Scientific Ireland
Suite 3, Plaza 212
Blanchardstown Corporate Park 2
Ballycoolin
Dublin 15

Website: www.ie.fishersci.com
Email: fsie.sales@thermofisher.com
Phone: +353 (0)1 885 5854

Invitrogen (Includes Gibco and Molecular Probes)

Website: www.invitrogen.com
Email: europeservices@lifetech.com

Jackson ImmunResearch

Address: Jackson ImmunResearch Europe Ltd.,
Unit 7
Acorn Business Centre
Oaks Drive, Newmarket
Suffolk
UK CB8 7SY

Website: www.jieurope.com/home.asp
Email: cuserv@jieurope.com
Phone: +44 (0)1638 782616

Lab Scan

Address: Poch S.A.
ul. Sowinskiego 11
44-101 Gliwice
Poland

Website: www.labscan.eu
Email: export@poch.com.pl
Phone: +48 32 2392181

Medical Supply Company

Address: Damastown
Mulhuddart
Dublin 15
Ireland

Website: www.medical-supply.ie
Email: info@medical-supply.ie
Phone: +353 (0)1 822 4222

Melford

Address: Melford Laboratories Ltd.
Bildeston Road
Chelsworth, Ipswich
Suffolk
IP7 7LE, UK

Website: www.melford.co.uk
Email: support@melford.co.uk
Phone: +44(0) 1449 74117

Merck (Includes Calbiochem and Millipore)

Address: MERCK SERONO
Citywest Business Campus
Dublin 24
Ireland

Website: www.merckgroup.com
Email: enquires_ie@merckserono.net
Phone: +353 (0)1 468 7590

Sarstedt

Address: Sarstedt Ltd.
Sinnottstown Lane
Drinagh
Wexford
Ireland

Website: www.sarstedt.com
Email: info@sarstedt.com
Phone: +353 (0)53 914 4922

Sigma-Aldrich

Address: Sigma-Aldrich Ireland Ltd.
Arklow
Ireland

Website: www.sigmaaldrich.com
Email: EIRCustSupport@sial.com
Phone: +353 (0)402 20370

Thermo-Scientific

Website: www.thermoscientific.com

Van Waters & Rogers (VWR)

Address: VWR International Ltd.
Orion Business Campus
Northwest Business Park
Ballycoolin
Dublin 15
Ireland

Website: ie.vwr.com
Email: info@ie.vwr.com
Phone: +353 (0)1 882 2332

Whatman

Website: www.whatman.com
Email: custservuk@ge.com
Phone: 1800 709 992

Chapter 3

GPI-PLC Interactions with Possible Partner Proteins

3.1. Introduction

3.1.1. The Localization and Regulation of the GPI-PLC Suggest it may interact with other Proteins

Two particular puzzles challenge our current understanding of the GPI-PLC. Although we can now be confident that the GPI-PLC is specifically localized in a linear array along the flagellar attachment zone, with at least some of the enzyme on the exterior of the cell, it is not understood how such a specific localization can be explained in the absence of any appropriate, recognizable, signal sequence in the enzyme's primary structure. Additionally, even though the GPI-PLC has been shown to behave like an integral membrane protein (Bülow and Overath, 1986, Fox *et al.*, 1986), the enzyme does not contain any region of sufficient hydrophobicity to allow for a plausible transmembrane domain. In the absence of these standard features, usually necessary for inserting an integral membrane protein into the cell membrane, it must be asked how the GPI-PLC associates itself with the trypanosomal membrane, particularly in such a specific fashion.

The second puzzle also arises from the GPI-PLC's localization. How can the GPI-PLC be found in the same cellular region as its substrate and yet not act on this substrate until particular conditions, such as hypotonic lysis of the cell, are met? Essentially, what normally prevents the GPI-PLC from shedding the protective VSG coat? Although many experimental conditions have been shown to induce GPI-PLC mediated release of the VSG, little is known about how the GPI-PLC is normally held in an inactive state.

The presence of one or more accessory proteins provides one potential solution to both of these puzzles. An accessory protein, for example, may interact with the GPI-PLC to regulate its ability to hydrolyze the VSG's GPI-anchor. This same accessory protein, or a second separate protein, may also be responsible for tethering the GPI-PLC to the flagellar membrane.

Results published earlier by this laboratory have shown that immunoprecipitation of the GPI-PLC from trypanosomal cell lysates, using a polyclonal rabbit IgG, results in the co-immunoprecipitation of numerous other proteins (Hanrahan *et al.*, 2009). Detection of some of these co-immunoprecipitated bands by autoradiography, after iodination of cell surface proteins, showed that at least some of the co-immunoprecipitated material represents surface proteins. These proteins were not co-immunoprecipitated when pre-immune rabbit serum was used in place of the rabbit anti-GPI-PLC antibody, nor were these proteins detected when the same immunoprecipitation procedure was undertaken with GPI-PCL^{-/-} cells. The surface iodinated proteins are therefore likely to have co-immunoprecipitated *via* an interaction with the GPI-PLC, rather than through non-specific interactions with the anti-GPI-PLC immunoglobulin or Protein-A conjugated Sepharose beads. But these co-immunoprecipitated proteins were never identified, so it currently remains unknown what proteins could be involved in the regulation and localization of the GPI-PLC of bloodstream form trypanosomes.

3.1.2. Aims

The ultimate aim of this chapter is to identify any proteins that may interact with the GPI-PLC. In particular, proteins that may account for the specific localization of the GPI-PLC, and proteins that could be involved in the regulation of the GPI-PLC are of interest. Since immunoprecipitation of the GPI-PLC has already showed some promise in detecting potential interacting proteins, the same basic approach will be used here.

Correct identification of proteins of interest is ultimately dependent on the availability of a robust protein identification procedure. Therefore, the first aim of this chapter is to validate a procedure for reliably identifying unknown proteins, primarily

proteins that will be visualized on a Coomassie stained gel. To identify unknown proteins for this work, a peptide mass fingerprinting approach will be adopted, using matrix assisted laser desorption ionization (MALDI) time of flight (ToF) mass spectrometry to measure accurately the masses of peptides generated from the trypsin, and chymotrypsin, digestion of unknown proteins.

3.2. Results

3.2.1. Cytochrome C as a Standard Protein for Establishing Initial MALDI-ToF Conditions

MALDI-ToF mass spectrometry provides highly precise mass data for a sample under study that, if sufficiently accurate, can be used to help identify an unknown sample or can identify the nature of a covalent modification to a protein. However the technique is highly sensitive to sample contamination, poor sample preparation, and incorrect calibration. Since such problems could unwittingly lead to the misidentification of a protein thought to interact with the GPI-PLC, it was absolutely crucial to ensure a robust MALDI-ToF methodology was established.

Cytochrome c was first chosen as a protein with which to test various protocols primarily because it has been used by numerous groups for optimizing experimental conditions; large amounts of data are therefore available concerning the use of cytochrome c in MALDI-ToF mass spectrometry. A mass spectrum of whole cytochrome c, dissolved in water ($3 \text{ mg} \cdot \text{ml}^{-1}$) before crystallizing with the matrix α -CHCA, was first obtained (figure 3.1) and yielded two primary peaks. The major peak, with a mass of 12,389.83 Da, represents cytochrome c ($> 99.95\%$ accuracy) that has been ionized by the addition of 1 proton. A second peak, with an apparent mass of 6,199.13 Da represents cytochrome c that has been ionized by two protons (the time of flight of an ion is proportional to the ion's mass divided by the ion's net charge).

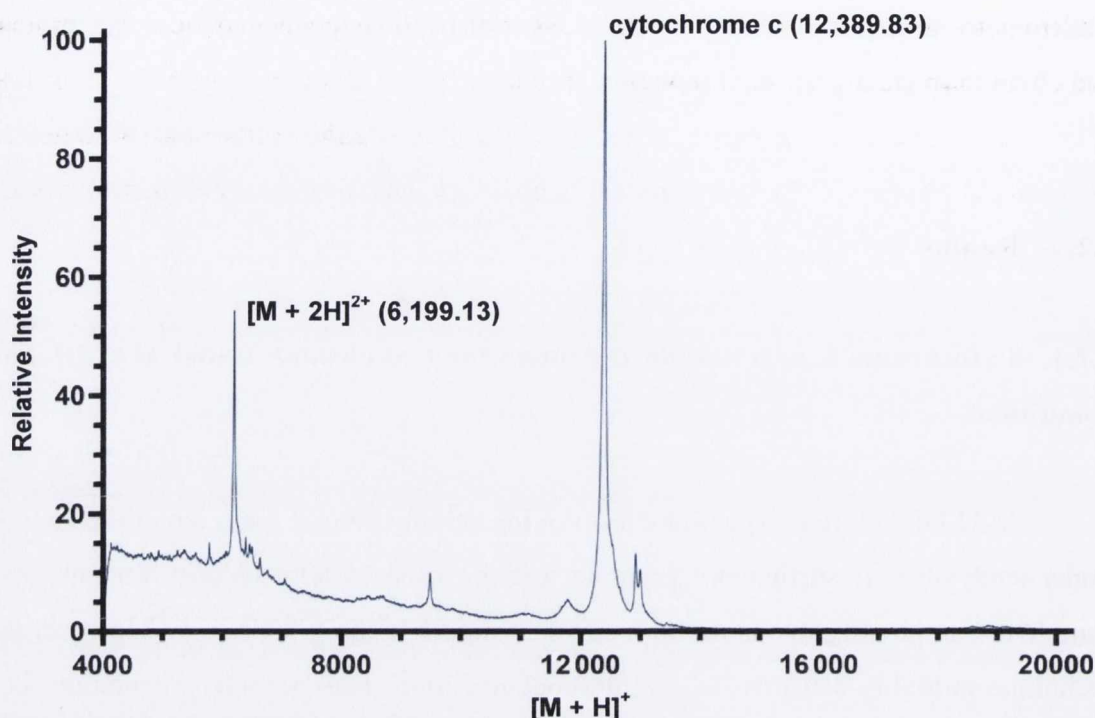


Figure 3.1. MALDI-ToF Mass Spectrum of Cytochrome C

Cytochrome c (from Equine heart muscle, accession number P0004), was dissolved in water and crystallized in the presence of α -CHCA using the “dried-droplet” method. A mass spectrum was obtained after irradiating the sample with a laser intensity of 56%. With the mass spectrometer set to linear detection mode, the acceleration voltage, grid voltage, and guide wire voltage, were set to 25,000 V, 92% and 0.05% respectively. The low mass gate was set to 4,000, preventing smaller masses from reaching the detector. A delay time of 325 ns was used. The peaks that correspond to cytochrome c ionized by the addition of one, or two, protons are labelled.

Although this type of basic mass spectrum provides valuable mass data for a protein in solution, it is not sufficient for identifying a protein. To identify a protein by mass spectrometry, either individual peptides of the protein must be sequenced (using tandem mass spectrometric methods), or the masses of enough peptides of a protein must be determined with sufficient accuracy to serve as a “mass fingerprint”. In this later case, the collected masses of even as few as three peptides from trypsin digested proteins can serve as a relatively unique identifier of each protein (Pappin *et al.*, 1993) and enable an identification to be made.

Cytochrome c ($4 \text{ mg} \cdot \text{ml}^{-1}$ in water) was therefore subjected to trypsin digestion to produce peptides whose masses could be compared to the hypothetical masses of peptides generated by *in silico* trypsin digests using the MS-Fit application of ProteinProspector. The mass spectrum of trypsin digested cytochrome c is shown in figure 3.2, and three of the major peak masses were correctly matched to the peptide masses of a hypothetical trypsin digest of equine cytochrome c (with each matched, measured, mass within 0.2 Da of the actual peptide mass).

3.2.2. Identification of the Standard Proteins Cytochrome C, Ovalbumin, and BSA by Peptide Mass Fingerprinting following in-gel Trypsin Digestion

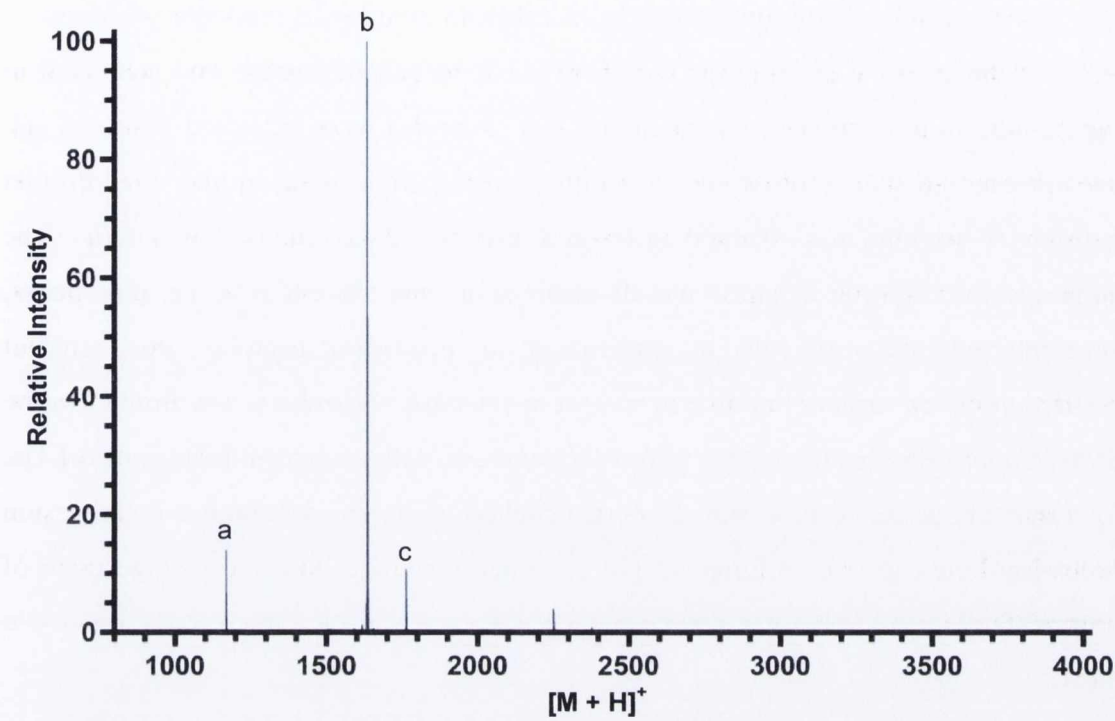
Identification of cytochrome c following digestion with trypsin verified a useful method for identifying potentially any unknown protein in solution. However it was vital that such a procedure, or variation of such a procedure, could also be used for identifying proteins that had been stained with Coomassie after running on a denaturing polyacrylamide gel. Although numerous procedures were attempted, ultimately the procedure of Shevchenko *et al.*, (2006) proved most effective.

The protocol described in section 2.4 for identifying proteins by MALDI-ToF mass spectrometry was therefore verified by testing the protocol on samples of cytochrome c, ovalbumin, and BSA, that had been electrophoresed on a polyacrylamide gel prior to trypsin digestion. Masses of the peptides resulting from the in-gel trypsin digestion of each protein were successfully matched to the correct protein by peptide mass fingerprinting. The mass spectra of in-gel trypsin digested cytochrome c, ovalbumin, and BSA, are shown in figures 3.3., 3.4, and 3.5 respectively.

Figure 3.2. MALDI-ToF Mass Spectrum of Trypsin Digested Cytochrome C

Cytochrome c was dissolved in 25 mM ammonium bicarbonate ($4 \text{ mg} \cdot \text{ml}^{-1}$) before adding 5 μl trypsin solution ($4 \text{ mg} \cdot \text{ml}^{-1}$, also in 25 mM ammonium bicarbonate). The solution was incubated overnight at 37°C before crystallizing a portion of the digested sample in the presence of α -CHCA using the “dried-droplet” method, as described in section 2.4.3. A mass spectrum was obtained as described in materials and methods. The resulting spectrum was de-isotoped and all peaks of at least 1% intensity, *i.e.* eight peaks, were entered into the MS-Fit application of ProteinProspector. No constant modifications, or variable modifications, were selected. Searching was limited to the *Equus Caballus* taxonomy of the SwissProt database, with an error tolerance of 0.2 Da. Three masses, peaks a, b and c, were matched to horse cytochrome c (accession P00004), shown in (b), yielding 24.8% sequence coverage with an average error of -0.0775 Da. The peptide detected are aligned with the cytochrome c sequence in (c).

(a)



(b)

Peak Label	Measured Mass (mono-isotopic)	Predicted Mass (mono-isotopic)	Sequence
a	1168.6480	1168.6222	(K)TGPNLHGLFGR(K)
b	1633.6838	1633.8189	(K)IFVQKCAQCHTVEK(G)
c	1761.7905	1761.9139	(K)KIFVQKCAQCHTVEK(G)

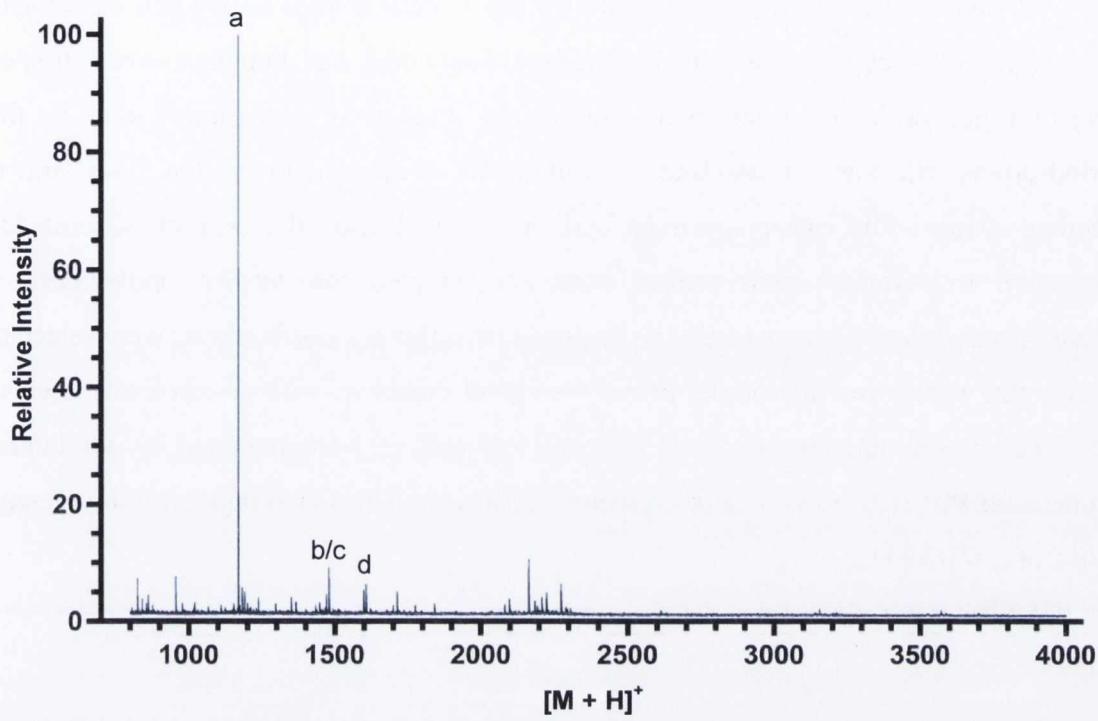
(c)

MGDVEKGKKIFVQKCAQCHTVEKGGGKHKTGPNLHGLFGRKTGQAPGFTYTDANKNKGITWKEETLMEYLE
KIFVQKCAQCHTVEK TGPNLHGLFGR
NPKKYIPGTKMIFAGIKKKTEREDLIAYLKATNE

Figure 3.3. MALDI-ToF Mass Spectrum of in-gel Trypsin Digested Cytochrome C

Cytochrome c (5 μ g) was cut from a 12.5% gel, destained, and subjected to trypsin digestion as described in section 2.4.1. Peptides were extracted from the gel, dried down, and then spotted onto a stainless steel sample plate by the dried-droplet method. A spectrum was obtained as detailed in section 2.4.5, and is shown in (a). The entire spectrum was de-isotoped and all peaks of at least 5% intensity, *i.e.* nine peaks, were entered into the MS-Fit application of ProteinProspector. No constant modifications, or variable modifications, were selected. Searching was limited to the *Equus Caballus* taxonomy of the SwissProt database, with an error tolerance of 0.1 Da. Four masses, peaks a, b, c and d, were matched to horse cytochrome c (accession P00004), shown in (b), yielding 37.1% sequence coverage with an average error of +0.0506 Da.

(a)



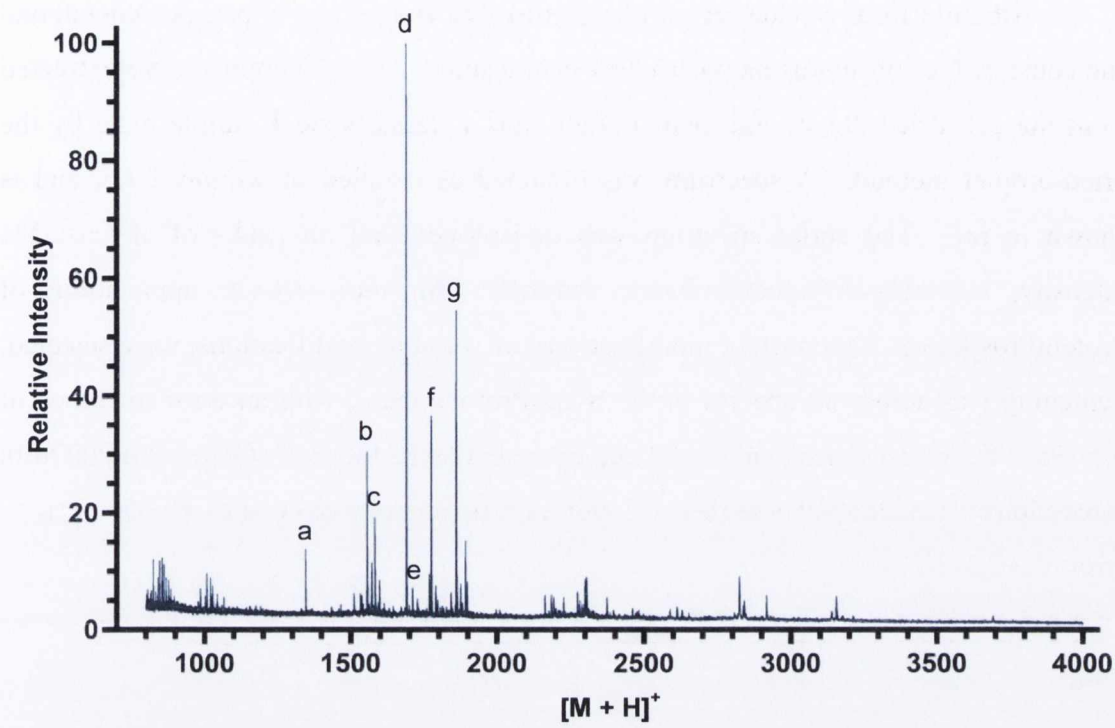
(b)

Peak Label	Measured Mass (mono-isotopic)	Predicted Mass (mono-isotopic)	Sequence
a	1168.6629	1168.6222	(K)TGPNLHGLFGR(K)
b	1478.8739	1478.8213	(K)KTEREDLIAYLK(K)
c	1478.8739	1478.8213	(K)TEREDLIAYLKK(A)
d	1598.8374	1598.7809	(R)KTGQAPGFTYTDANK(N)

Figure 3.4. MALDI-ToF Mass Spectrum of in-gel Trypsin Digested Ovalbumin

Albumin from chicken egg white (5 μ g) was cut from a 12.5% gel, de-stained, and subjected to trypsin digestion as described in section 2.4.1. Peptides were extracted from the gel, dried down, and then spotted onto a stainless steel sample plate by the dried-droplet method. A spectrum was obtained as detailed in section 2.4.5, and is shown in (a). The entire spectrum was de-isotoped and all peaks of at least 5% intensity, *i.e.* twenty eight peaks, were entered into the MS-Fit application of ProteinProspector. No constant modifications, or variable modifications, were selected. Searching was across all species in the SwissProt database, with an error tolerance of 0.1 Da. Seven masses, peaks a, b, c, d, e, f and g, were matched to ovalbumin (accession P01012), shown in (b), yielding 22.8% sequence coverage with an average error of +0.0436 Da.

(a)



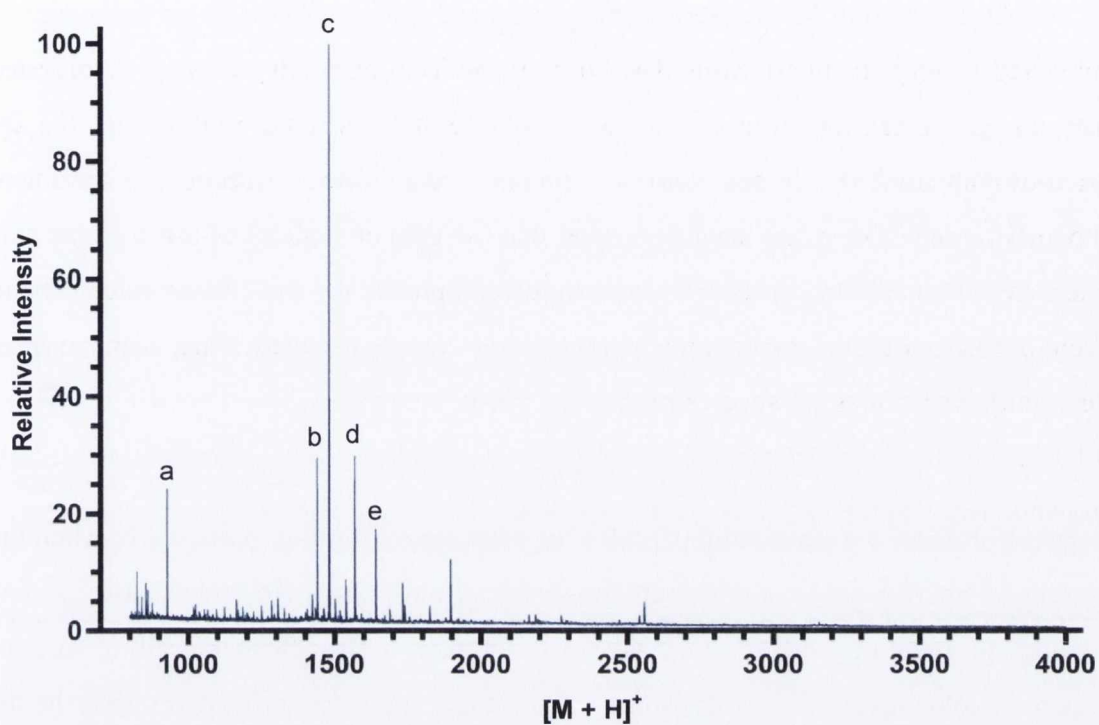
(b)

Peak Label	Measured Mass (mono-isotopic)	Predicted Mass (mono-isotopic)	Sequence
a	1345.7720	1345.7375	(K)HIATNAVLFFGR(C)
b	1555.7678	1555.7210	(K)AFKDEDTQAMPFR(V)
c	1581.7658	1581.7213	(K)LTEWTSSNVMEER(K)
d	1687.8812	1687.8398	(R)GGLEPINFQTAADQAR(E)
e	1709.8508	1709.8163	(K)LTEWTSSNVMEERK(I)
f	1773.9479	1773.8991	(K)ISQAVHAAHAEINEAGR(E)
g	1859.0206	1858.9658	(R)ELINSWVESQTNGIIR(N)

Figure 3.5. MALDI-ToF Mass Spectrum of in-gel Trypsin Digested BSA

Albumin from bovine serum (5 μ g) was cut from a 12.5% gel, de-stained, and subjected to trypsin digestion as described in section 2.4.2. Peptides were extracted from the gel, dried down, and then spotted onto a stainless steel sample plate by the dried-droplet method. A spectrum was obtained as detailed in section 2.4.5, and is shown in (a). The entire spectrum was de-isotoped and all peaks of at least 5% intensity, *i.e.* eleven peaks, were entered into the MS-Fit application of ProteinProspector. No constant modifications, or variable modifications, were selected. Searching was across all species in the SwissProt database, with an error tolerance of 0.1 Da. Five masses, peaks a, b, c, d and e, were matched to BSA (accession P02769), shown in (b), yielding 9.9% sequence coverage with an average error of +0.0564 Da.

(a)



(b)

Peak Label	Measured Mass (mono-isotopic)	Predicted Mass (mono-isotopic)	Sequence
a	927.5276	927.4934	(K)YLYEIAR(R)
b	1439.8707	1439.8118	(R)RHPEYAVSVLLR(L)
c	1479.8496	1479.7954	(K)LGEYGFQNALIVR(Y)
d	1567.8015	1567.7427	(K)DAFLGSFLYEYSR(R)
e	1640.0137	1639.9377	(R)KVPQVSTPTLVEVSR(S)

3.2.3. Identification of the Standard Proteins Cytochrome C, Ovalbumin, and BSA by Peptide Mass Fingerprinting following Chymotrypsin Digestion

A notable problem with MALDI-ToF mass spectrometry of trypsin digested samples, is the greater intensity that peptides with a C-terminal arginine residue are detected compared to peptides with a C-terminal lysine residue. Although this problem is poorly understood, it has been suggested that the greater basicity of the arginine side chain lends itself to preferred ionization compared to the lysine side chain. Consequently, arginine-terminating peptides are over-represented when using trypsin for peptide mass fingerprinting (Kraus *et al.*, 1999).

One solution to this limitation of MALDI-ToF mass spectrometry is to use a different protease for generating peptides for mass fingerprinting, particularly when the protein of interest may contain high lysine to arginine ratios (in which case the few peptides terminating with arginine would be preferentially detected, leading to poor sequence coverage). Chymotrypsin appears to be an under-utilized protease in the peptide mass fingerprinting arena, but its use was examined here. The use of chymotrypsin was expected to increase the balance of sequence coverage obtained over traditional trypsin-based approaches and, therefore, significantly increase the confidence of correct protein identification.

The protocol presented earlier for in-gel trypsin digestion was therefore also validated using chymotrypsin. The proteins cytochrome c, ovalbumin, and BSA, were each successfully identified following in-gel chymotrypsin digestion. In each case, a greater number of peptides of appropriate mass were produced using chymotrypsin, and a greater sequence coverage was achieved compared to that obtained with trypsin digests; an increase in sequence coverage between 2.1% (for BSA) and 18.2% (for cytochrome c) was observed. Additionally, no detection bias towards any particular chymotrypsin cleavage products was observed. The mass spectra of in-gel chymotrypsin digested cytochrome c, ovalbumin, and BSA, are shown in figures 3.6, 3.7, and 3.8 respectively. The combined sequence coverages obtained from trypsin and chymotrypsin digests are shown in figure 3.9.

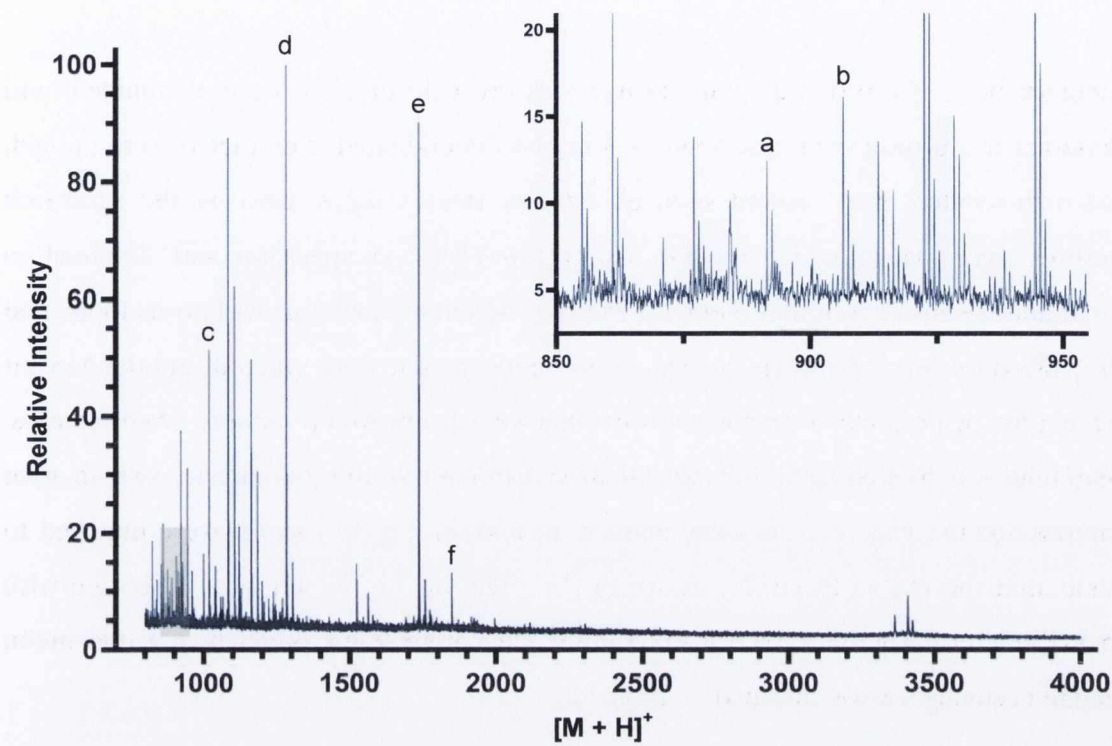
3.2.4. MALDI-ToF Mass Spectra can be Obtained from as little as 16 ng of Coomassie Stained Protein

A series of BSA dilutions were electrophoresed on a 12.5% polyacrylamide gel in order to test the effectiveness of the protein identification procedure, particularly with faint protein bands. Since the GPI-PLC is not a very abundant protein in trypanosomes, it is probable that only a low amount of any accessory or regulatory protein is present in the cell also. Each of the BSA dilutions prepared were matched to BSA, even when the Coomassie stained protein band was barely visible on the gel; BSA was identifiable at the lowest amount loaded (16 ng), (figure 3.10). Although there is a noticeable increase in the level of background noise seen in the mass spectra of very faint protein bands (*i.e.* less than 256 ng of protein present) it is expected that this could be improved, if necessary, by reducing the concentration of trypsin used for the in-gel digests. It may also be possible to reduce background noise in these cases by reducing the concentration of α -CHCA present in the matrix solution.

Figure 3.6. MALDI-ToF Mass Spectrum of in-gel Chymotrypsin Digested Cytochrome C

Cytochrome c (5 μ g) was cut from a 12.5% gel, de-stained, and subjected to chymotrypsin digestion as described. Peptides were extracted from the gel, dried down, and then spotted onto a stainless steel sample plate by the sandwich method before washing twice with 5 μ l 0.1% TFA. A spectrum was obtained as detailed in section 2.4.5, and is shown in (a). The entire spectrum was de-isotoped and all peaks of at least 5% intensity, *i.e.* thirty six peaks, were entered into the MS-Fit application of ProteinProspector. No constant modifications, or variable modifications, were selected. Searching was limited to the *Equus Caballus* taxonomy of the SwissProt database, with an error tolerance of 0.1 Da. Six masses, peaks a, b, c, d, e and f, were matched to horse cytochrome c (accession P00004), shown in (b), yielding 55.2% sequence coverage with an average error of -0.0548 Da. For more clearly showing peaks a and b, the green shaded region is shown enlarged.

(a)



(b)

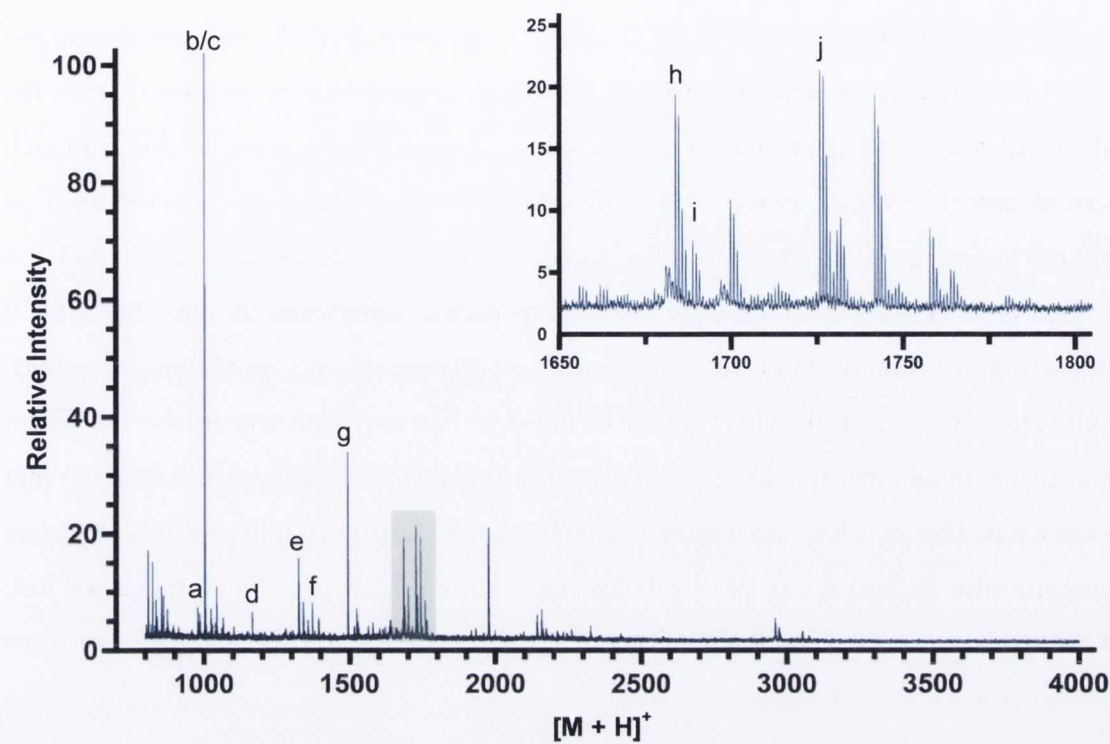
Peak Label	Measured Mass (mono-isotopic)	Predicted Mass (mono-isotopic)	Sequence
a	891.4384	891.4934	(Y)LENPKKY(I)
b	906.4437	906.5117	(Y)IPGTKMIF(A)
c	1018.4962	1018.5429	(F)GRKTGQAPGF(T)
d	1282.5893	1282.6539	(F)GRKTGQAPGFTY(T)
e	1734.8820	1734.9749	(F)AGIKKKTEREDLIAY(L)
f	1847.9478	1847.9498	(Y)TDANKNKGITWKEETL(M)

Figure 3.7. MALDI-ToF Mass Spectrum of in-gel Chymotrypsin Digested Ovalbumin

Albumin from chicken egg white (5 µg) was cut from a 12.5% gel, de-stained, and subjected to chymotrypsin digestion as describe. Peptides were extracted from the gel, dried down, and then spotted onto a stainless steel sample plate by the sandwich method before washing twice with 5 µl 0.1% TFA. A spectrum was obtained as detailed in section 2.4.5, and is shown in (a). The entire spectrum was de-isotoped and all peaks of at least 5% intensity, *i.e.* twenty eight peaks, were entered into the MS-Fit application of ProteinProspector. No constant modifications, or variable modifications, were selected. Searching was across all species in the SwissProt database, with an error tolerance of 0.1 Da. Ten masses, peaks a, b, c, d, e, f, g, h, i and j, were matched to ovalbumin (accession P01012), shown in (b), yielding 26.2% sequence coverage with an average error of -0.0654 Da. For more clearly showing peaks h to j, the green shaded region is shown enlarged.

Label	Measured Mass (amu)	Protein Name	Sequence
a	100.071	Ovalbumin	YIPKSTNIPK
b	100.071	Ovalbumin	(YIPKSTNIPK)
c	100.071	Ovalbumin	(YIPKSTNIPK)
d	100.071	Ovalbumin	(YIPKSTNIPK)
e	100.071	Ovalbumin	(YIPKSTNIPK)
f	100.071	Ovalbumin	(YIPKSTNIPK)
g	100.071	Ovalbumin	(YIPKSTNIPK)
h	100.071	Ovalbumin	(YIPKSTNIPK)
i	100.071	Ovalbumin	(YIPKSTNIPK)
j	100.071	Ovalbumin	(YIPKSTNIPK)

(a)



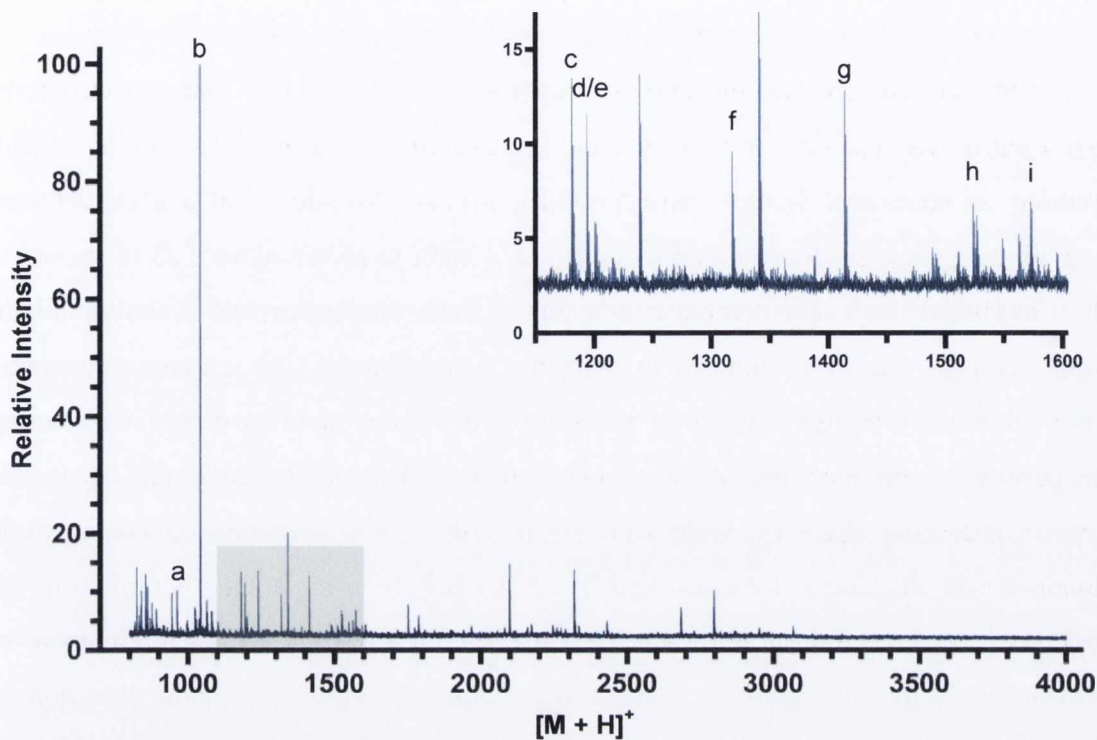
(b)

Peak Label	Measured Mass (mono-isotopic)	Predicted Mass (mono-isotopic)	Sequence
a	978.4805	978.5407	(F)KGLWEKAF(K)
b	1002.4713	1002.5156	(F)RADHPFLF(C)
c	1002.4713	1002.5367	(Y)RGGLEPINF(Q)
d	1165.5505	1165.6000	(L)YRGGLEPINF(Q)
e	1324.6142	1324.6752	(Y)LPRMKMEEKY(N)
f	1371.6134	1371.6804	(L)KVHHANENIFY(C)
g	1492.7011	1492.7682	(Y)AEERYPILPEYL(Q)
h	1683.7987	1683.8734	(W)TSSNVMEERKIKVY(L)
i	1688.8087	1688.8966	(L)RDILNQITKPNDVY(S)
j	1725.7531	1725.8299	(F)RVTEQESKPVQMMY(Q)

Figure 3.8. MALDI-ToF Mass Spectrum of in-gel Chymotrypsin Digested BSA

Albumin from bovine serum (5 μ g) was cut from a 12.5% gel, destained, and subjected to chymotrypsin digestion as described. Peptides were extracted from the gel, dried down, and then spotted onto a stainless steel sample plate by the sandwich method before washing twice with 5 μ l 0.1% TFA. A spectrum was obtained as detailed in section 2.4.5, and is shown in (a). The entire spectrum was deisotoped and all peaks of at least 5% intensity, *i.e.* thirty peaks, were entered into the MS-Fit application of ProteinProspector. No constant modifications, or variable modifications, were selected. Searching was limited to the *Bos Taurus* taxonomy in the SwissProt database, with an error tolerance of 0.1 Da. Ten masses, peaks a, b, c, d, e, f, g, h, i and j, were matched to BSA (accession P02769), shown in (b), yielding 13.0% sequence coverage with an average error of +0.0837 Da. For more clearly showing peaks c to i, the green shaded region is shown enlarged.

(a)



(b)

Peak Label	Measured Mass (mono-isotopic)	Predicted Mass (mono-isotopic)	Sequence
a	944.5413	944.4697	(Y)SRRHPEY(A)
b	1041.6351	1041.5588	(Y)EIARRHPY(F)
c	1180.7364	1180.6473	(Y)GFQNALIVRY(T)
d	1193.7341	1193.6525	(L)RLAKEYEATL(E)
e	1193.7341	1193.6565	(Y)VPKAFDEKLF(T)
f	1317.7997	1317.7062	(Y)LYEIARRHPY(F)
g	1413.8549	1413.7597	(Y)SRRHPEYAVSVL(L)
h	1526.9387	1526.8438	(Y)SRRHPEYAVSVLL(R)
i	1548.9081	1548.8380	(F)DKLKHLVDEPQNL(I)
j	1572.8778	1572.7904	(F)DEHVKLVLNELTEF(A)

Figure 3.9. Tryptic and Chymotryptic Peptides of Cytochrome C, Ovalbumin, and BSA, Detected During MALDI-ToF Mass Spectrometry

Shown opposite are the peptides detected by MALDI-ToF mass spectrometry following in-gel digestion of cytochrome c (a), ovalbumin (b), and BSA (c), by trypsin (peptides in blue) and chymotrypsin (peptides in red). An additional peptide detected following in-solution digestion of cytochrome c with trypsin (figure 3.2) is shown in blue, and underlined. The complete sequence of each standard protein is shown in bold type. Trypsin digestion alone of cytochrome c resulted in 37% sequence coverage, while chymotrypsin digestion alone resulted in 55.2% sequence coverage. Combining the peptides from both digestions resulted in a total sequence coverage of 66.7%. Trypsin digestion alone of ovalbumin resulted in 22.8% sequence coverage, while chymotrypsin digestion alone resulted in 26.2% sequence coverage. Combining the peptides from both digestions of ovalbumin resulted in a final sequence coverage of 43.5%. Only 9.9% sequence coverage was obtained following trypsin digestion of BSA, while 13% coverage was obtained with chymotrypsin digestion of BSA. In total, 18.1% sequence coverage was obtained combining the results of both these digests.

GPI-PLC Interactions with Possible Partner Proteins

(a)

MGDVEKGKKIFVQKCAQCHTVEKGGKHKGTGPNLHGLFGRKTGQAPGFTYTDANKNKGITWKEETLMEYLENPKKIYPGTMIFAGIKK
KIFVQKCAQCHTVEK
TGPNLHGLFGRKTGQAPGFTYTDANK
GRKTGQAPGFTYTDANKNKGITWKEETL LENPKKIYPGTMIFAGIKK
KTEREDLIAYLKATNE
KTEREDLIAYLKK
KTEREDLIAY

(b)

MGSIGAASMEFCFDVFKELKVHHANENIFYCPIAIMSALAMVYLGAkdSTRtQINKVVRFDKLPFGDSIEAQCGTSVNVHSSLRDIL
KVHHANENIFY DIL
NQITKPNdVYSfSLASRLyAEERYpILPEYLQCVKELyRGGLEPINFQTAADQARELINSWVESQTNgiIRNVLQpSSVDSQTAmlV
NQITKPNdVY AEERYpILPEYL YRGGLEPINF
GGLEPINFQTAADQARELINSWVESQTNgiIR
NAIVFKGLWEKAFKDEDtQAMPFRVTEQESKpVQMMYQIGLFRVASMASEKMKILELPFASGTMSMLVLLPDEVSGLEQLESiINFEK
AFKDEDtQAMPFR
KGLWEKAF RVTEQESKpVQMMY
LTEWtSSNVMEERKIKVYLPRMKMEEKYNLTsvLMAGITdVfSSANLSGISSAESLKISQAVHAAHAEINEAGREVVGSAEAGVDA
LTEWtSSNVMEERK ISQAVHAAHAEINEAGR
TSSNVMEERKIKVYLPRMKMEEKY
ASVSEEFRA DHpFLFCIKHIATNAVLFfGRcVSP
HIATNAVLFfGR
RADHPFLF

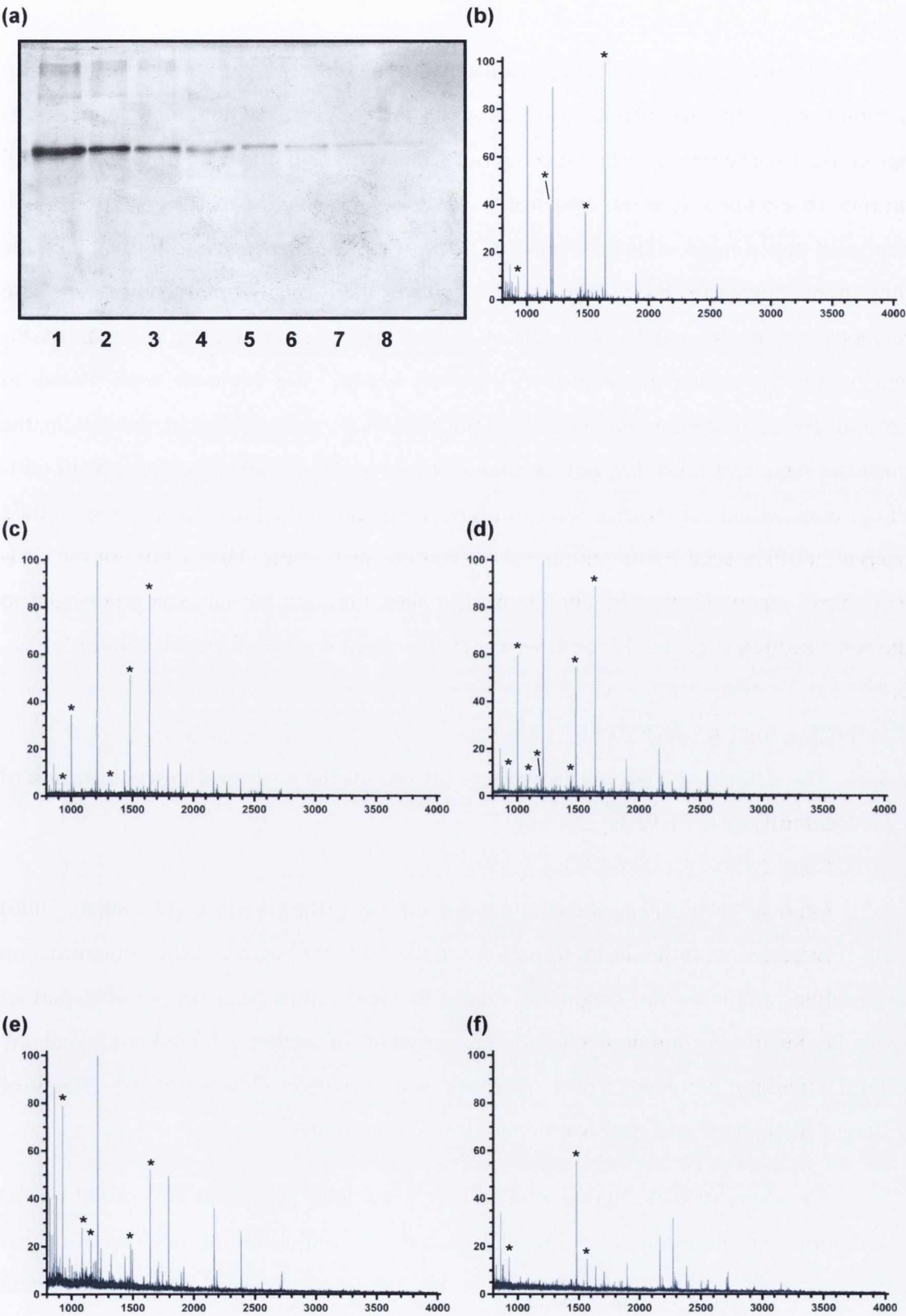
(c)

MKWVtFiSllllLFSSAYSrGVfRRDTHKSEIAHRfKDLGEEHfKGLVLIAFSQYLQqCPfDEHVKLvNELTEfAKTCVADESHAGCEK
DEHVKLvNELTEf
SLHTLFGDELCKVASLREtYGDmADCCEKQEPERNECFLSHKDDSPDLPKLKPDfNTLCDEFKADEKKfWgKYLYEIARRHPYfYAPE
YLYEIAR
YLYEIARRHPY
LLYyANKYngVfQeCCQAEdKGACLLPKIETMREKVLASSARQRLCASIQKfGERALKAWSVARLSQKFpKAEfVEVTKLVTDLTkV
HKECCHGDILLECADDRADLAKYICDNQDTISSKLKECCDKPLLEKSHCIAEVEKDAIPENLPPLTADFAEDKDVCkNYQeAKDAFLGS
DAFLGS
FLYeySRRHPEYAVSVLLRLAKEYEATLEeCCAKDDPHACYSTVfDKLKHlVDEPQNLiKQNCdQfEKLGeyGFQNALIVRYTRKVPQ
FLYeySRRHPEYAVSVLLR LGeyGFQNALIVR KVPQ
SRRHPEYAVSVLLRLAKEYEATL DKLKHlVDEPQNL GFQNALIVRY
VSTPTLVEVSRSLGKVGRCTKPESERMPCTEDYLSLiLNRlCVLHEKTPVSEKVTKCTESLVNRRPCFSALTpDEtYVPKAFDEK
VSTPTLVEVSR VPKAFDEK
LFTFHADICTLPdTEKQIKKQtALVELLKHKPKATEEQlKtVMENfVAFVDKCCAADDKEACFAVEGPKLVVSTQtALA
LF

Figure 3.10. MALDI-TOF MS Identification of BSA Dilutions

Samples (2.05 μ g, 1.02 μ g, 512 ng, 256 ng, 128 ng, 64 ng, 32 ng and 16 ng) of albumin from bovine serum were run on a 12.5% denaturing gel (lanes 1-8, respectively, shown in 'a'). Gel pieces corresponding to 2.048 μ g, 1.024 μ g, 256 ng, 64 ng and 16 ng of BSA were subjected to the usual de-staining, trypsin digestion and peptide extraction procedures. Peptides were spotted onto the target MALDI plate by the "dried-droplet" method and spectra were obtained as described in materials and methods. A spectrum for each of the analyzed BSA bands is given in (b), (c), (d), (e) and (f), each of which correspond to BSA amounts of 2.048 μ g, 1.024 μ g, 256 ng, 64 ng and 16 ng respectively. Spectra were processed in the usual manner, with the resulting peak masses of at least 5% intensity entered into the MS-Fit application of ProteinProspector. No variable or constant modifications were selected, and peptide masses were checked through the SwissProt database, limited to the *Bos Taurus* taxonomy. Peptides successfully matched to BSA are marked '*'. The percentage of the total sequence covered by the peptides varied from 11%, for 250 ng of BSA, to 5.4%, for 32 ng of BSA. Mean error ranged from 0.0739 Da, for 32 ng BSA, to -0.00768 Da, for 1 μ g BSA. The loading scheme for the gel in (a) is given below:

Lane 1:	2.05 μ g BSA
Lane 2:	1.02 μ g BSA
Lane 3:	512 ng BSA
Lane 4:	256 ng BSA
Lane 5:	128 ng BSA
Lane 6:	64 ng BSA
Lane 7:	32 ng BSA
Lane 8:	16 ng BSA



3.2.5. Immunoprecipitation of the GPI-PLC

To investigate the interaction of the GPI-PLC of bloodstream form trypanosomes with possible accessory proteins, the enzyme was immunoprecipitated as described by Hanrahan (Hanrahan *et al.*, 2009). The GPI-PLC was successfully immunoprecipitated, and ran as a band just above the 36 kDa marker on a 15% gel, shown in figure 3.11. A protein band of identical molecular weight is observable in the immunoprecipitate from GPI-PLC^{-/-} cells, however the intensity of the band in this case is very much reduced and likely reflects non-specific protein binding to the anti-GPI-PLC antibody, or to the Protein-A agarose beads. No proteins were found to specifically co-immunoprecipitate with the GPI-PLC, as any protein present in the immunoprecipitate from WT cells is also observed in the immunoprecipitate from GPI-PLC^{-/-} cells. Identical proteins were immunoprecipitated when detergents were omitted from the buffers used for the immunoprecipitation wash steps. The identity of the GPI-PLC band was confirmed by peptide mass fingerprinting after subjecting the band to trypsin digestion (figure 3.12) and, separately, to chymotrypsin digestion (figure 3.13).

3.2.6. The Effects of Different Lysis Conditions on the co-Immunoprecipitation of Proteins with the GPI-PLC

Original protocols used for immunoprecipitating the GPI-PLC (Hanrahan, 2006) were formulated with the desire to pull-down the GPI-PLC with as little contamination as possible, and were not originally sought to yield information on possible partner proteins. Since the immunoprecipitation experiments in section 3.2.5 did not reveal any potential partner proteins, a new approach was required. Therefore the effects of different lysis conditions, and wash conditions, were studied.

The experimental approach taken here (described in section 2.5) rested on the assumption that protein-protein interactions that survived an initial incubation in lysis buffer, would survive later wash steps in the same buffer. If protein-protein interactions were abolished by the lysis buffer incubation, then no combination of buffers in subsequent wash steps could re-establish the interaction. Therefore a selection of lysis buffers, each containing a different detergent, was made that were

used at all stages of an immunoprecipitation experiment. Immunoprecipitation of the GPI-PLC was attempted using Tes buffers containing one of each of the detergents n-octylglucoside (n-octyl- β -D-glucopyranoside), n-dodecyl- β -D-maltoside, Triton[®] X-100, CHAPS, SDS, and cetyltrimethylammonium bromide (CTAB); each detergent was used at a concentration close to the minimum critical micellar concentration (CMC), and at a concentration slightly above the CMC.

Immunoprecipitations of the GPI-PLC in the presence of the non-ionic detergents n-octylglucoside, n-dodecyl- β -D-maltoside, and Triton[®] X-100, are shown in plate 3.1. The GPI-PLC could be readily immunoprecipitated after lysing cells in the presence of 1.2 mM Triton[®] X-100, however no additional proteins were specifically co-immunoprecipitated in this circumstance. The GPI-PLC was also successfully immunoprecipitated after cells were lysed in Tes buffer containing n-octylglucoside at concentrations of 20 mM and 30 mM. However, a large amount of additional proteins were non-specifically co-immunoprecipitated under this condition and no protein that specifically co-immunoprecipitated with the GPI-PLC was found. A band identical in apparent molecular weight to the GPI-PLC was immunoprecipitated from GPI-PLC^{-/-} cells when 30 mM n-octylglucoside was used, however this unknown protein band had a much reduced intensity compared to that of the GPI-PLC. In the presence of Triton[®] X-100 and n-octylglucoside, the GPI-PLC migrated as a band with an approximate apparent molecular weight of 39 kDa (or 39.45 kDa in the presence of Triton[®] X-100, and 38.6 kDa in the presence of n-octylglucoside).

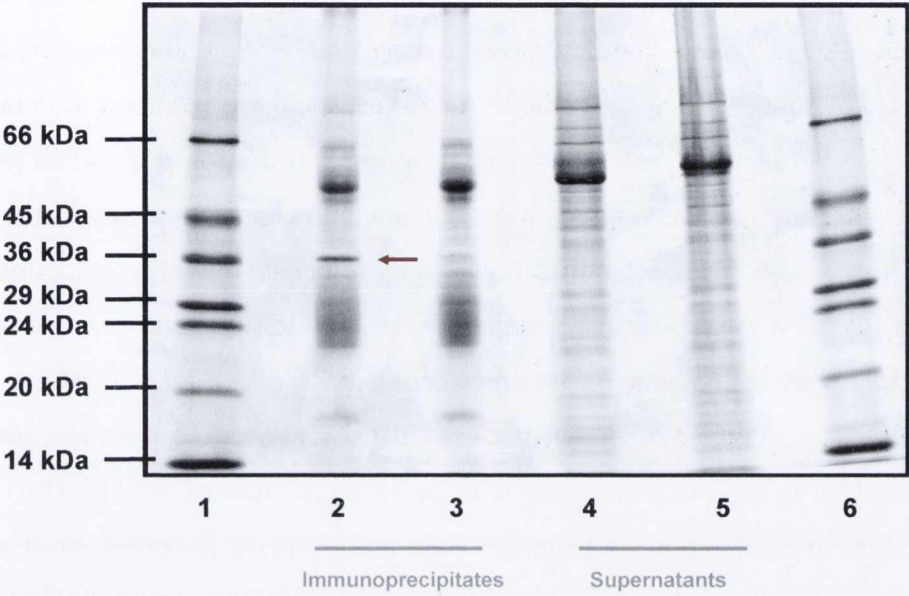
Despite similar chemical properties to n-octylglucoside, the GPI-PLC could not be immunoprecipitated in the presence of n-dodecyl- β -D-maltoside. Numerous other proteins were non-specifically immunoprecipitated from both WT and GPI-PLC^{-/-} cells in the presence of n-dodecyl- β -D-maltoside. Additionally, the GPI-PLC could not be immunoprecipitated in the case of the ionic detergents SDS and CTAB, and even the lowest concentrations of SDS used (7 mM) was sufficient to greatly disrupt the binding of anti-GPI-PLC IgG to the Protein-A agarose (plate 3.2). Immunoprecipitation of the GPI-PLC was also unsuccessful when CHAPS was used as the principal detergent.

Figure 3.11. Standard Immunoprecipitation of the GPI-PLC did not Detect Interacting Proteins

Cell lysates were prepared, from cultured bloodstream form trypanosomes, as described in section 2.5.2 but using 50 mM Tris buffer (pH 7.4), containing 150 mM NaCl, 0.1% (v/v) Nonidet P-40, 0.5% (w/v) CHAPS and the protease inhibitors PMSF, TLCK and leupeptin (at normal concentrations), in place of a Tes-based lysis buffer. Lysates were incubated overnight with anti-GPI-PLC conjugated Protein-A agarose as described. A sample of the supernatant following overnight immunoprecipitation was taken for SDS-PAGE before the Protein-A agarose beads were washed with 1 ml 50 mM Tris buffer (pH 7.4), containing 100 mM NaCl, +/- 0.2% (w/v) CHAPS. The Protein-A beads were then washed once with 1 ml 50 mM Tris buffer (pH 7.4), +/- 0.1% (v/v) Triton[®] X-100. Immunoprecipitated material was prepared for SDS-PAGE as described in section 2.5.3 and electrophoresed on 15% polyacrylamide gels shown opposite. The gel in (a) shows immunoprecipitated protein that was washed in the presence of detergent, while the gel in (b) shows immunoprecipitated protein that was washed in buffer containing no detergent. The GPI-PLC is highlighted with a red arrow. The loading scheme for both gels is identical, and is given below:

Lane 1:	Molecular weight standards (Sigma-Aldrich)
Lane 2:	Immunoprecipitate (I.P.) from WT cells (1×10^9 cell equivalents)
Lane 3:	I.P. from GPI-PLC ^{-/-} cells (1×10^9 cell equivalents)
Lane 4:	Supernatant protein from WT cells (2.5×10^6 cell equivalents)
Lane 5:	Supernatant protein from GPI-PLC ^{-/-} cells (2.5×10^6 cell equivalents)
Lane 6:	Molecular weight standards (Sigma-Aldrich)

(a)



(b)

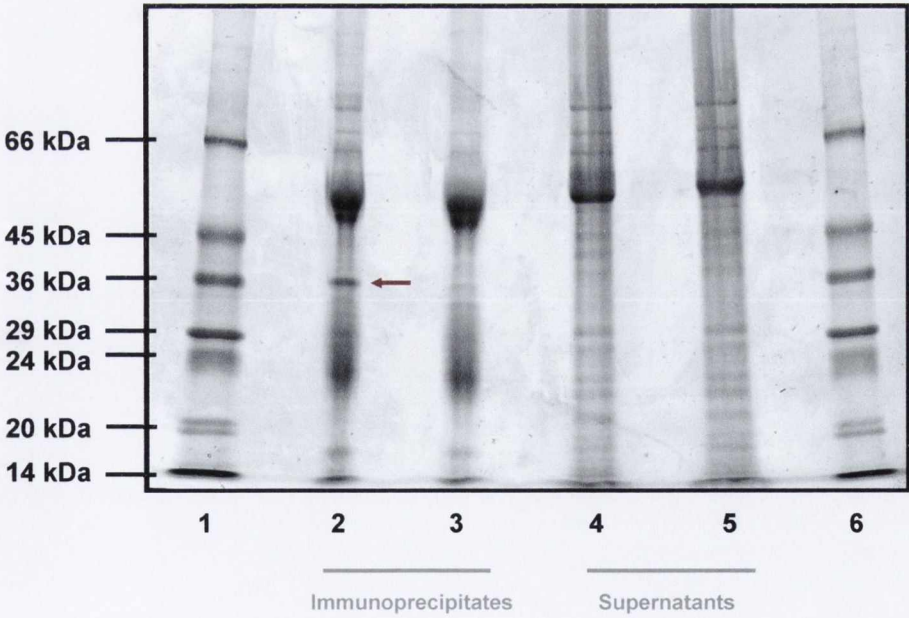
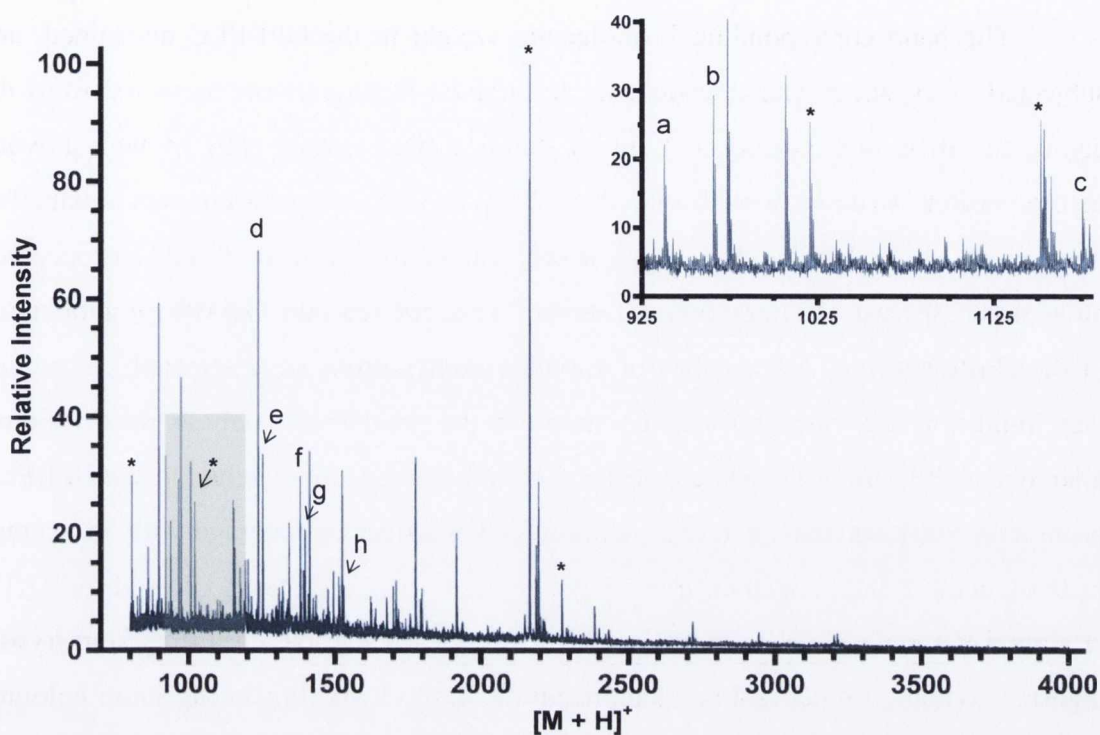


Figure 3.12. MALDI-ToF Mass Spectrum of Trypsin Digested GPI-PLC

The band corresponding in molecular weight to the GPI-PLC de-stained, and subjected to trypsin digestion as described. Peptides were extracted from the gel, dried down, and then spotted onto a stainless steel sample plate by the sandwich method before washing twice with 5 μ l 0.1% (v/v) TFA. A spectrum was obtained as detailed in section 2.4.5, and is shown in (a). The entire spectrum was de-isotoped and all peaks of at least 5% intensity, *i.e.* fifty three, were entered into the MS-Fit application of ProteinProspector. No constant modifications were selected, however oxidation of methionine residues was allowed. Searching was limited to the *Trypanosoma* taxonomy in the SwissProt database, with an error tolerance of 0.2 Da. Eight masses, peaks a, b, c, d, e, f, g and h, were matched to the GPI-PLC (accession P09194), shown in (b), yielding 17.0% sequence coverage with an average error of +0.112 Da. For clarity, the green shaded region containing peaks a, b, and c, is shown inset as a separate graph. Five peaks that were matched to bovine trypsin (accession P00760) are labelled '*'.


(a)



(b)

Peak Label	Measured Mass (mono-isotopic)	Predicted Mass (mono-isotopic)	Sequence
a	937.6128	937.5214	(R)QLLDHGV(R)(Y)
b	965.6379	965.5415	(R)FLTVGVSSR(W)
c	1175.6426	1175.5361	(R)MNISPDQENK(I)
d	1236.7420	1236.6405	(K)MYVTQAIGTPR(N)
e	1252.7200	1252.6354	(K)MYVTQAIGTPR(N)†
f	1380.7270	1380.6001	(K)WSPQSWMSDTR(S)
g	1396.7355	1396.5950	(K)WSPQSWMSDTR(S)†
h	1529.8670	1529.7206	(K)WFYDLNVNGVMR(G)†

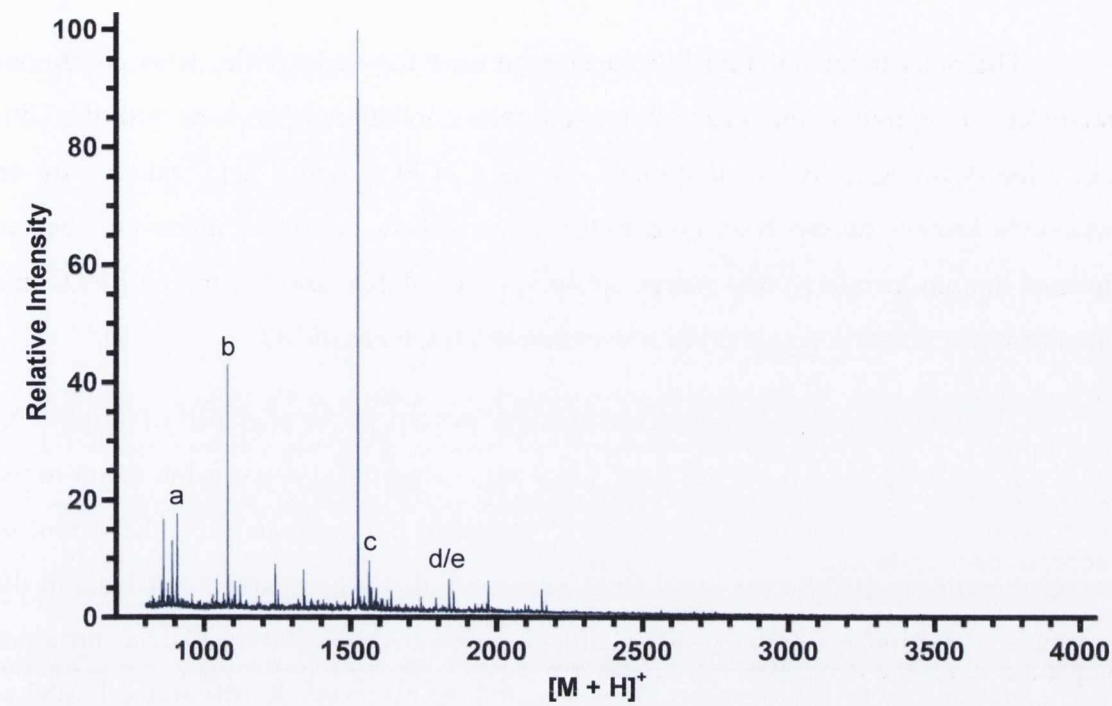
* Peak matched to peptide from proteolyzed bovine trypsin

† Peptide contains an oxidized methionine residue

Figure 3.13. MALDI-ToF Mass Spectrum of Chymotrypsin Digested GPI-PLC

The band corresponding in molecular weight to the GPI-PLC de-stained, and subjected to chymotrypsin digestion as described. Peptides were extracted from the gel, dried down, and then spotted onto a stainless steel sample plate by the sandwich method before washing twice with 5 μ l 0.1% (v/v) TFA. A spectrum was obtained as detailed in section 2.4.5, and is shown in (a). The entire spectrum was de-isotoped and all peaks of at least 5% intensity, *i.e.* eleven, were entered into the MS-Fit application of ProteinProspector. No constant or variable modifications were selected. Searching was limited to the *Trypanosoma* taxonomy in the SwissProt database, with an error tolerance of 0.1 Da. Five masses, peaks a, b, c, d and e, were matched to the GPI-PLC (accession P09194), shown in (b), yielding 14.8% sequence coverage with an average error of -0.0852 Da. Together, trypsin and chymotrypsin digestions allowed for 27.1% sequence coverage of the GPI-PLC, shown in (c) with peptides resulting from trypsin digestion coloured blue, and peptides resulting from chymotrypsin digestion coloured red.

(a)



(b)

Peak Label	Measured Mass (mono-isotopic)	Predicted Mass (mono-isotopic)	Sequence
a	907.4692	907.5105	(W)QSTRRIF(L)
b	1079.5579	1079.5884	(L)RSLPPYVTF(L)
c	1562.6852	1562.7856	(L)TVGVSSRWGRCQNL(S)
d	1835.8003	1835.8956	(L)DLRMNISPDQENKIY(T)
e	1835.8003	1835.9585	(L)TTPLCNLWQSTRRIF(L)

(c)

MFGGVKWSPQSWMSDTRSSIEKKCIGQVYMGVGAHNAGTHGIQMFSPFGLDAPEKLRSLPPYVTFLLRFLTVGVSSRWGRCQNL SIRQL
WSPQSWMSDTR RSLPPYVTF FLTVGVSSR QL

LDHGVRYLDLRMNISPDQENKIYTTHFHISVPLQEVLDVKDFLTPASANEFVILDFLHFYGFNESHMTMKRFVEELQALEEFYIPTT
LDHGVR MNISPDQENK TVGVSSRWGRCQNL

VSLTTPLCNLWQSTRRIFLVVRPYVEYPYARLRVSKSIWVNQMEINDLLDRLEELMTRDLEDVSIGGVPSKMYVTQAIGTPRNDF
TTPLCNLWQSTRRIF MYVTQAIGTPR

AVAACCGACPGSHPDLYSAAKHKNPHLLKWFYDLNVNGVMRGERVTIRRGNNTHGNILLDFVQEGTCTVKGVDPKPMNAVALCVHLNT
WFYDLNVNGVMR

NQTARS

3.2.7. Lysis of Bloodstream form Trypanosomes Activates the GPI-PLC

The activity of the GPI-PLC was examined for each of the lysis conditions performed in section 3.2.6. The ability to co-immunoprecipitate proteins with the GPI-PLC, for example, may be dependent on the GPI-PLC being held inactive by an accessory protein during lysis conditions. One would speculate, therefore, that an optimal immunoprecipitation strategy might require solubilization of the GPI-PLC in a condition that failed to result in the conversion of mfVSG to sVSG.

The GPI-PLC could be best immunoprecipitated in the presence of Triton[®] X-100 or n-octylglucoside, however these non-ionic detergents also proved to result in the most prominent activation of the GPI-PLC (assessed approximately by the extent of sVSG present in cell lysates). GPI-PLC activation also accompanied cell lysis in the presence of 0.8 mM n-dodecyl- β -D-maltoside. Meanwhile, detergent concentrations that were too low to lyse trypanosomes (*i.e.* 0.2 mM Triton[®] X-100 and 0.1 mM n-dodecyl- β -D-maltoside) also failed to cause significant activation of the GPI-PLC. Lysis of trypanosomes in these non-ionic detergents is shown in plate 3.3.

In the case of the ionic detergents (shown in plate 3.4) cell lysis occurred in each case, however the amount of sVSG produced appeared less than that which occurred in the presence of the uncharged detergents. In particular, no sVSG was found in the extract of cells lysed with 7 mM SDS, likely due to denaturation and inactivation of the GPI-PLC in this condition.

Plate 3.1. Immunoprecipitation of the GPI-PLC using non-Ionic Detergents

Trypanosomes, purified from an infected rat, were washed twice in Tes buffer before lysing in Tes buffer containing protease inhibitors and one of the detergents: Triton[®] X-100 (0.1 mM or 1.2 mM), n-octylglucoside (20 mM or 30 mM), or n-dodecyl- β -D-maltoside (0.1 mM or 0.8 mM). Lysates were centrifuged at 15,000 g for fifteen minutes, at 4 °C, and the resulting supernatant was incubated overnight in the presence of anti-GPI-PLC conjugated Protein-A agarose as described in section 2.5.3. Immunoprecipitates were prepared for SDS-PAGE, as described, and run on 15% polyacrylamide gels shown opposite. In each case, the GPI-PLC is highlighted by a red arrow. The loading scheme for each gel is shown below:

Gel (a):	Lane 1:	Molecular weight standards (Thermo-Scientific)
	Lane 2:	I.P. from WT cells lysed with 0.1 mM Triton [®] X-100
	Lane 3:	I.P. from GPI-PLC ^{-/-} cells lysed with 0.1 mM Triton [®] X-100
	Lane 4:	I.P. from WT cells lysed with 1.2 mM Triton [®] X-100
	Lane 5:	I.P. from GPI-PLC ^{-/-} cells lysed with 1.2 mM Triton [®] X-100
	Lane 6:	Molecular weight standards (Thermo-Scientific)
Gel (b):	Lane 1:	Molecular weight standards (Thermo-Scientific)
	Lane 2:	I.P. from WT cells lysed with 20 mM n-octylglucoside
	Lane 3:	I.P. from GPI-PLC ^{-/-} cells lysed with 20 mM n-octylglucoside
	Lane 4:	I.P. from WT cells lysed with 30 mM n-octylglucoside
	Lane 5:	I.P. from GPI-PLC ^{-/-} cells lysed with 30 mM n-octylglucoside
	Lane 6:	Molecular weight standards (Thermo-Scientific)
Gel (c):	Lane 1:	Molecular weight standards (Thermo-Scientific)
	Lane 2:	I.P. from WT cells lysed with 0.1 mM n-dodecyl- β -D-maltoside
	Lane 3:	I.P. from GPI-PLC ^{-/-} cells lysed with 0.1 mM n-dodecyl- β -D-maltoside
	Lane 4:	I.P. from WT cells lysed with 0.8 mM n-dodecyl- β -D-maltoside
	Lane 5:	I.P. from GPI-PLC ^{-/-} cells lysed with 0.8 mM n-dodecyl- β -D-maltoside
	Lane 6:	Molecular weight standards (Thermo-Scientific)

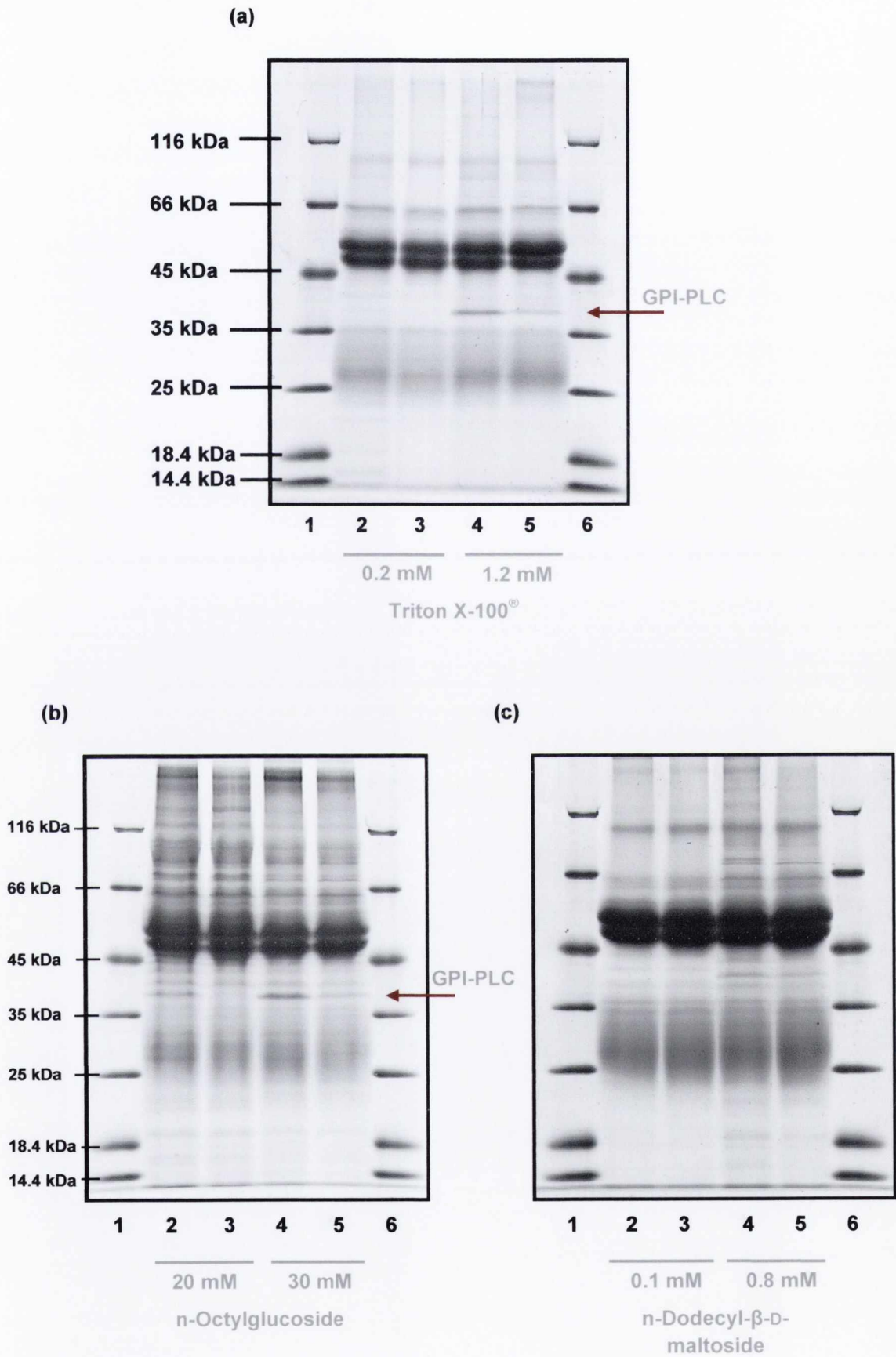


Plate 3.2. Immunoprecipitation of the GPI-PLC using Zwitterionic and Ionic Detergents

Trypanosomes, purified from an infected rat, were washed twice in Tes buffer before lysing in Tes buffer containing protease inhibitors and one of the detergents: CHAPS (6 mM or 12 mM), SDS (7 mM or 12 mM), or CTAB (1 mM or 1.5 mM). Lysates were centrifuged at 15,000 g for fifteen minutes, at 4 °C, and the resulting supernatant was incubated overnight in the presence of anti-GPI-PLC conjugated Protein-A agarose as described in section 2.5.3. Immunoprecipitates were prepared for SDS-PAGE, as described, and run on 15% polyacrylamide gels shown opposite. In each case, the GPI-PLC is highlighted by a red arrow. The loading scheme for each gel is shown below:

Gel (a):	Lane 1:	Molecular weight standards (Thermo-Scientific)
	Lane 2:	I.P. from WT cells lysed with 6 mM CHAPS
	Lane 3:	I.P. from GPI-PLC ^{-/-} cells lysed with 6 mM CHAPS
	Lane 4:	I.P. from WT cells lysed with 12 mM CHAPS
	Lane 5:	I.P. from GPI-PLC ^{-/-} cells lysed with 12 mM CHAPS
	Lane 6:	Molecular weight standards (Thermo-Scientific)
Gel (b):	Lane 1:	Molecular weight standards (Thermo-Scientific)
	Lane 2:	I.P. from WT cells lysed with 7 mM SDS
	Lane 3:	I.P. from GPI-PLC ^{-/-} cells lysed with 7 mM SDS
	Lane 4:	I.P. from WT cells lysed with 12 mM SDS
	Lane 5:	I.P. from GPI-PLC ^{-/-} cells lysed with 12 mM SDS
	Lane 6:	Molecular weight standards (Thermo-Scientific)
Gel (b):	Lane 1:	Molecular weight standards (Thermo-Scientific)
	Lane 2:	I.P. from WT cells lysed with 1 mM CTAB
	Lane 3:	I.P. from GPI-PLC ^{-/-} cells lysed with 1 mM CTAB
	Lane 4:	I.P. from WT cells lysed with 1.5 mM CTAB
	Lane 5:	I.P. from GPI-PLC ^{-/-} cells lysed with 1.5 mM CTAB
	Lane 6:	Molecular weight standards (Thermo-Scientific)

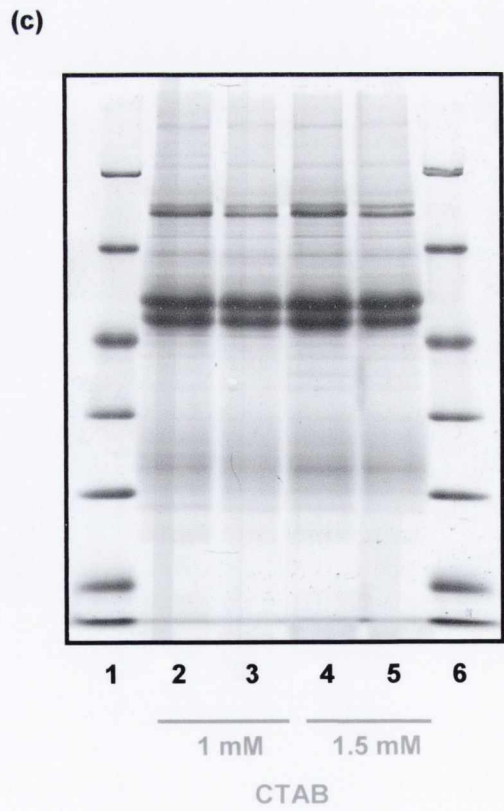
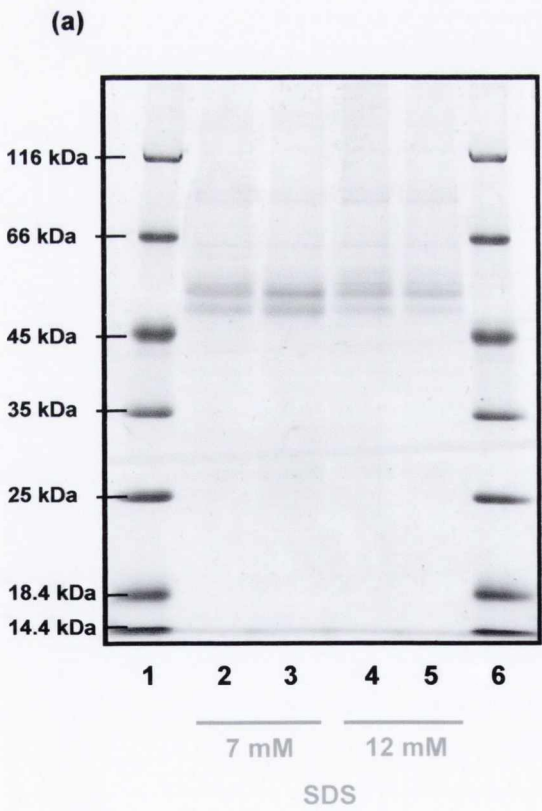
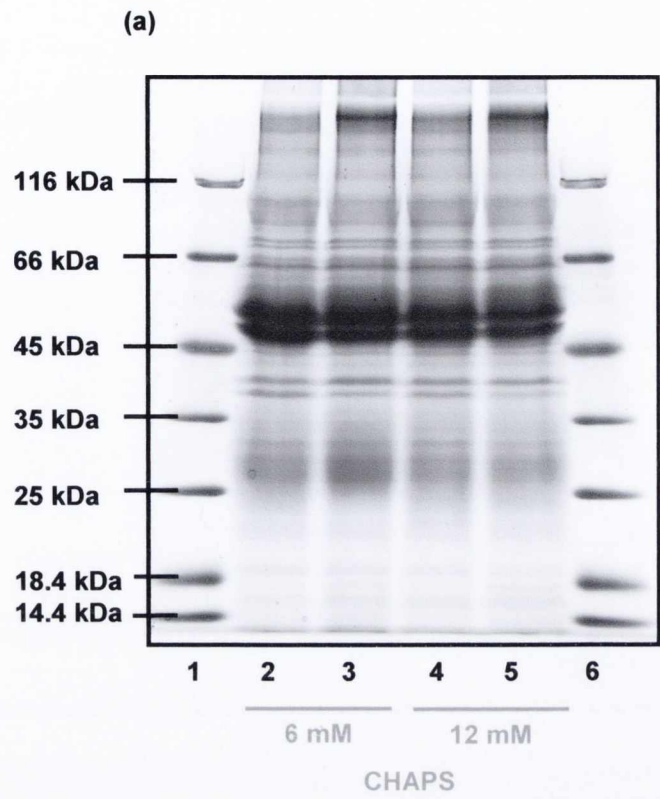


Plate 3.3. GPI-PLC Activity during Cell Lysis with non-Ionic Detergents

Trypanosomes, purified from an infected rat, were washed twice in standard Tes buffer before lysing in Tes buffer containing protease inhibitors and one of the detergents: Triton[®] X-100 (0.1 mM or 1.2 mM), n-octylglucoside (20 mM or 30 mM), or n-dodecyl- β -D-maltoside (0.1 mM or 0.8 mM). Lysates were centrifuged at 15,000 g for fifteen minutes, at 4 °C, before preparing a sample of the resulting supernatant for SDS-PAGE. WT MITat 1.2 cells, lysed by boiling in sample buffer, were used as a control to show mfVSG (black arrow). sVSG is highlighted with a red arrow and reflects the GPI-PLC in an “activated” state. In each gel, 2.5×10^6 cell equivalents were loaded per track. The loading scheme for each gel is shown below:

Gel (a):	Lane 1:	Molecular weight standards (Thermo-Scientific)
	Lane 2:	WT MITat 1.2 SDS lysates
	Lane 3:	Cells lysed with 0.1 mM Triton [®] X-100
	Lane 4:	Cells lysed with 1.2 mM Triton [®] X-100
	Lane 5:	Molecular weight standards (Thermo-Scientific)

Gel (b):	Lane 1:	Molecular weight standards (Thermo-Scientific)
	Lane 2:	WT MITat 1.2 SDS lysates
	Lane 3:	Cells lysed with 20 mM n-octylglucoside
	Lane 4:	Cells lysed with 30 mM n-octylglucoside
	Lane 5:	Molecular weight standards (Thermo-Scientific)

Gel (c):	Lane 1:	Molecular weight standards (Thermo-Scientific)
	Lane 2:	WT MITat 1.2 SDS lysates
	Lane 3:	Cells lysed with 0.1 mM n-dodecyl- β -D-maltoside
	Lane 4:	Cells lysed with 0.8 mM n-dodecyl- β -D-maltoside
	Lane 5:	Molecular weight standards (Thermo-Scientific)

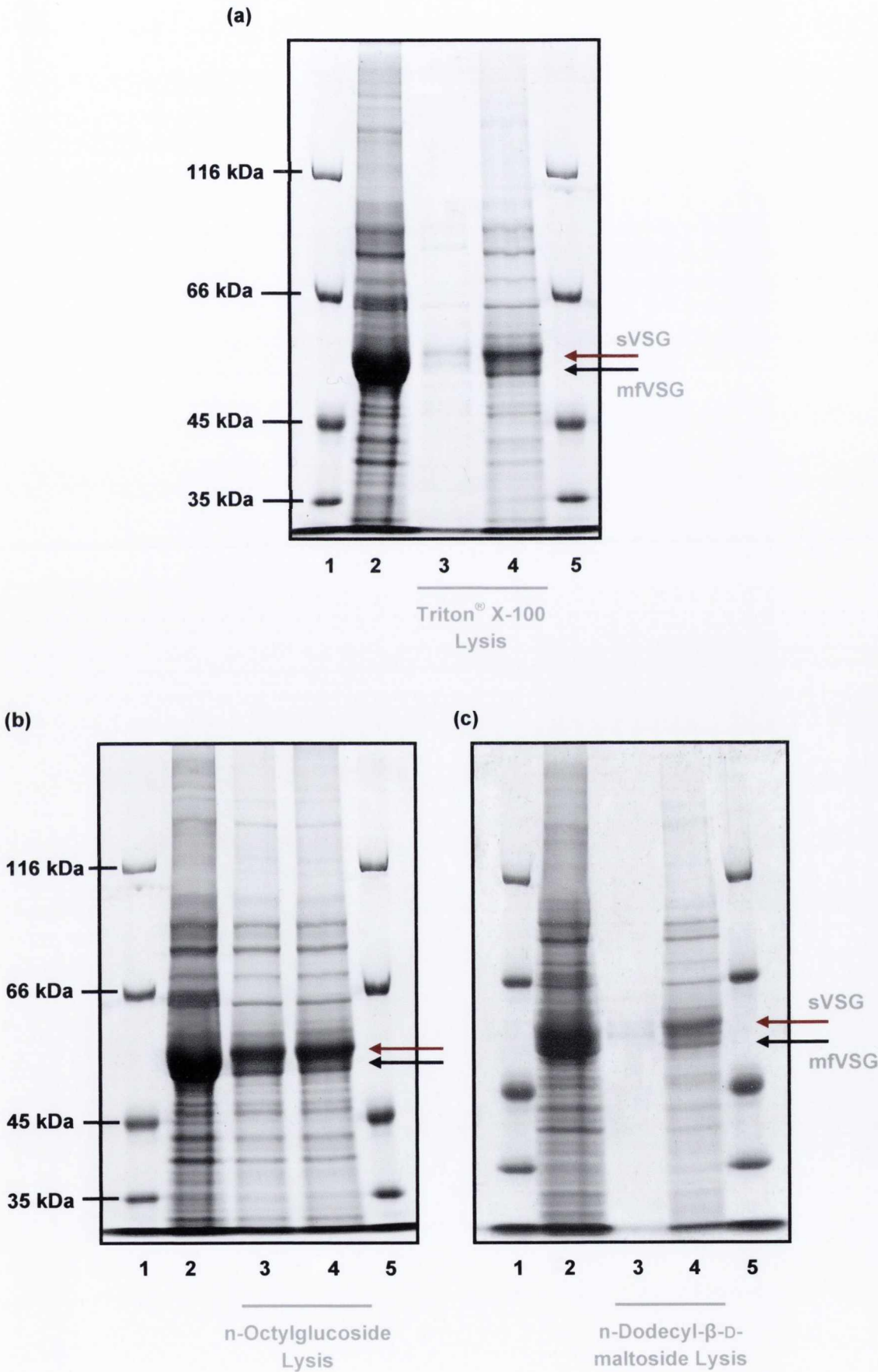


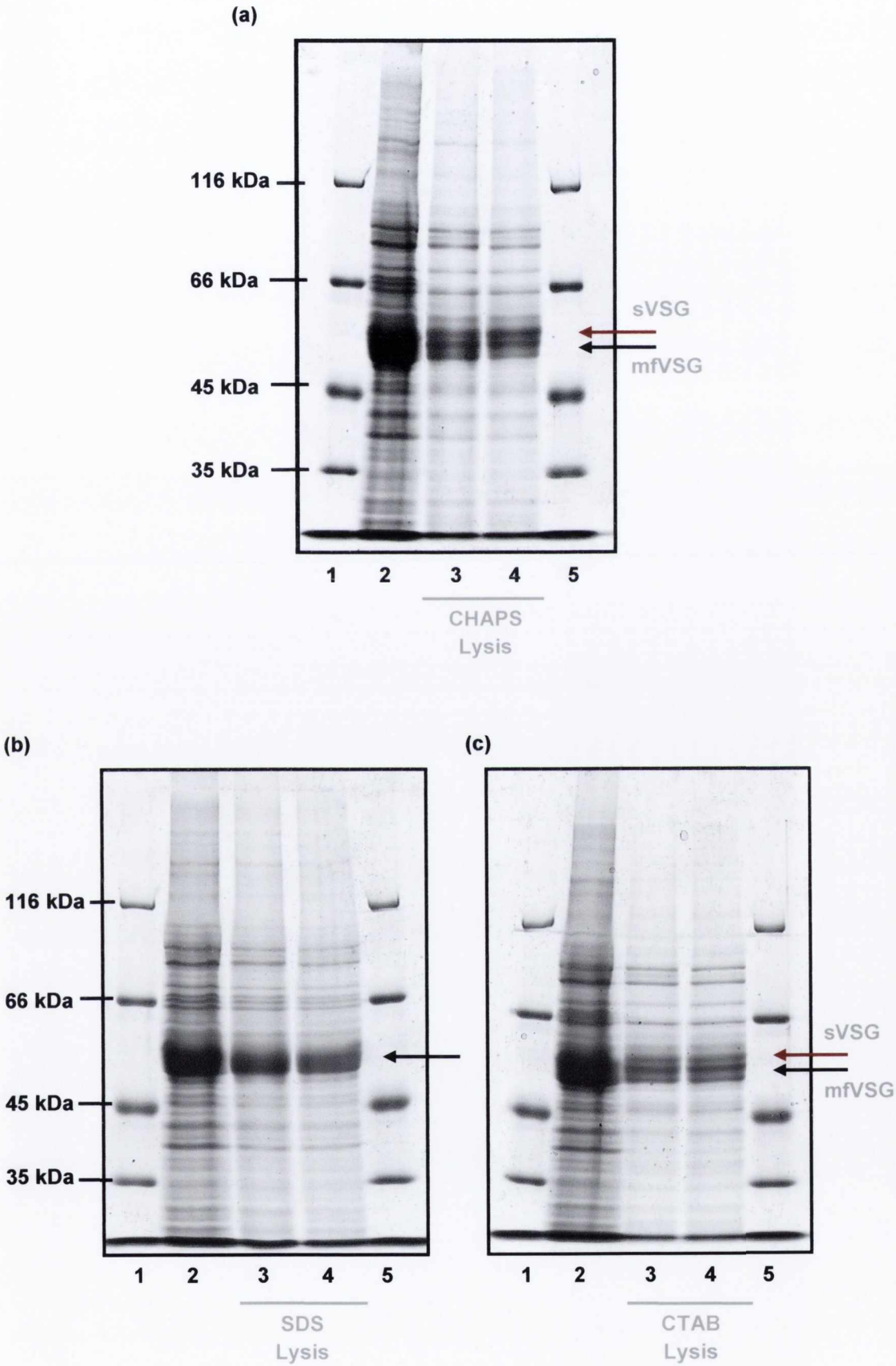
Plate 3.4. GPI-PLC Activity during Cell Lysis with Zwitterionic and Ionic Detergents

Trypanosomes, purified from an infected rat, were washed twice in standard Tes buffer before lysing in Tes buffer containing protease inhibitors and one of the detergents: CHAPS (6 mM or 12 mM), SDS (7 mM or 12 mM), or CTAB (1 mM or 1.5 mM). Lysates were centrifuged at 15,000 g for fifteen minutes, at 4 °C, before preparing a sample of the resulting supernatant for SDS-PAGE. WT MITat 1.2 cells, lysed by boiling in sample buffer, were used as a control to show mfVSG (black arrow). sVSG is highlighted with a red arrow and reflects the GPI-PLC in an “activated” state. In each gel, 2.5×10^6 cell equivalents were loaded per track. The loading scheme for each gel is shown below:

Gel (a):	Lane 1:	Molecular weight standards (Thermo-Scientific)
	Lane 2:	WT MITat 1.2 SDS lysates
	Lane 3:	Cells lysed with 6 mM CHAPS
	Lane 4:	Cells lysed with 12 mM CHAPS
	Lane 5:	Molecular weight standards (Thermo-Scientific)

Gel (b):	Lane 1:	Molecular weight standards (Thermo-Scientific)
	Lane 2:	WT MITat 1.2 SDS lysates
	Lane 3:	Cells lysed with 7 mM SDS
	Lane 4:	Cells lysed with 12 mM SDS
	Lane 5:	Molecular weight standards (Thermo-Scientific)

Gel (b):	Lane 1:	Molecular weight standards (Thermo-Scientific)
	Lane 2:	WT MITat 1.2 SDS lysates
	Lane 3:	Cells lysed with 1 mM CTAB
	Lane 4:	Cells lysed with 1.5 mM CTAB
	Lane 5:	Molecular weight standards (Thermo-Scientific)



3.2.8. Developments to the basic GPI-PLC Immunoprecipitation Protocol using n-Octylglucoside

The condition in which the GPI-PLC was immunoprecipitated in the presence of n-octylglucoside showed some promise in co-immunoprecipitating additional proteins, once the number of non-specific co-immunoprecipitates could be reduced. This procedure was therefore repeated with some modifications aimed at increasing the specificity of the technique. The effect of the GPI-PLC inhibitor, pCMPS, on the immunoprecipitation procedure was also studied; activation of the GPI-PLC during lysis conditions produces diglyceride, a detergent which could potentially disrupt interactions between the GPI-PLC and a putative partner protein. To reduce contamination of immunoprecipitated material with general cellular protein, the wash steps were increased to three 1 ml washes (in the same buffer that was used to generate the cell lysate). Finally, to increase the amount of GPI-PLC present, and to increase the amount of any potential partner protein present, the volume of cell lysate incubated with the anti-GPI-PLC conjugated Protein A agarose beads was increased to 1.5 ml (*i.e.* 1.5×10^9 cell equivalents).

Ultimately, rather than aiding the co-immunoprecipitation of potential partner proteins, the use of pCMPS actually prevented immunoprecipitation of the GPI-PLC itself (plate 3.5). It was observed during the lysis of bloodstream form trypanosomes in the presence of 10 mM pCMPS a large precipitate developed. This white precipitate occupied a volume of approximately 300 – 400 μ l, and had a consistency comparable to a light, wet, wool. In experiments this precipitate was avoided, and it was therefore necessary to prepare at least 2.5 ml of lysates in this condition to ensure enough lysate was present for the incubation with antibody conjugated Protein-A beads. Curiously, this precipitate was not observed in cases where a slightly tan coloured pCMPS powder was used (data not shown), but was always observed when a white powder was used. Both tan and white coloured powders appear within specifications of the manufacturer (a product information sheet is available under Sigma-Aldrich product number C4503). The reason for the differences observed is not known, but the observation is included here to aid future observations; all of the results presented in this thesis were taken from experiments using white powders of pCMPS.

The immunoprecipitation of the GPI-PLC from the n-octylglucoside lysate of 1.5×10^9 WT cells shows some differences to the control immunoprecipitation in GPI-PLC^{-/-} cells. Firstly, a number of co-immunoprecipitated proteins are visible in the immunoprecipitate from WT cells (in the absence of pCMPS); two to three protein bands are present amongst the smear of protein represent the IgG light chain, and additional protein bands are present at approximately 43 kDa and 19 kDa. However the co-immunoprecipitated band at 43 kDa is also visible in immunoprecipitates from WT and GPI-PLC^{-/-} cells that were lysed in the presence of pCMPS, and therefore probably represents a non-specific contamination. The protein band at approximately 19 kDa, although not present in the control immunoprecipitation from GPI-PLC^{-/-} cells in this instance, was present in all earlier immunoprecipitations performed in the presence of n-octylglucoside (shown in plate 4.1).

It is presently unclear whether the co-immunoprecipitated protein bands present between 35 kDa and 23 kDa are partly attributable to the IgG light chain, which runs as a broad smear between 18.4 kDa and 25 kDa. Further experiments are clearly required to resolve this issue and will require further studies on the trypanosomal proteins that can non-specifically interact with Protein-A agarose beads in the presence of 30 mM n-octylglucoside. If these proteins are precipitated through non-specific interactions then these interactions are likely to be relatively strong, as even after a single 1 ml wash in lysis buffer little contamination remained in the supernatant (plate 3.5). The migration of these proteins after β -mercaptoethanol has been omitted from the sample buffer should also be attempted.

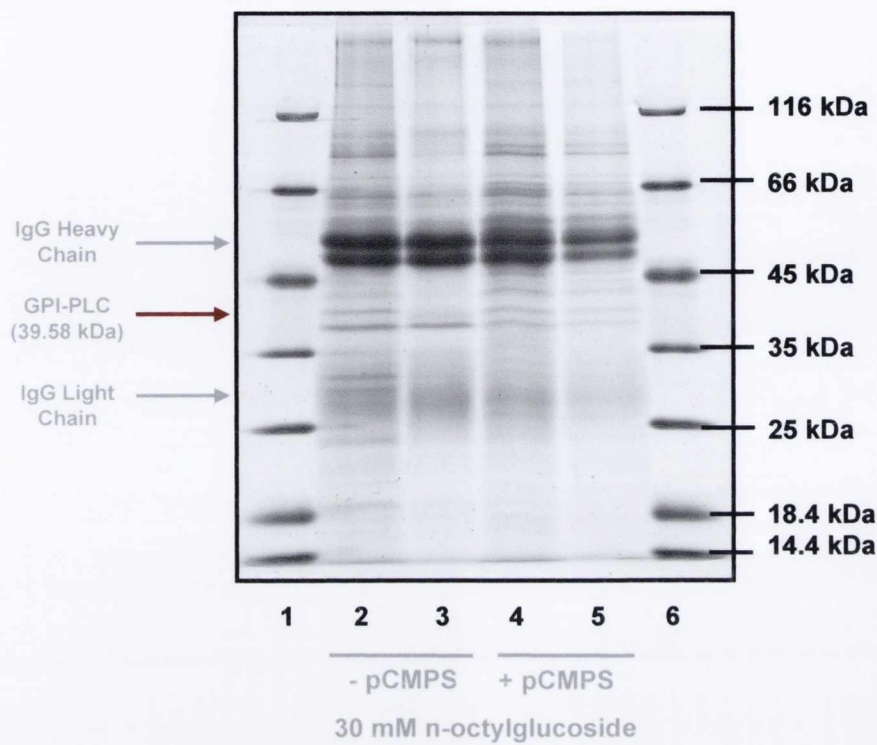
Keeping these concerns in mind, the detection of these proteins could simply have been dependent on the increase in cell numbers used for these last immunoprecipitation experiments. Additionally, the ease with which the GPI-PLC can be reproducibly immunoprecipitated as a 39 kDa protein band, using a standard Tes buffer containing 20 mM to 30 mM n-octylglucoside, highlight that these detergent conditions should be the principle conditions for considering further optimizations in the search for interacting proteins through immunoprecipitation based approaches.

Plate 3.5. GPI-PLC Immunoprecipitation using pCMPS and n-Octylglucoside

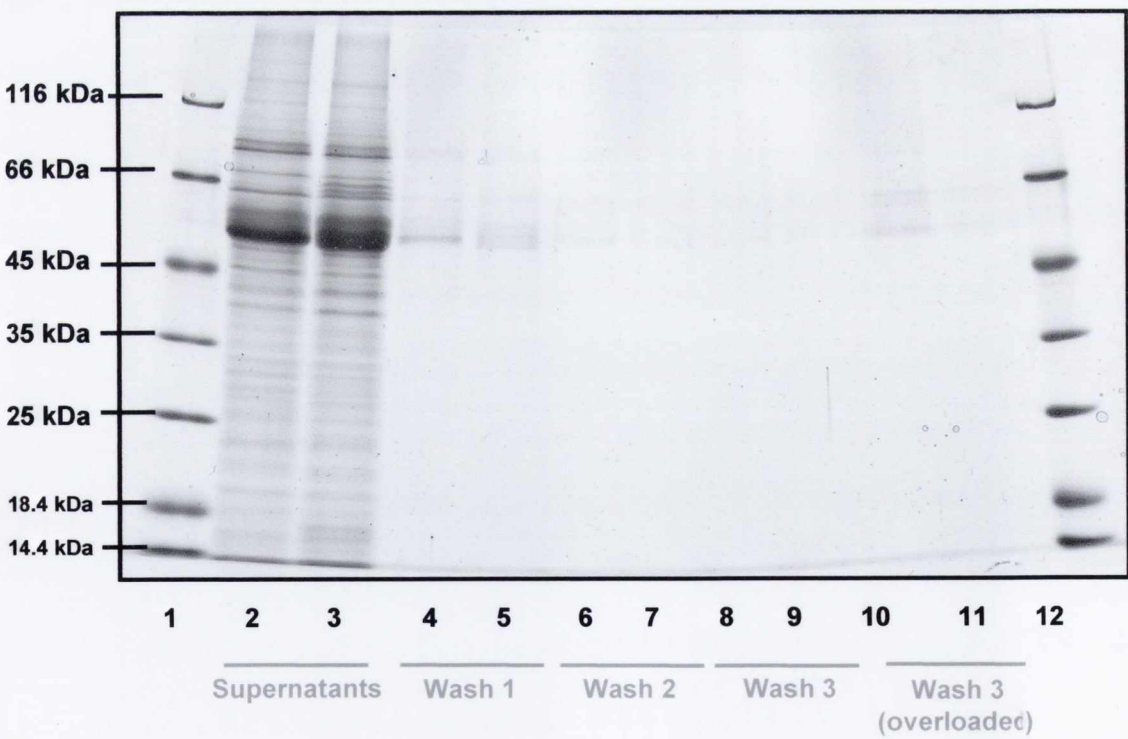
Trypanosomes, purified from an infected rat, were washed twice in Tes buffer before lysing in Tes buffer containing protease inhibitors, 30 mM n-octylglucoside, and 10 mM pCMPS. Lysates were centrifuged at 15,000 g for fifteen minutes, at 4 °C, and 1.5 ml of the resulting supernatant was incubated overnight in the presence of anti-GPI-PLC conjugated Protein-A agarose as described in section 2.5.3. Immunoprecipitates were prepared for SDS-PAGE, as described, and run on 15% polyacrylamide gels shown opposite. Immunoprecipitated GPI-PLC is highlighted by a red arrow. A second gel was run (gel b), loaded with samples of each wash step (with 3.13 µl of sample equivalent to 2.5×10^6 cells). A second portion of the final wash step (wash 3) was over-loaded into lanes ten and eleven to ensure minimal contamination was present. The loading scheme for each gel is shown below:

Gel (a):	Lane 1:	Molecular weight standards (Thermo-Scientific)
	Lane 2:	I.P. from WT cells lysed with 30 mM n-octylglucoside
	Lane 3:	I.P. from GPI-PLC ^{-/-} cells lysed with 30 mM n-octylglucoside
	Lane 4:	I.P. from WT cells lysed with 30 mM n-octylglucoside in the presence of 10 mM pCMPS
	Lane 5:	I.P. from GPI-PLC ^{-/-} cells lysed with 30 mM n-octylglucoside in the presence of 10 mM pCMPS
	Lane 6:	Molecular weight standards (Thermo-Scientific)
Gel (b):	Lane 1:	Molecular weight standards (Thermo-Scientific)
	Lane 2:	Supernatant following overnight incubation of lysates with IgG-conjugated beads (3.13 µl, - pCMPS)
	Lane 3:	Supernatant following overnight incubation of lysates with IgG-conjugated beads (3.13 µl, + pCMPS)
	Lane 4:	Sample of wash 1 (3.13 µl - pCMPS)
	Lane 5:	Sample of wash 1 (3.13 µl + pCMPS)
	Lane 6:	Sample of wash 2 (3.13 µl - pCMPS)
	Lane 7:	Sample of wash 2 (3.13 µl + pCMPS)
	Lane 8:	Sample of wash 3 (3.13 µl - pCMPS)
	Lane 9:	Sample of wash 3 (3.13 µl + pCMPS)
	Lane 10:	Sample of wash 3 (25 µl - pCMPS)
	Lane 11:	Sample of wash 3 (25 µl + pCMPS)
	Lane 12:	Molecular weight standards (Thermo-Scientific)

(a)



(b)



3.3. Discussion

The first aim of this chapter was successfully achieved; a robust procedure for confidently identifying Coomassie stained proteins, present in polyacrylamide gels, was validated. The standard procedure, optimized for using trypsin to generate peptides for mass fingerprinting and based largely on the protocol of Shevchenko *et al.* (2006), was shown to work also with chymotrypsin. Using chymotrypsin did not produce peptides with any associated detection biases, as was found with proteins that were digested with trypsin, and also resulted in excellent sequence coverage of the protein under study. Additionally, using this second protease for generating additional peptides for mass fingerprinting is expected to greatly enhance the confidence in obtaining correct protein identifications by, firstly, confirming identifications achieved with tryptic digests of proteins and, secondly, by increasing the total sequence coverage obtained for identified proteins. Although mass spectra obtained from chymotrypsin digests of proteins presented here were comparatively “noisy” compared to the mass spectra obtained from tryptic digests, with a lower percentage of significant mass peaks matched to the protein under study, this could likely be improved by correctly titrating the chymotrypsin to a more appropriate concentration.

Immunoprecipitation of the GPI-PLC was successful when Triton[®] X-100, or n-octylglucoside, was used as the detergent of choice (once the concentration of detergent used was sufficient to solubilize cells, and once pCMPS was omitted from the protocol). Surprisingly, n-dodecyl- β -D-maltoside, which shares similar chemical characteristics to n-octylglucoside, was not compatible with GPI-PLC immunoprecipitation. An important aspect of co-immunoprecipitation experiments is that they can yield information on the detergent conditions that disrupt protein-protein interactions, or influence denaturation of the target or partner proteins. Determining the difference between n-octylglucoside and n-dodecyl- β -D-maltoside that ultimately decides the success of the immunoprecipitation procedure may be vital in understanding the interactions of the GPI-PLC with potential partner proteins, or might yield information on the interaction of the GPI-PLC with different detergents. The GPI-PLC, for example, may bind sufficient amounts of n-dodecyl- β -D-maltoside to limit the amount of anti-GPI-PLC antibody that can bind. Developing an understanding of the interactions between the GPI-PLC and various detergents may prove invaluable

in understanding the association between the GPI-PLC and the trypanosomal membrane.

A large number of commercially available analogues of n-octylglucoside and n-dodecyl- β -D-maltoside exist to make such a study feasible. Two sets of experiments could be performed, and the immunoprecipitation of the GPI-PLC studied in each. Comparing immunoprecipitations with both non-polar detergent head groups (*i.e.* the malto(pyranose)side *versus* gluco(pyrano)side detergent head groups) while varying the detergent tail length would determine the crucial detergent factors that influence immunoprecipitation of the GPI-PLC. Detergent tail lengths covering hexyl (*i.e.* a tail length of C₆H₁₃), octyl (C₈H₁₇), nonyl (C₉H₁₉) and dodecyl (C₁₂H₂₅) pyranosides are readily available. Similarly, keeping the octyl tail chain of the detergent constant, while varying the head group may be useful – n-octyl- β -D-galactopyranoside, n-octyl- β -D-thioglucopyranoside and n-octanoylsucrose, are readily available detergents that could yield information on the carbohydrate moieties that promote or disrupt protein-protein interactions (including interactions between the GPI-PLC and anti-GPI-PLC IgG).

Immunoprecipitation of the GPI-PLC was also achieved using Hanrahan's Nonidet P-40 and CHAPS based buffer recipe (Hanrahan, personal communication). Triton[®] X-100 (octylphenoxypolyethoxyethanol) is structurally very similar to Nonidet P-40 [also octylphenoxypolyethoxyethanol, (note that to this author, Triton[®] X-100 and Nonidet P-40 essentially only represent different trade names for the same detergent, with perhaps some differences in the ratios of the different polymer chain lengths present in each mixture)], so it is perhaps unsurprising that both detergents can be used to immunoprecipitate the GPI-PLC. However, during this work no specific co-immunoprecipitating proteins were found using a Nonidet P-40 and CHAPS based buffer, a Triton[®] X-100 based buffer, or an n-octylglucoside based buffer.

It is currently unclear why specific interactions were detected by Hanrahan *et al.*, (2009) using an NP-40 (nonylphenoxypolyethoxyethanol) and CHAPS based buffer, while these interactions were not observed here. One possible explanation is that the detection of co-immunoprecipitated surface-iodinated proteins is more sensitive than a Coomassie-based approach, however only the later approach is readily compatible with the subsequent MALDI-ToF mass spectrometry necessary for protein

identification. Visualization of co-immunoprecipitates by Coomassie staining was therefore a necessary adaption to earlier protocols. It also remains possible, although unlikely, that differences between “nonyl”phenoxypolyethoxyethanol (NP-40), and “octyl”phenoxypolyethoxyethanol (Nonidet P-40 and Triton[®] X-100) are sufficient to cause the observed differences, and therefore repeating an immunoprecipitation experiment in the presence of NP-40 should be conducted.

A necessary step, however, in attempting additional immunoprecipitation conditions is to first produce more anti-GPI-PLC IgG, as current stocks have been exhausted. A procedure to generate a recombinant fusion protein of maltose binding protein (MBP) protein and the GPI-PLC, which can be used for the production of anti-GPI-PLC immunoglobulins, is presented in appendix A. This MBP-GPI-PLC should also be considered a powerful tool in confirming co-immunoprecipitations. Using MBP-GPI-PLC mixed with GPI-PLC^{-/-} cell lysates should result in the co-immunoprecipitation of any partner proteins observed in WT cell lysates, once the precise detergent conditions have been optimized. Further uses of this recombinant MBP-GPI-PLC in probing for protein-protein interactions will be considered in chapter six.

Chapter 4

GPI-PLC-Mediated VSG Release during the De-energization of Bloodstream Form Trypanosomes

4.1. Introduction

4.1.1. Experimental Release of the VSG from Trypanosomes

The VSG of *T. brucei* has been the subject of intense research in the trypanosome field. Although the release of the VSG has been studied enthusiastically for a number of decades, our understanding of the mechanisms underlying release of the VSG from the trypanosome membrane remains largely incomplete. What can be said, with confidence, is that the GPI-PLC is responsible for hydrolyzing the GPI-anchor of the VSG under a number of experimental conditions, and hydrolysis of this GPI-anchor causes a release of the VSG into the extracellular medium. However the identity of any signalling molecule responsible for directly activating the GPI-PLC has remained elusive. Similarly, no member of a putative signalling pathway culminating in the activation of the GPI-PLC has been found.

Currently there is evidence that the GPI-PLC is regulated by Ca^{2+} ions; it has been shown that in the presence of the calcium ionophore A-23187, Ca^{2+} stimulates a release of the VSG (Bowles and Voorheis, 1982). Later evidence by Jackson (1983) implicated the GPI-PLC in this VSG release process by showing that the VSG released during the treatment of trypanosomes with A-23187 and Ca^{2+} migrated with a higher apparent molecular weight (assessed by SDS-PAGE), analogous to sVSG. Therefore, the fact that Ca^{2+} ions can activate an enzyme without any known calcium-binding domain suggests an exciting model: that other, calcium-binding, proteins are involved in regulating the GPI-PLC. However, there is still no evidence that such a hypothetical

protein interacts directly with the GPI-PLC, and it remains speculative as to at what point precisely Ca^{2+} might act in the VSG release process.

Although it may broadly be said that “stress” conditions activate the GPI-PLC, such as low hydrogen ion concentration (Rolin *et al.*, 1996) or removal of glucose from the extracellular medium (Brabazon, 1999), the involvement of Ca^{2+} in these circumstances is not clear. It should be noted though, that low pH has been demonstrated to cause an increase in intracellular Ca^{2+} in *T. brucei* (Ruben *et al.*, 1991), and it is certainly conceivable that de-energizing trypanosomes could result in Ca^{2+} leakage from intracellular stores.

Currently, stress conditions that result in VSG release can be categorized into two broad groups. First are those stress conditions that also result in cell rupture, such as osmotic shock (Bowles and Voorheis, 1982; Cardoso de Almeida and Turner, 1983). Second are those conditions in which the VSG is released while the cells remain intact, such treatment with A-23187 and Ca^{2+} (Bowles and Voorheis, 1982), exposure to low hydrogen ion concentration (Rolin *et al.*, 1996) and de-energization (Hanrahan *et al.*, 2009). While the GPI-PLC has been implicated in each of these processes, no protein that regulates the GPI-PLC under these experimental conditions has been found.

4.1.2. Aims

The possibility that Ca^{2+} is involved in regulating GPI-PLC activity is certainly an attractive one, and a possibility that seems to have received only little attention to date. The availability of GPI-PLC^{-/-} mutant cells provides great potential for detailed studies on the activation and regulation of the GPI-PLC, and it seems timely that such a study be carried out. Therefore, the aim of this chapter is to study the VSG release process in an effort to more greatly understand the role that Ca^{2+} plays in regulating the GPI-PLC of *T. brucei*. In particular, the release of VSG during de-energization of trypanosomes will be monitored, and the release of surface proteins will be characterized. Additionally, the GPI-PLC-mediated release of VSG during trypanosomal de-energization is being studied to determine whether the GPI-PLC interacts with any Ca^{2+} binding protein.

4.2. Results

4.2.1. The De-energization of Bloodstream Form Trypanosomes in Phosphate Buffer Causes a Release of the VSG

Using the basic protocol set out by Brabazon (1999), bloodstream form trypanosomes were de-energized by incubating at a density of 1×10^9 cells·ml⁻¹ in hyperosmotic (approximately 450 mOsm) phosphate buffer containing 25 mM 2-deoxy-D-glucose. Samples taken at chosen time points were cold-quenched in five volumes of buffer before centrifuging as described in materials and methods and measuring the absorbance of the supernatants of each sample at 280 nm. As other workers had speculated over the production of hypoxanthine during the de-energization process, the absorbance of each supernatant at 260 nm was also measured (figure 4.1). Samples of the released protein were also visualized by SDS-PAGE (figure 4.1).

Although the general trend of protein release observed in figure 4.1 is almost identical to other workers (Brabazon, 1999; Hanrahan 2006), there are a number of important distinctions that can be observed. The absorbance at 260 nm of each supernatant sample was consistently higher than the absorbencies measured at 280 nm, and both measurements followed the same trend. While hypoxanthine may undoubtedly contribute to the absorbance measurements, it seems unlikely that the production of hypoxanthine (measured at 260 nm) should follow precisely the same trend as the release of VSG from the trypanosome surface (measured at 280 nm). Furthermore, this experiment revealed that VSG is not the only protein released during the de-energization process; many proteins are released over a time course identical to that of the VSG. From figure 4.1 it is seen that VSG release begins following thirty to forty minutes of de-energization, while the release of additional proteins, the increase in absorbance at 280 nm, and the increase in absorbance at 260 nm, follow precisely the same trend.

Since the released protein visualized by SDS-PAGE looked like general cell lysis, and because the time-course of the protein release correlated with the half lives in aqueous solution of two of the protease inhibitors used (*i.e.* PMSF and TLCK), the de-energization procedure was repeated. In this instance however, fresh aliquots of PMSF and TLCK were added to the de-energization incubation every ten minutes (figure 4.3).

This precaution failed to rescue the apparent cell breakdown, so the protein release cannot be an artefact resulting from the hydrolysis, and inactivation, of PMSF or TLCK.

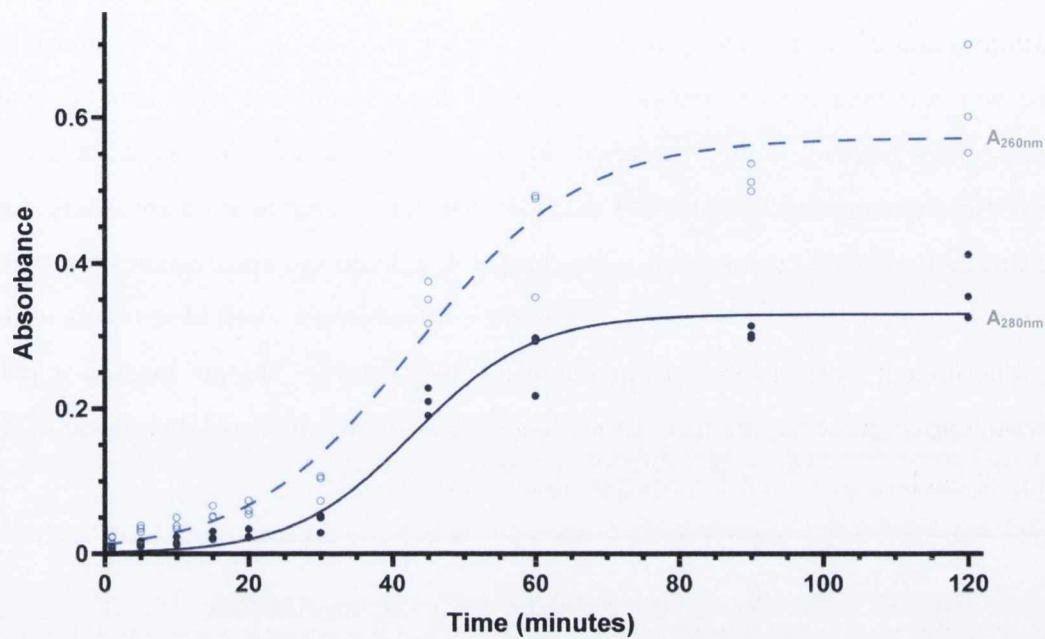
To test if the GPI-PLC was necessary for the release of protein during trypanosomal de-energization, GPI-PLC^{-/-} cells were subjected to the same de-energization process. Surprisingly, trypanosomes lacking this enzyme also released similar proteins over a similar time course, with the exception being that no VSG was detected in the extracellular medium following de-energization (figure 4.3).

Figure 4.1. Protein Release from WT Cells Incubated in a De-energizing Phosphate Buffer

WT MITat 1.2 cells were de-energized in phosphate buffer, containing protease inhibitors and 25 mM 2-deoxy-**D**-glucose, for two hours. Samples of the de-energized cells were taken at the time points shown, cold quenched in five volumes of phosphate buffer, and immediately centrifuged at 18,000 *g* for 45 seconds. The supernatants were further centrifuged at 15,000 *g* for 15 minutes (and at 4 °C) and the absorbencies at 260 nm and 280 nm were measured. The recorded absorbencies are shown in (a), with the broken blue line and empty circles showing the absorbance of each supernatant at 260 nm, and the solid blue line with filled circles showing the absorbance at 280. A portion of each supernatant was also kept for SDS-PAGE and run on a 10% gel, shown in (b). The gel loading scheme is given below (2.5 x 10⁶ cell equivalents were loaded on each track).

Lane 1:	Molecular weight standards (Sigma-Aldrich)
Lane 2:	0 minute supernatant
Lane 3:	1 minute supernatant
Lane 4:	5 minute supernatant
Lane 5:	10 minute supernatant
Lane 6:	15 minute supernatant
Lane 7:	20 minute supernatant
Lane 8:	30 minute supernatant
Lane 9:	45 minute supernatant
Lane 10:	60 minute supernatant
Lane 11:	90 minute supernatant
Lane 12:	120 minute supernatant

(a)



(b)

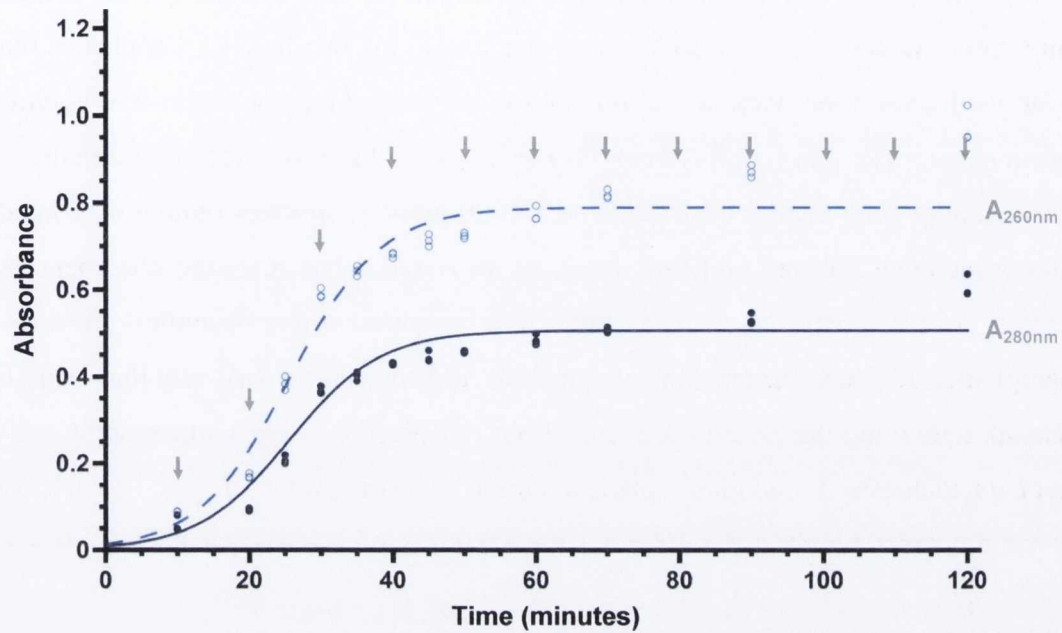


Figure 4.2. Protein Release from WT Cells Incubated in a De-energizing Phosphate Buffer with Additions of Protease Inhibitors Throughout

WT MITat 1.2 cells were de-energized in phosphate buffer, containing protease inhibitors and 25 mM 2-deoxy-D-glucose. Further aliquots of PMSF were added at the time points indicated with arrows. Samples of the de-energized cells were taken at the time points shown, cold quenched in five volumes of phosphate buffer, and immediately centrifuged at 18,000 *g* for 45 seconds. The supernatants were further centrifuged at 15,000 *g* for 15 minutes (and at 4 °C) and the absorbencies measured at 260 nm (dashed, light blue trace) and at 280 nm (unbroken, dark blue trace). Further sample of each supernatant were prepared for SDS-PAGE. The gel loading scheme is shown below (note that the time points loaded are slightly different than those in figure 4.1). In each case, 2.5×10^6 cell equivalents were loaded.

Lane 1:	Molecular weight standards (Sigma-Aldrich)
Lane 2:	0 minute supernatant
Lane 3:	10 minute supernatant
Lane 4:	20 minute supernatant
Lane 5:	25 minute supernatant
Lane 6:	30 minute supernatant
Lane 7:	35 minute supernatant
Lane 8:	40 minute supernatant
Lane 9:	50 minute supernatant
Lane 10:	60 minute supernatant
Lane 11:	90 minute supernatant
Lane 12:	SDS lysate

(a)



(b)

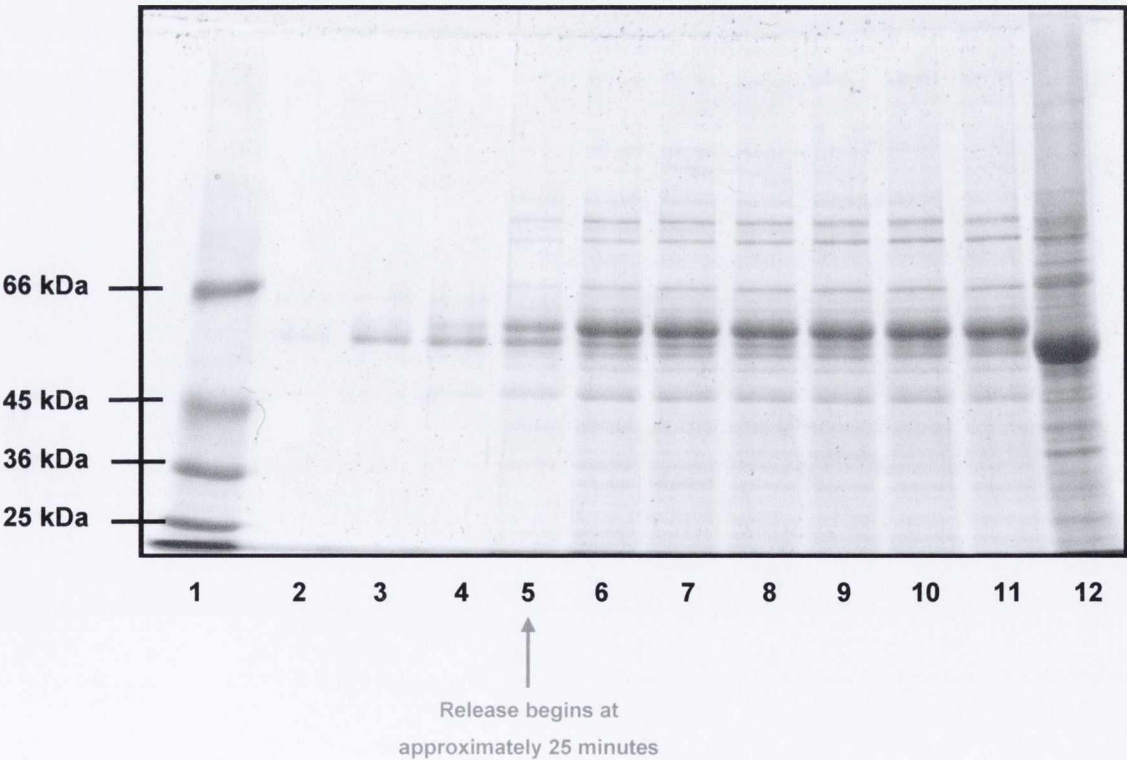
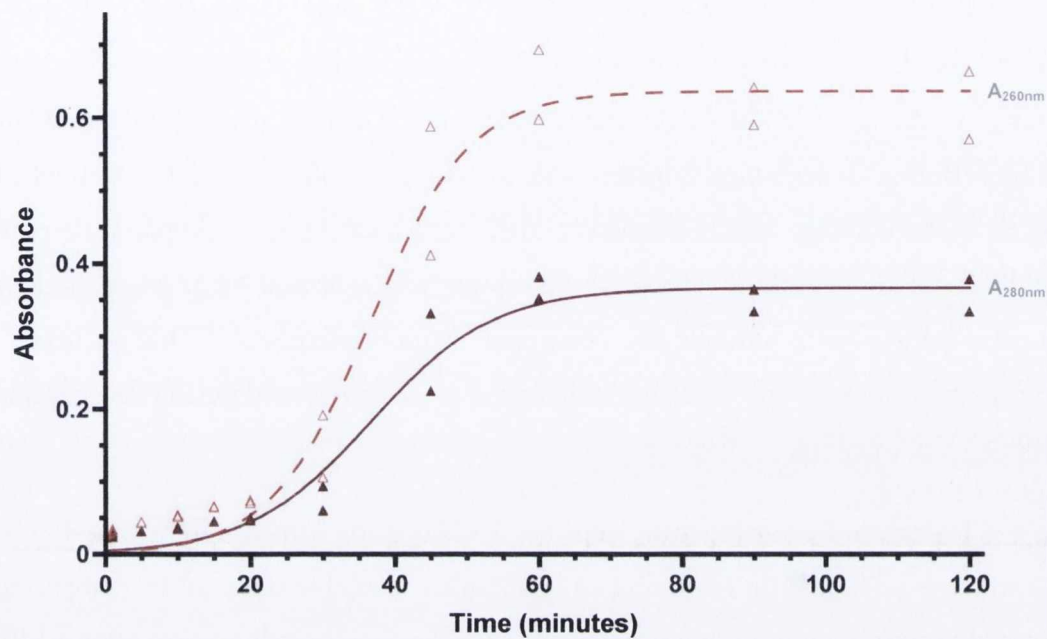


Figure 4.3. Protein Release from GPI-PLC^{-/-} Cells Incubated in a De-energizing Phosphate Buffer

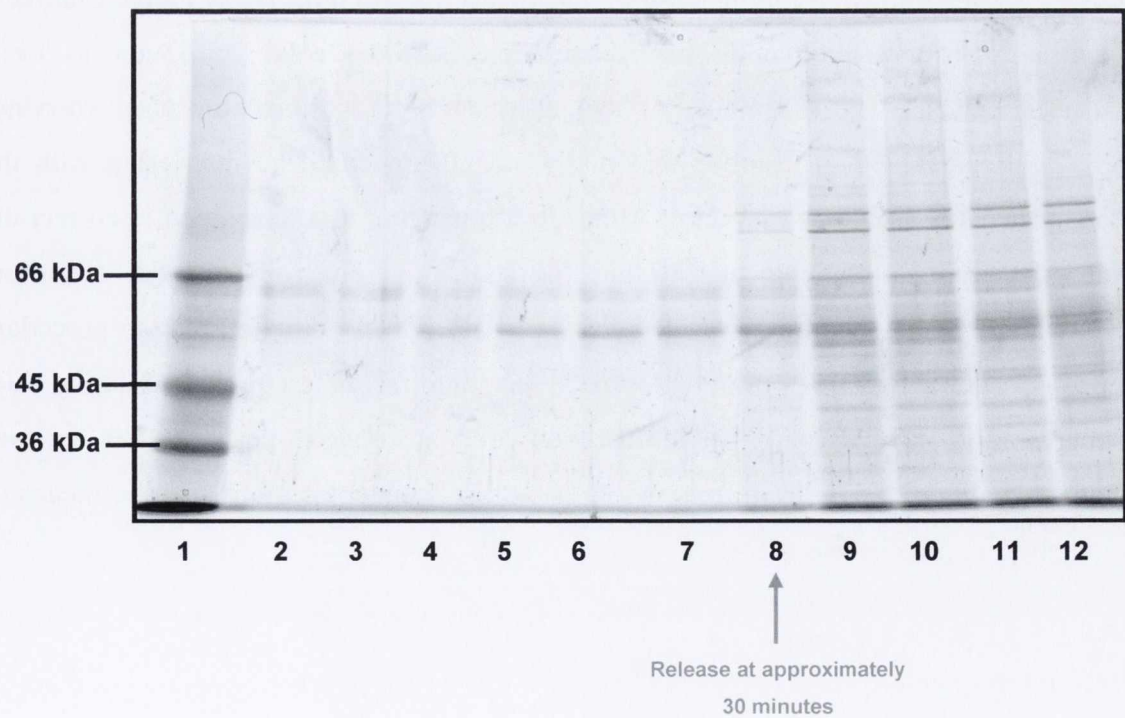
GPI-PLC^{-/-} MITat 1.2 cells were de-energized in phosphate buffer, containing protease inhibitors and 25 mM 2-deoxy-D-glucose, for two hours. Samples of the de-energized cells were taken at the time points shown, cold quenched in five volumes of phosphate buffer, and immediately centrifuged at 18,000 *g* for 45 seconds. The supernatants were further centrifuged at 15,000 *g* for 15 minutes (and at 4 °C) and the absorbencies at 260 nm and 280 nm were measured. The recorded absorbencies are shown in (a), with the broken, light red line and empty triangles showing the absorbance of each supernatant at 260 nm, and the solid, dark red line with filled triangles showing the absorbance at 280 nm. A portion of each supernatant was also kept for SDS-PAGE and electrophoresed on a 10% gel, shown in (b). The gel loading scheme is given below (2.5 x 10⁶ cell equivalents were loaded on each track).

Lane 1:	Molecular weight standards (Sigma-Aldrich)
Lane 2:	0 minute supernatant
Lane 3:	1 minute supernatant
Lane 4:	5 minute supernatant
Lane 5:	10 minute supernatant
Lane 6:	15 minute supernatant
Lane 7:	20 minute supernatant
Lane 8:	30 minute supernatant
Lane 9:	45 minute supernatant
Lane 10:	60 minute supernatant
Lane 11:	90 minute supernatant
Lane 12:	120 minute supernatant

(a)



(b)



4.2.2. Testing the VSG Release Protocol

That the quantitative release of protein, followed at 280 nm, was almost identical in de-energized WT and GPI-PLC^{-/-} cells was unexpected. It was also unexpected that so many proteins appeared to be released over the de-energization time course. Great care had been taken in the experimental design to avoid contamination of de-energization supernatant samples with insoluble cellular material. A quick, high speed, centrifugation step immediately after cold-quenching a sample time-point to rapidly remove the bulk of cells from the sample was followed by a longer, high speed centrifugation step to remove any remaining insoluble material. This sequence was expected to minimize any contamination of the proteins released during de-energization with general, insoluble, cell debris.

The second centrifugation step (*i.e.* 15,000 **g** for fifteen minutes, at 4 °C) was shown to be sufficient for removing any remaining insoluble material by comparing the released protein present in this supernatant with the protein still present after a 140,000 **g** centrifugation step for one hour (also at 4 °C). Shown in figure 4.4, the protein remaining in the supernatant after each centrifugation procedure is identical.

After following the increase in absorbance at 260 nm, it was thought unlikely that a release or production of hypoxanthine was the sole contributor to these measurements. The magnitude of the absorbance at 260 nm was also worrying; material released that absorbed maximally at 260 nm could be interfering with the quantification of released protein. Although a procedure was developed to correct the absorbance measurements at 280 nm for contamination from material absorbing maximally at neighbouring wavelengths (see Appendix B), this correction procedure was not adopted for this work. Absorbance spectra of the material released during a de-energization experiment revealed that only one absorbance peak at 260 nm was detected (figure 4.5). Therefore, it was necessary to adopt a more specific means of quantifying protein release.

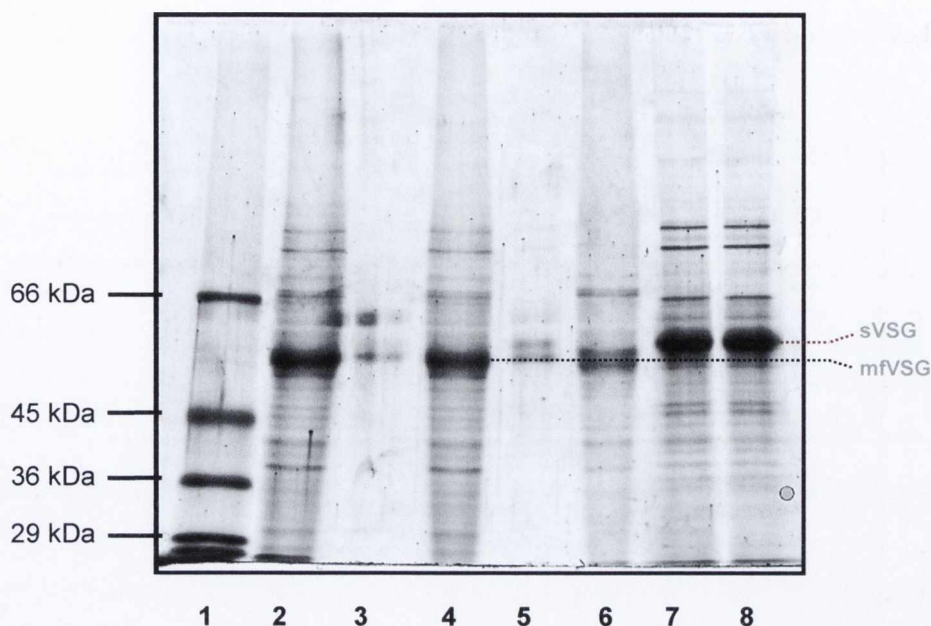


Figure 4.4. Released Protein Present in the Supernatant following De-energization is Soluble

WT MITat 1.2 cells were de-energized in phosphate buffer containing protease inhibitors and 25 mM 2-deoxy-D-glucose. Samples of the de-energized cells were taken after zero, thirty, and sixty minutes, cold quenched, and immediately centrifuged at 18,000 *g* for 45 seconds. The resulting pellets were kept for SDS-PAGE whilst the supernatants were further centrifuged at 15,000 *g* for 15 minutes (and at 4 °C) before keeping for SDS-PAGE. A duplicate sample of the 60 minute supernatant was instead centrifuged at 140,000 *g* for 1 hour to ensure all protein present in the released supernatant represented soluble protein. The loading scheme is given below (2.5 x 10⁶ cell equivalents were loaded on each lane of a 10% gel).

Lane 1:	molecular weight standards (Sigma)
Lane 2:	0 minutes, pellet
Lane 3:	0 minutes, supernatant
Lane 4:	30 minutes, pellet
Lane 5:	30 minutes, supernatant
Lane 6:	60 minutes, pellet
Lane 7:	60 minutes, supernatant (following 15,000 <i>g</i> centrifugation)
Lane 8:	60 minutes, supernatant (following 140,000 <i>g</i> centrifugation)

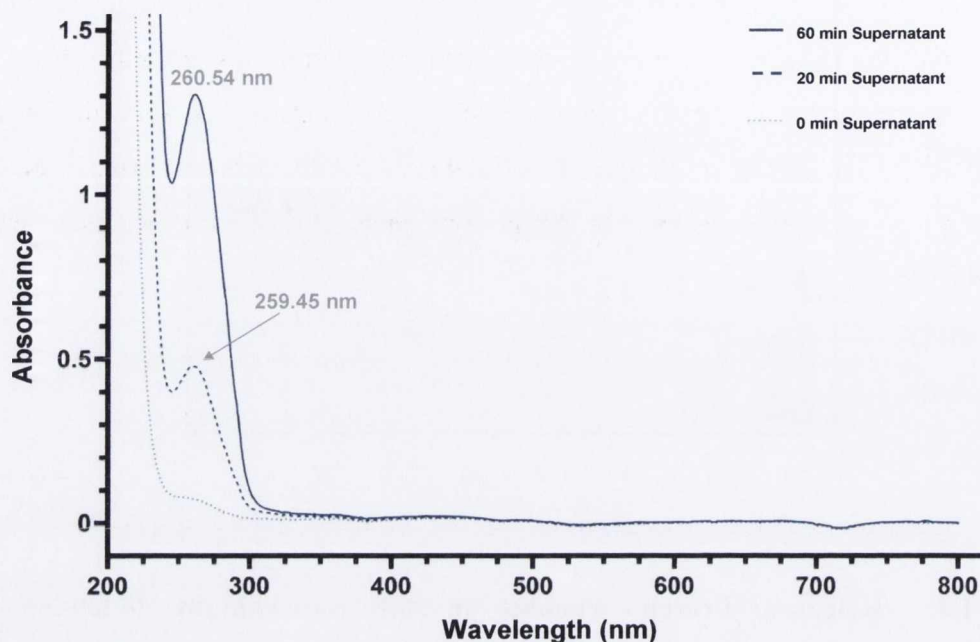


Figure 4.5. Absorbance Spectra of Material Released During Trypanosomal De-energization

WT trypanosomes were de-energized in a Tes-based buffer containing 25 mM 2-deoxy-D-glucose as described in materials and methods. Samples were taken at zero minutes, 20 minutes, and 60 minutes and old quenched in five volumes of hyperosmotic Tes buffer before centrifuging at 18,000 *g* for 45 seconds. The resulting supernatant was again centrifuged, at 15,000 *g* for 15 minutes and at 4 °C. Absorbance spectra for each of the resulting supernatants are shown, with the absorbance maxima labelled for the 20 minute supernatant sample and for the 60 minute supernatant sample.

4.2.3. Suitability of the Pierce 660 nm Protein Assay Reagent

Of a number of available protein assay reagents, a determining factor in choosing the Pierce 660 nm protein assay reagent for quantifying protein release was this reagent's compatibility with metal chelating agents such as EGTA, which were to be used in future de-energization experiments. Furthermore, purines such as hypoxanthine and adenosine were shown here to have no effect on the assay reagent (figure 4.6).

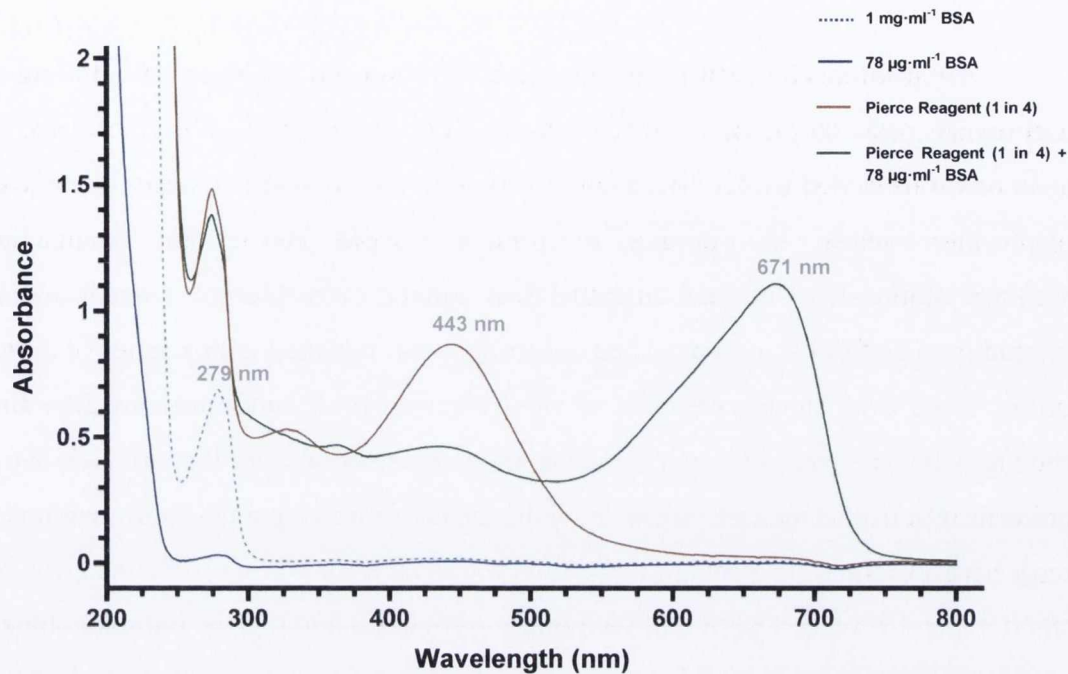
However, while the Pierce 660 nm protein assay reagent is compatible with EGTA, the reagent is not as sensitive as other dye-based protein assays. From absorbance measurements of de-energized samples, protein was found to be released to a final concentration of approximately $325 \mu\text{g}\cdot\text{ml}^{-1}$ (assuming an A_{280} of 1.0 is approximately equal to $1 \text{ mg}\cdot\text{ml}^{-1}$ of protein), or $260 \mu\text{g}\cdot\text{ml}^{-1}$ following application of the correction procedure outlined in Appendix B. Therefore, it was tested if the Pierce 660 nm protein assay reagent was compatible with sample volumes higher than the $10 \mu\text{l}$ recommended by the manufacturer.

Shown in figure 4.7, over an appropriate range of protein concentrations, the Pierce 660 nm protein assay reagent still responded linearly when $15 \mu\text{l}$ of sample, $20 \mu\text{l}$ of sample, and even $30 \mu\text{l}$ of sample was mixed with the assay reagent (in proportions always resulting in a final, total volume of $160 \mu\text{l}$). However there was a noticeable divergence from linearity when $50 \mu\text{l}$ of sample was mixed with $110 \mu\text{l}$ of assay reagent. Additionally, there was only a minimal decrease in efficiency of the reagent's detection of protein when $20 \mu\text{l}$ of sample was mixed with $140 \mu\text{l}$ of the reagent. Efficiency of the reagent then continued to decrease as the sample volume was increased past $20 \mu\text{l}$. These data confirmed that it was perfectly acceptable, and comfortably within the assay reagent's working limits, to instead mix $20 \mu\text{l}$ of sample with $140 \mu\text{l}$ of the Pierce 660 nm protein assay reagent. The maintenance of a linear response to protein concentration under these conditions satisfies any concerns that the trends of protein released presented during this chapter are artefacts of a protein assay reagent being used outside of the recommended assay conditions.

Figure 4.6. Absorbance Spectra of the Pierce 660 nm Protein Assay Reagent; Adenosine and Hypoxanthine have no Effect on the Reagent

An absorbance spectrum of the Pierce 660 nm protein assay reagent yields an absorbance peak at 443 nm. After mixing with a $5 \text{ mg}\cdot\text{ml}^{-1}$ solution of BSA this absorbance peak was shifted to 671 nm (shown in 'a'). In order to ensure the majority of the Pierce reagent's absorbance spectrum was within the measurable range of the spectrophotometer, solutions containing the reagent were diluted 1 in 4. The final concentration of BSA present in the Pierce reagent and BSA mixture is therefore $78 \text{ }\mu\text{g}\cdot\text{ml}^{-1}$. Absorbance spectra of $1 \text{ mg}\cdot\text{ml}^{-1}$ and $78 \text{ }\mu\text{g}\cdot\text{ml}^{-1}$ solutions of BSA are also shown in (a) as a comparison. The absorbance spectrum of the Pierce reagent was not affected by relevant concentrations of hypoxanthine or adenosine ($40 \text{ }\mu\text{M}$ and $35 \text{ }\mu\text{M}$ respectively), (b).

(a)



(b)

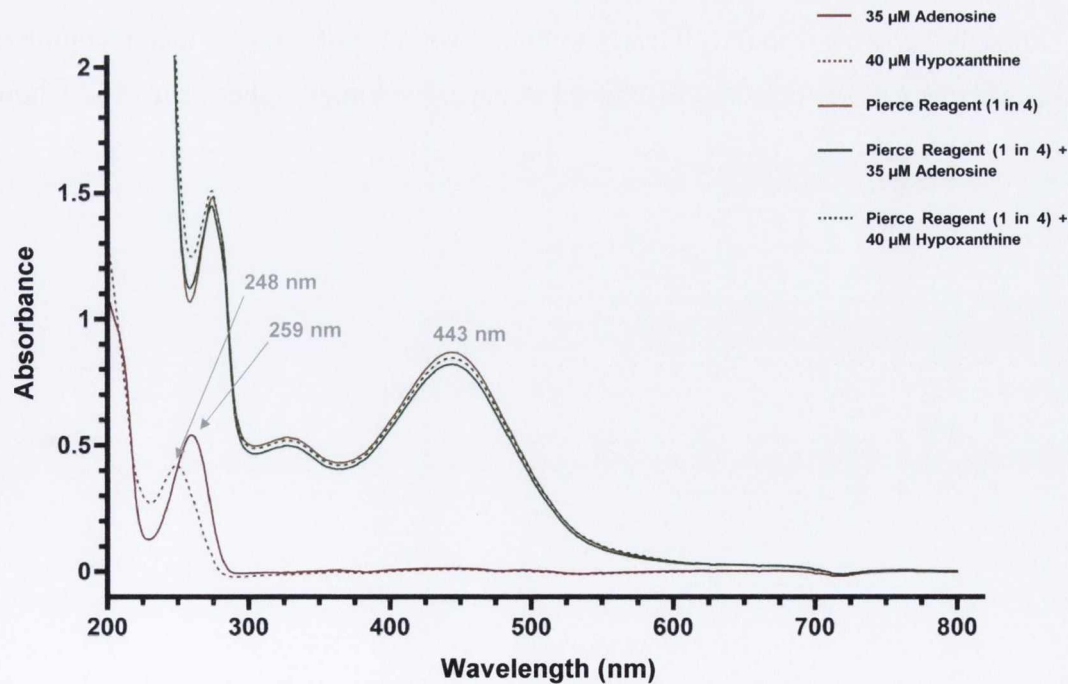
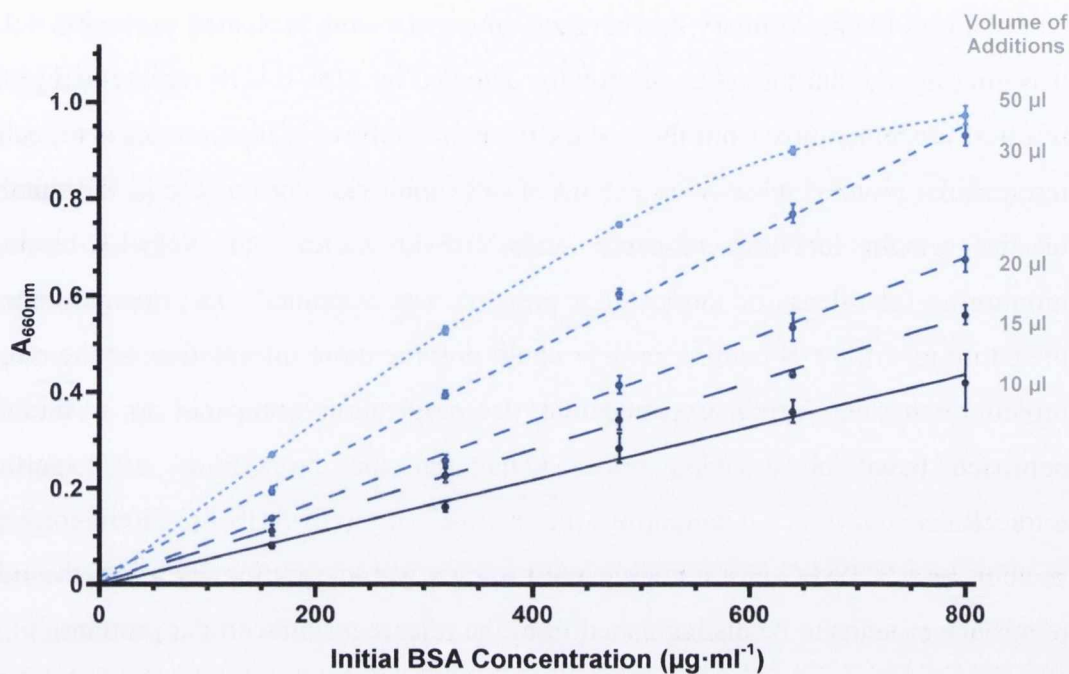


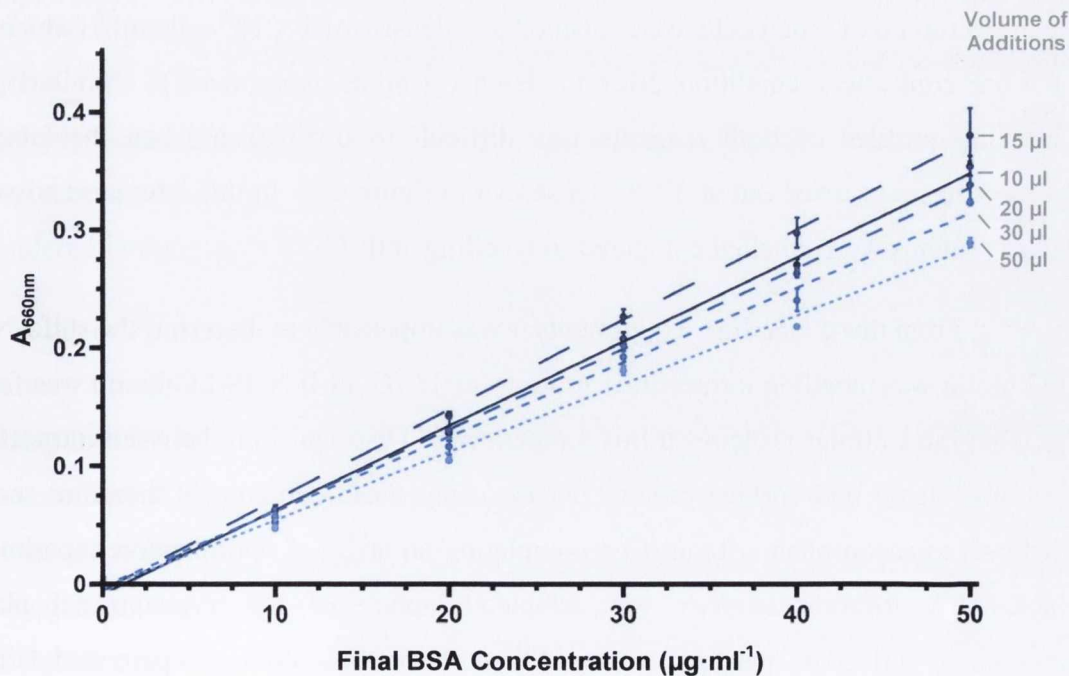
Figure 4.7. Response of the Pierce 660 nm Protein Assay Reagent is Linear Adding up to Three Times the Normal Volume of Sample

Stock solutions of BSA were prepared to $160\ \mu\text{g}\cdot\text{ml}^{-1}$, $320\ \mu\text{g}\cdot\text{ml}^{-1}$, $480\ \mu\text{g}\cdot\text{ml}^{-1}$, $620\ \mu\text{g}\cdot\text{ml}^{-1}$ and $800\ \mu\text{g}\cdot\text{ml}^{-1}$. Different volumes (10 μl to 50 μl) of each of these stock solutions were added to the Pierce 660 nm Protein Assay Reagent to test if the reagent responded linearly to volumes of protein sample above the manufacturer's recommendation (a). In each case the final volume of each assay reagent and BSA mixture was 160 μl (as normal). The experiment was repeated with a series of dilutions of the initial BSA stocks, prepared to yield identical BSA concentrations after finally mixing with the protein assay reagent (b). Each condition for (a) and (b) was performed in triplicate; data points show the mean of three separate determinations, and error bars represent the standard deviation (where no error bar is visible the error bar is smaller than the representation of the point). In both (a) and (b), the unbroken blue line represents the standard condition of mixing 10 μl protein sample with 150 μl of Pierce 660 nm protein assay reagent. The reagent was found to respond linearly to most stock BSA solutions, but there was a divergence from linearity when 50 μl of higher BSA concentrations were used (*i.e.* at 50 μl of stock $620\ \mu\text{g}\cdot\text{ml}^{-1}$ and $800\ \mu\text{g}\cdot\text{ml}^{-1}$ BSA, which results in final BSA concentrations of $194\ \mu\text{g}\cdot\text{ml}^{-1}$ and $250\ \mu\text{g}\cdot\text{ml}^{-1}$ respectively). Although the assay responds linearly to higher volumes of samples than recommended, the efficiency of the response is reduced at sample volumes higher than 15 μl (shown in 'b').

(a)



(b)



4.2.4. Distinguishing Between the Release of Surface Proteins and Intracellular Proteins

From the preliminary de-energization experiments presented in section 4.2.1, it was unclear whether the released proteins detected by SDS-PAGE represented proteins released predominantly from the surface of intact cells, or if the proteins also included intracellular proteins released as a result of cell rupturing. For this thesis, the suitability of the protein labelling reagents sulfo-NHS-LC-biotin and NHS-LC-biotin for monitoring the release of intracellular proteins was examined. In principle, labelling the entire repertoire of cellular protein could provide more information on the range of proteins released during trypanosomal de-energization compared to a traditional approach based on assaying released material for the activity of a particular intracellular enzyme. Comparing the release of sulfo-NHS-LC-biotin-conjugated proteins and NHS-LC-biotin-conjugated proteins would additionally allow the release of surface proteins to be distinguished from the release of intracellular proteins.

However, when bloodstream form trypanosomes were labelled (as described in section 2.8.3) at 0 °C, few proteins other than the VSG were detected. As shown in figure 4.8, labelling profiles of NHS-LC-biotin and sulfo-NHS-LC-biotin were almost identical when cells were labelled at 2.5×10^7 cells·ml⁻¹ (as recommended by the manufacturers) or when cells were labelled at a density of 1×10^9 cells·ml⁻¹ (which was a more convenient condition prior to de-energization experiments). Similarly, the labelling profiles of both reagents was difficult to distinguish when the labelling procedure was carried out at 37 °C, as shown in figure 4.9. In this later case however, more proteins were labelled compared to labelling at 0 °C.

From these labelling experiments it was impossible to discern if the sulfo-NHS-LC biotin was labelling intracellular proteins at 37 °C, or if NHS-LC-biotin was failing to label intracellular proteins at this temperature. Distinguishing between intracellular protein release and surface protein release using these compounds therefore seemed difficult to accomplish without first completing an array of optimization experiments. Instead, a different strategy was adopted: rupture of the trypanosomal plasma membrane during de-energization would be monitored by blotting supernatant samples for the presence of the intracellular marker enzyme AAT (control blots probing for AAT in SDS lysates were presented earlier in figure 2.1).

4.2.5. De-energization of Bloodstream form Trypanosomes Causes a Release of the VSG in Addition to Intracellular Proteins

The general de-energization protocol was repeated, but replacing hyperosmotic phosphate buffer with a hyperosmotic Tes buffer (as described in materials and methods). This change to a Tes based buffer was to accommodate later studies on the effects of added calcium on the de-energization process, as precipitation of calcium (as calcium phosphate) in a phosphate buffer would likely lead to difficulties in interpreting any results. Shown in figure 4.10, de-energization of WT bloodstream form trypanosomes caused a similar trend of protein release as observed in phosphate buffer. To ensure the release process was followed as thoroughly as possible, the absorbencies of each time point were measured at 260 nm, 280 nm, and at 660 nm after mixing with the Pierce 660 nm protein assay reagent. The same general trends of protein release are observed in WT and GPI-PLC^{-/-} cells incubated in identical conditions, however the variation in the time of onset of protein release is greater in GPI-PLC^{-/-} cells compared to WT cells. Release of material from GPI-PLC^{-/-} cells is shown in figure 4.11.

Large variation in the data is particular evident for the onset of protein release from cells incubated in the presence of 10 mM glucose. Inclusion of 10 mM glucose in the basic hyperosmotic Tes buffer delays protein release for about twenty to forty minutes. The differences in the precise time of the onset of protein release is likely to be largely dependent on the precise energy state of the cells prior to initiating the de-energization, or energization, incubation. Factors that could effect this variation will be considered in the discussion.

Once protein release had essentially completed (at approximately sixty minutes), the released protein concentration in the WT de-energization condition had reached 1.64 mg·ml⁻¹ according to the Pierce 660 nm assay, or 3.83 mg·ml⁻¹ according to a corrected A_{280nm} value. These values represent 29% and 68% of the total cellular protein respectively. For GPI-PLC^{-/-} cells, total protein release (at eighty minutes) plateaus at approximately 1.35 mg·ml⁻¹ according to the Pierce 660 nm assay (25% of total cellular protein), or 2.87 mg·ml⁻¹ according to a corrected A_{280nm} value (51% of total cellular protein).

Figure 4.8. Comparative labelling of Trypanosomes with Sulfo-NHS-LC-Biotin and NHS-LC-Biotin at 0 °C

Bloodstream form trypanosomes were washed twice in Tes buffer before adjusting to the desired concentration and incubating with 2 mM sulfo-NHS-LC-biotin or 2 mM NHS-LC-biotin in Tes buffer containing glucose, and on ice, as described in section 2.8.3. Both blots were exposed to the same sheet of light sensitive film to ensure results were directly comparable.

Blot (a): Cells were labelled at a density of 2.5×10^7 cells.ml⁻¹, at 0 °C, with each of the labelling reagents. Different numbers of cell equivalents were loaded on each track as listed below:

Lane 1: sulfo-NHS-LC-biotin labelled cells (5×10^5 cells loaded)

Lane 2: NHS-LC-biotin labelled cells (5×10^5 cells loaded)

Lane 3: sulfo-NHS-LC-biotin labelled cells (1×10^6 cells loaded)

Lane 4: NHS-LC-biotin labelled cells (1×10^6 cells loaded)

Blot (b): Cells were labelled at a density of 1×10^9 cells.ml⁻¹, at 0°C, with each of the labelling reagents. Different numbers of cell equivalents were loaded on each track as listed below:

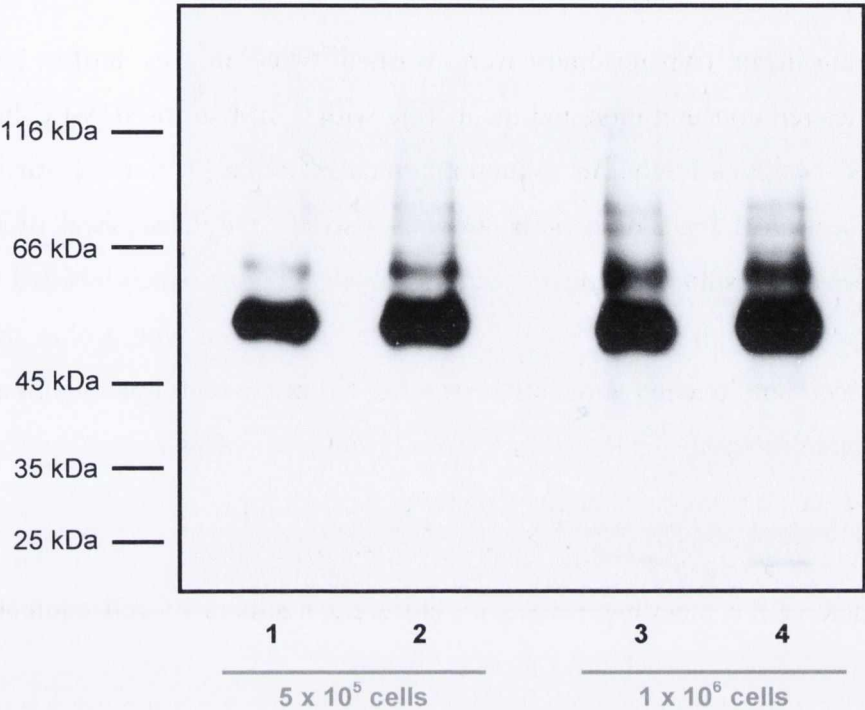
Lane 1: sulfo-NHS-LC-biotin labelled cells (5×10^5 cells loaded)

Lane 2: NHS-LC-biotin labelled cells (5×10^5 cells loaded)

Lane 3: sulfo-NHS-LC-biotin labelled cells (1×10^6 cells loaded)

Lane 4: NHS-LC-biotin labelled cells (1×10^6 cells loaded)

(a)



(b)

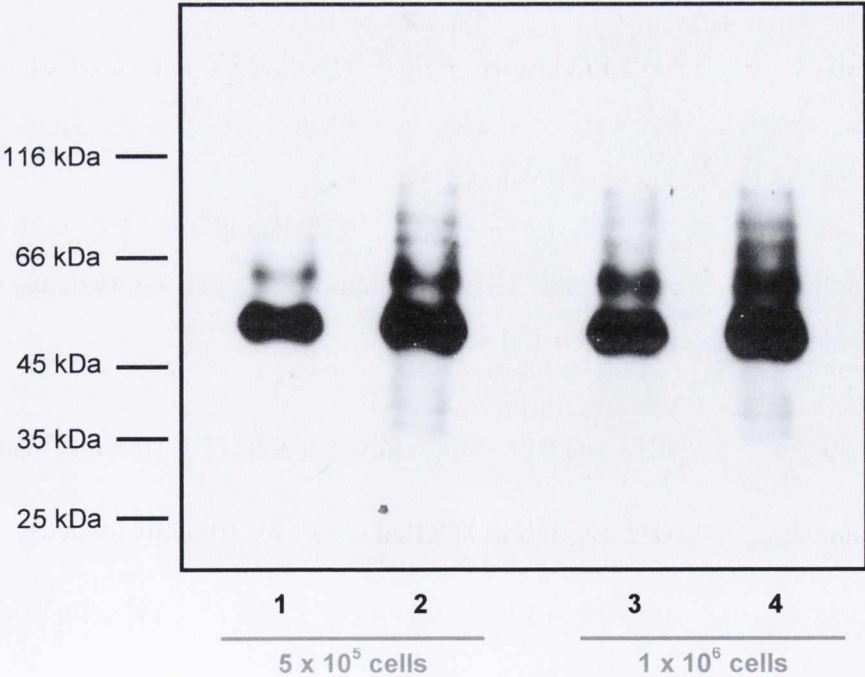


Figure 4.9. Comparative labelling of Trypanosomes with Sulfo-NHS-LC-Biotin and NHS-LC-Biotin at 37 °C

Bloodstream form trypanosomes were washed twice in Tes buffer before adjusting to the desired concentration and incubating with 2 mM sulfo-NHS-LC-biotin or 2 mM NHS-LC-biotin in Tes buffer containing glucose, and at 37 °C (with stirring), as described in section 2.8.3. Both blots were exposed to the same sheet of light sensitive film to ensure results were directly comparable. Proteins which labelled with NHS-LC-biotin and not with sulfo-NHS-LC-biotin are highlighted with a blue arrow, while proteins which only reacted with sulfo-NHS-LC-biotin are highlighted with a red arrow.

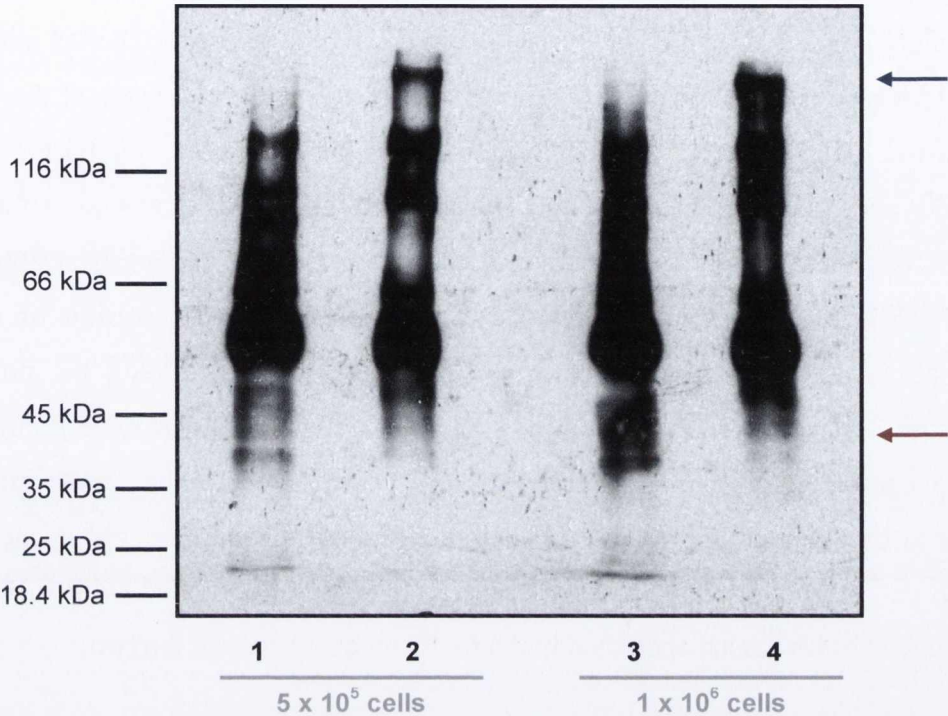
Blot (a): Cells were labelled at a density of 2.5×10^7 cells.ml⁻¹, at 37 °C, with each of the labelling reagents. Different numbers of cell equivalents were loaded on each track as listed below:

- Lane 1: sulfo-NHS-LC-biotin labelled cells (5×10^5 cells loaded)
- Lane 2: NHS-LC-biotin labelled cells (5×10^5 cells loaded)
- Lane 3: sulfo-NHS-LC-biotin labelled cells (1×10^6 cells loaded)
- Lane 4: NHS-LC-biotin labelled cells (1×10^6 cells loaded)

Blot (b): Cells were labelled at a density of 1×10^9 cells.ml⁻¹, at 37°C, with each of the labelling reagents. Different numbers of cell equivalents were loaded on each track as listed below:

- Lane 1: sulfo-NHS-LC-biotin labelled cells (5×10^5 cells loaded)
- Lane 2: NHS-LC-biotin labelled cells (5×10^5 cells loaded)
- Lane 3: sulfo-NHS-LC-biotin labelled cells (1×10^6 cells loaded)
- Lane 4: NHS-LC-biotin labelled cells (1×10^6 cells loaded)

(a)



(b)

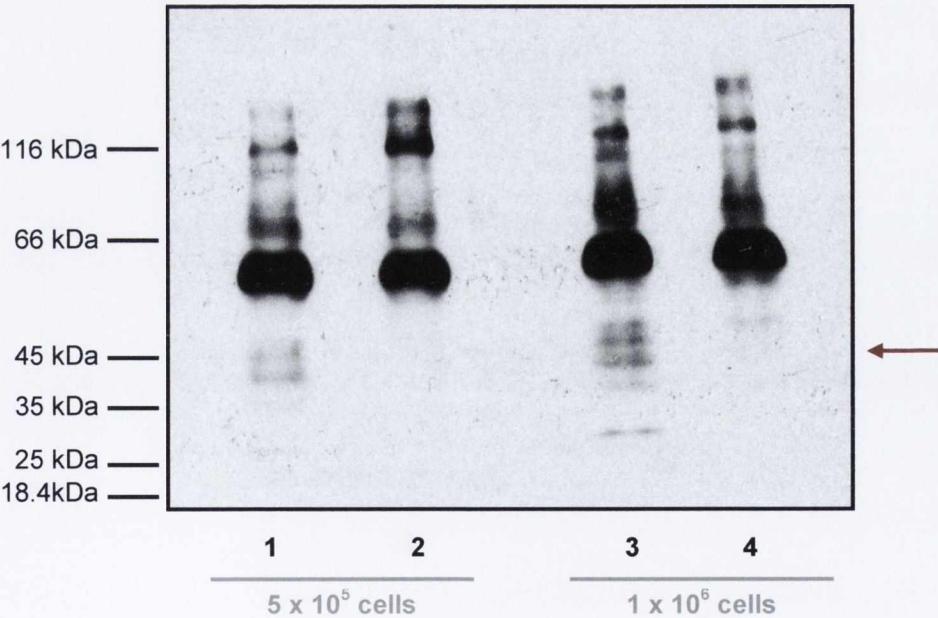


Figure 4.10. De-energization of WT Cells in Tes Buffer

WT MITat 1.2 cells were de-energized in hyperosmotic Tes buffer, containing protease inhibitors and 25 mM 2-deoxy-D-glucose (dark blue traces) or 10 mM glucose (dashed, light blue traces), for two hours. Samples of the cells were taken at the time points shown, cold quenched in five volumes of hyperosmotic Tes buffer, and immediately centrifuged at 18,000 *g* for 45 seconds. The supernatants were further centrifuged at 15,000 *g* for 15 minutes (and at 4 °C) and the absorbencies at 260 nm (shown in 'a') and 280 nm (shown in 'b') were measured. A portion of each supernatant in two of the experiments was also kept for determining the protein concentration using the Pierce 660 nm protein assay reagent (shown in 'c'). In 'c', protein concentrations have been corrected to represent the protein concentration released into the extracellular medium (*i.e.* the concentration prior to cold quenching the samples). Each energization experiment in 'c' has been plotted with different symbols (open circles versus open diamonds) to distinguish between the two.



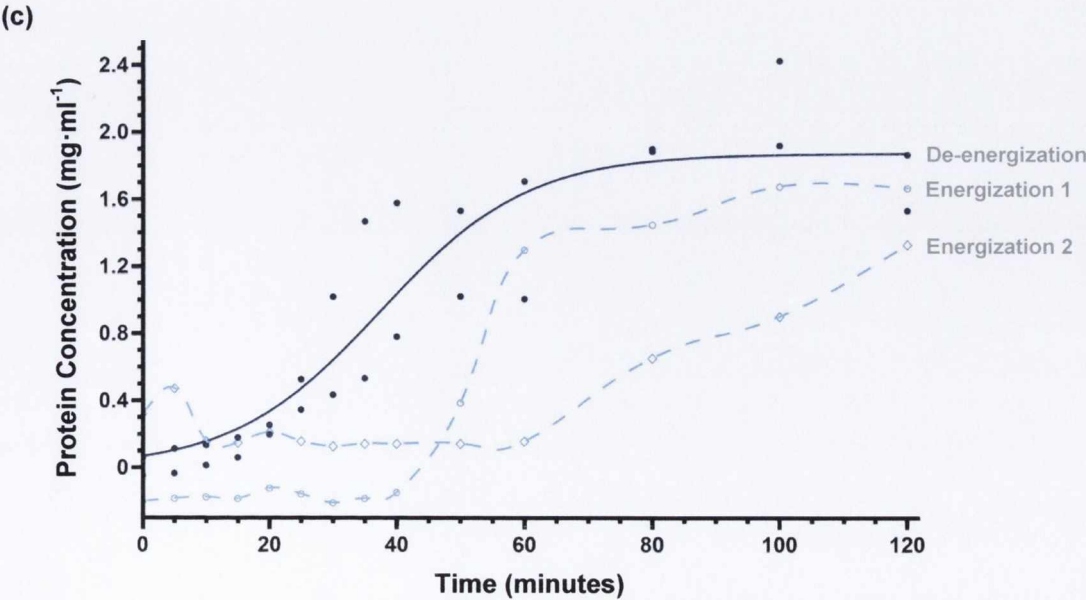
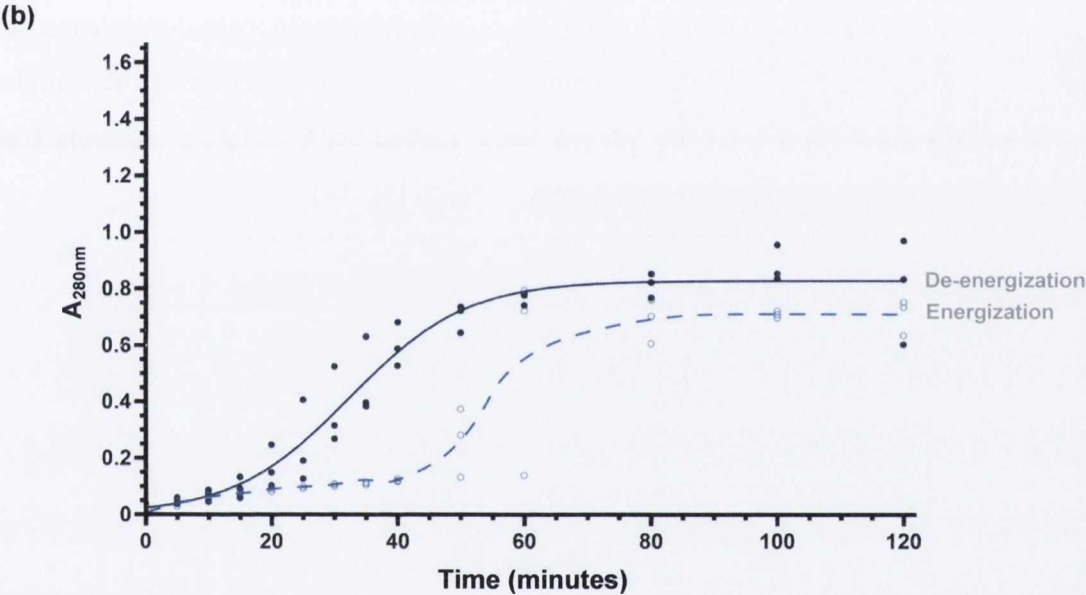
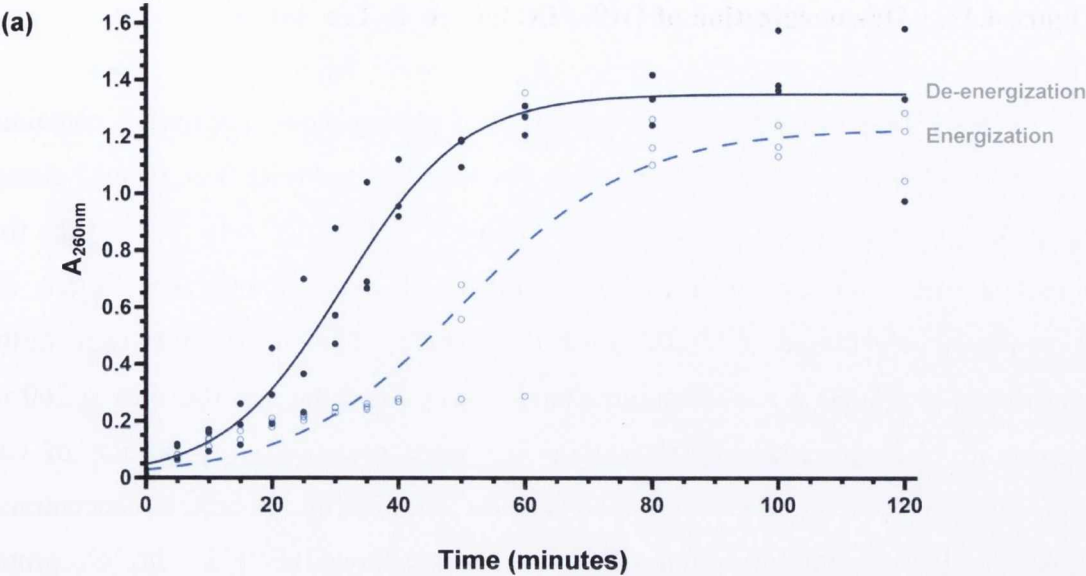
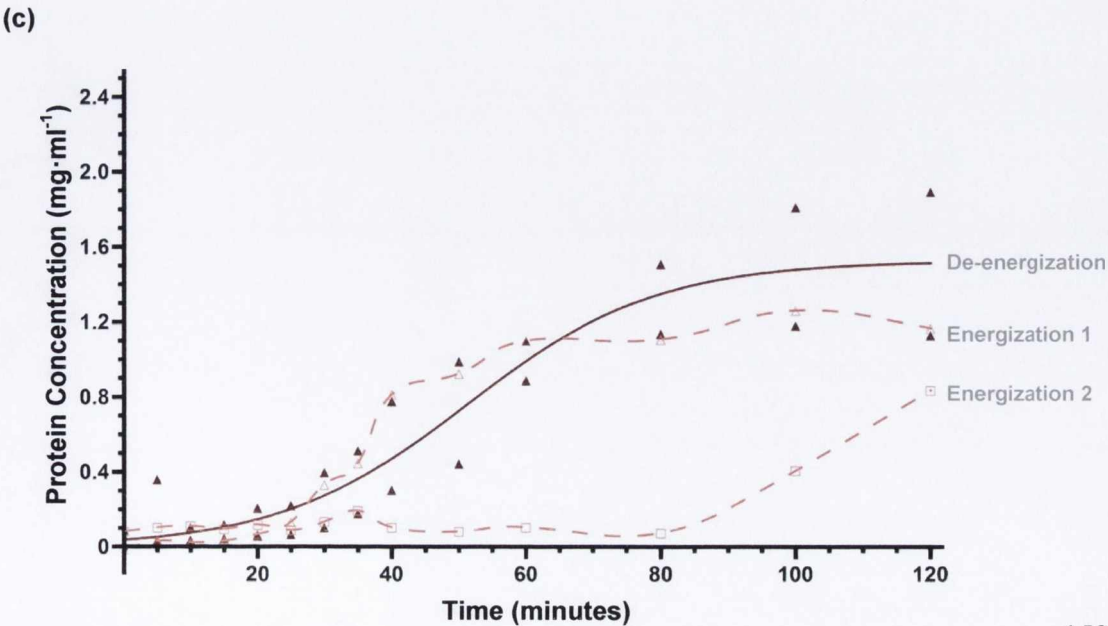
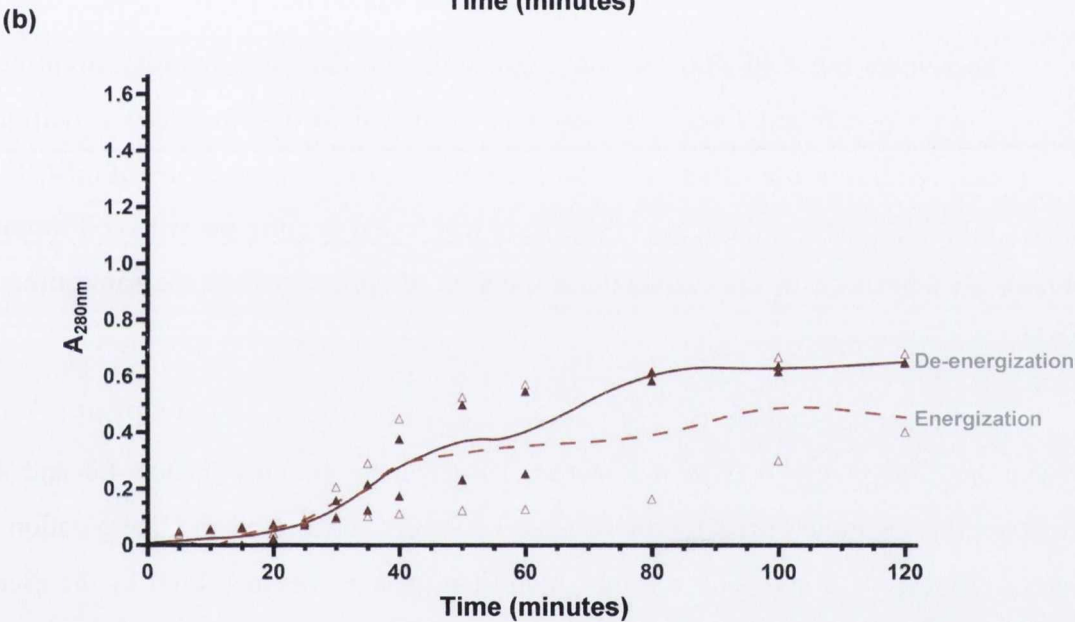
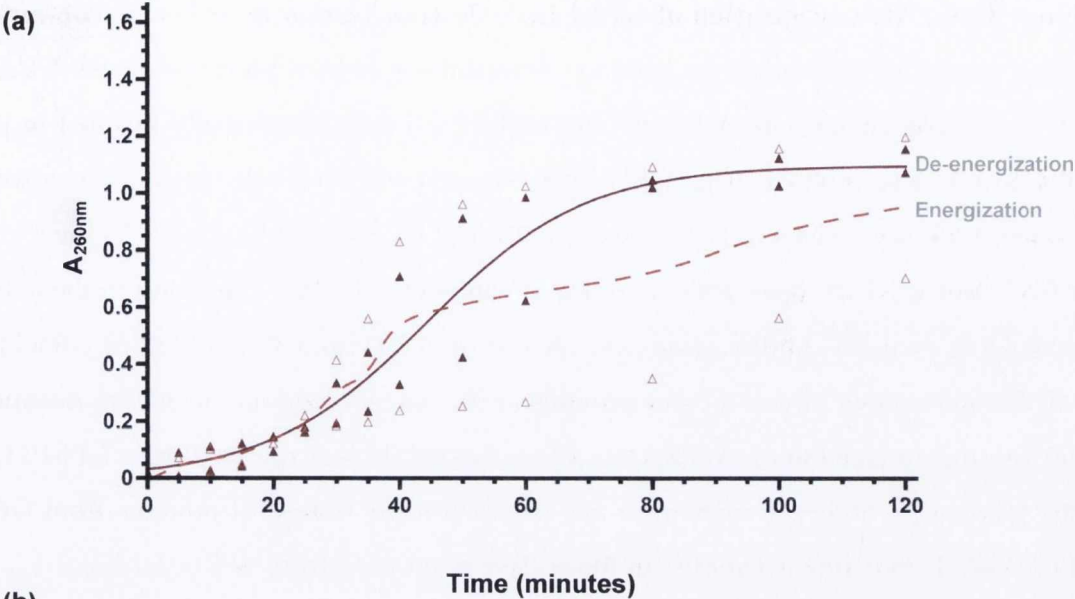


Figure 4.11. De-energization of GPI-PLC^{-/-} Cells in Tes Buffer

WT MITat 1.2 cells were de-energized in hyperosmotic Tes buffer, containing protease inhibitors and 25 mM 2-deoxy-D-glucose (dark red traces) or 10 mM glucose (dashed, light red traces), for two hours. Samples of the cells were taken at the time points shown, cold quenched in five volumes of hyperosmotic Tes buffer, and immediately centrifuged at 18,000 *g* for 45 seconds. The supernatants were further centrifuged at 15,000 *g* for 15 minutes (and at 4 °C) and the absorbencies at 260 nm (shown in 'a') and 280 nm (shown in 'b') were measured. A portion of each supernatant in the experiments was also kept for determining the protein concentration using the Pierce 660 nm protein assay reagent (shown in 'c'). In 'c', protein concentrations have been corrected to represent the protein concentration released into the extracellular medium (*i.e.* the concentration prior to cold quenching the samples). Each energization experiment in 'c' has been plotted with different symbols (open triangles versus open squares) to distinguish between the two.



SDS-PAGE of samples obtained from de-energization experiments confirmed earlier suspicions that cells were leaking intracellular proteins. Shown in plates 4.1 and 4.2, the de-energization of WT cells and GPI-PLC^{-/-} cells respectively resulted in the release of a large number of proteins from the cells. In both cell types, the cytosolic enzyme AAT was detected in the released material by Western blotting. As the levels of AAT increased in supernatant samples the amounts of AAT remaining in the pellet fractions decreased. Furthermore, the release of AAT from trypanosomes coincides with the appearance of many other proteins in the de-energization incubation medium, and with the conversion of mfVSG to sVSG. No sVSG was released from GPI-PLC^{-/-} cells (plate 4.2), however apart from this distinction the release of proteins from GPI-PLC^{-/-} cells is indistinguishable from the release of proteins from WT cells.

Therefore, the GPI-PLC is not responsible for the vast majority of protein release from bloodstream form trypanosomes incubated in de-energizing conditions. Under such conditions the GPI-PLC is only responsible for the conversion of mfVSG to sVSG. GPI-PLC-mediated release of the VSG was found to correlate with cell rupture, with sVSG appearing in the extracellular medium at approximately the same time as AAT.

In Coomassie-stained gels of samples taken from WT cell and GPI-PLC^{-/-} cell incubations, no protein release was detected over sixty minutes (plates 4.3 and 4.4 respectively). Samples used for these gels, however, correspond to 'Energization 2' traces in figures 4.10 and 4.11, during which little protein was measured by the Pierce 660 nm reagent over the same time course.

Plate 4.1. De-energization of WT Bloodstream Form Trypanosomes causes a Release of many Proteins, including the Intracellular Protein Alanine Aminotransferase

WT trypanosomes collected from the blood of an infected rat were de-energized in Tes buffer as described in section 2.6.2. At selected time points, released protein (*i.e.* supernatants following centrifugation of de-energized cell sample), and protein remaining bound to the cell (*i.e.* pellets following centrifugation of de-energized cell sample), was run on a 10% denaturing gel and stained with Coomassie (2.5×10^6 cell equivalents were loaded per lane). Bands corresponding to sVSG and mfVSG are highlighted. Replica gels were transferred to nitrocellulose membranes and probed with anti-AAT antibody as described in section 2.3.7 (2.0×10^6 cell equivalents were loaded per lane). Detected AAT is labelled (specificity of the anti AAT antibody is shown in figure 2.1. in materials and methods). The loading scheme is given below:

Lane 1:	molecular weight standards (Thermo Scientific)
Lane 2:	WT whole cell SDS lysate
P & S Lanes:	pellet (P) and supernatant (s) samples taken at the indicated times
Lane 23:	WT whole cell SDS lysate
Lane 24:	molecular weight standards (Thermo Scientific)

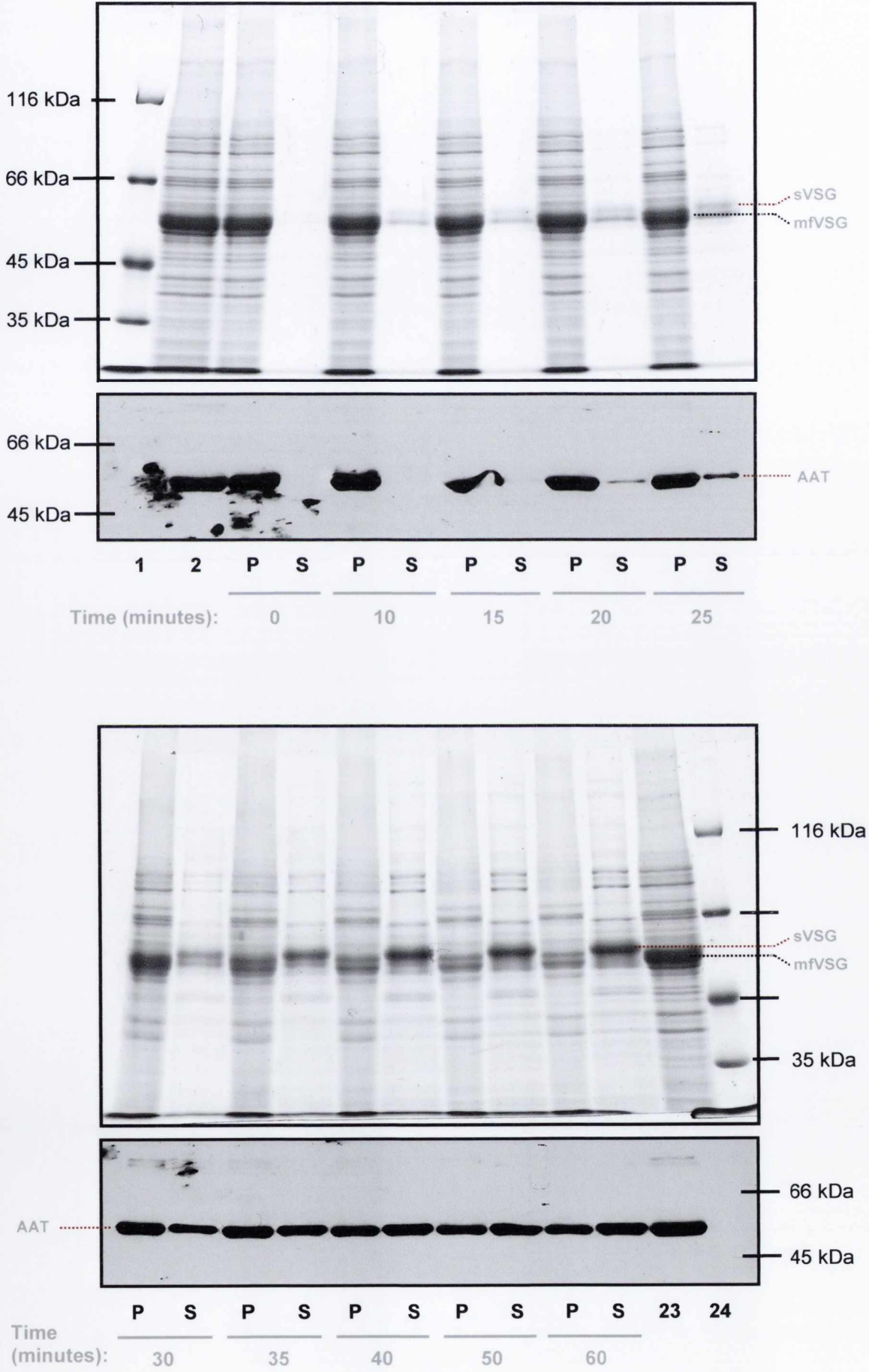


Plate 4.2. De-energization of GPI-PLC^{-/-} Bloodstream Form Trypanosomes causes a release of many Proteins, but mfVSG is not converted to sVSG

GPI-PLC^{-/-} trypanosomes collected from the blood of an infected rat were de-energized in Tes buffer as described in section 2.6.2. At selected time points, released protein (*i.e.* supernatants following centrifugation of de-energized cell sample), and protein remaining bound to the cell (*i.e.* pellets following centrifugation of de-energized cell sample), was run on a 10% denaturing gel and stained with Coomassie (2.5 x 10⁶ cell equivalents were loaded per lane). Replica gels were transferred to nitrocellulose membranes and probed with anti-AAT antibody as described in section 2.3.7 (2.0 x 10⁶ cell equivalents were loaded per lane). Detected AAT is labelled. The loading scheme is given below:

- Lane 1: molecular weight standards (Thermo Scientific)
- Lane 2: GPI-PLC^{-/-} whole cell SDS lysate
- P & S Lanes: pellet (P) and supernatant (s) samples taken at the indicated times
- Lane 23: GPI-PLC^{-/-} whole cell SDS lysate
- Lane 24: molecular weight standards (Thermo Scientific)

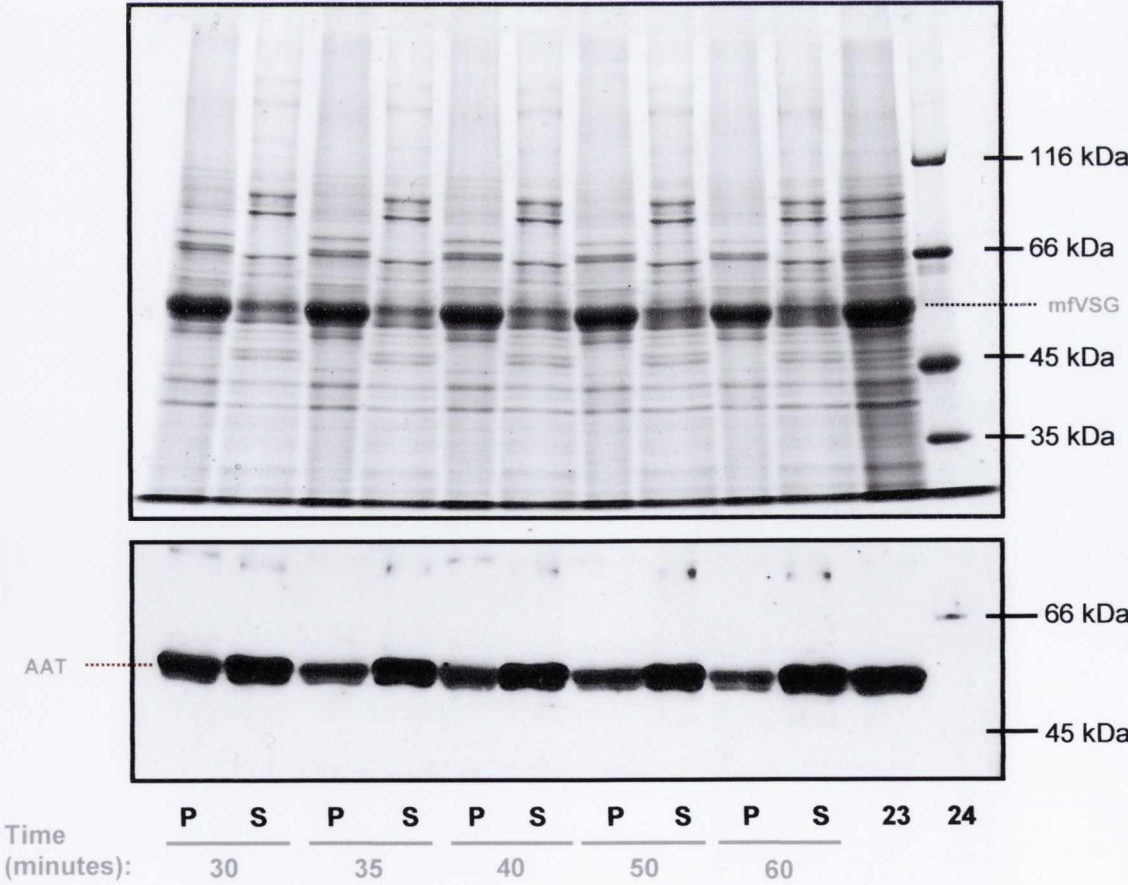


Plate 4.3. Energization of WT Bloodstream Form Trypanosomes

WT trypanosomes collected from the blood of an infected rat were incubated in Tes buffer containing glucose as described in section 2.6.2. At selected time points, released protein (*i.e.* supernatants following centrifugation of cell sample), and protein remaining bound to the cell (*i.e.* pellets following centrifugation of cell sample), was run on a 10% denaturing gel and stained with Coomassie (2.5×10^6 cell equivalents were loaded per lane). There is only a barely detectable level of protein present in each supernatant sample, and in no case was there any significant conversion of mfVSG to sVSG. Replica gels were transferred to nitrocellulose membranes and probed with anti-AAT antibody as described in section 2.3.7 (2.0×10^6 cell equivalents were loaded per lane). Detected AAT is labelled. The loading scheme is given below:

- Lane 1: molecular weight standards (Thermo Scientific)
- Lane 2: WT whole cell SDS lysate
- P & S Lanes: pellet (P) and supernatant (s) samples taken at the indicated times
- Lane 23: WT whole cell SDS lysate
- Lane 24: molecular weight standards (Thermo Scientific)

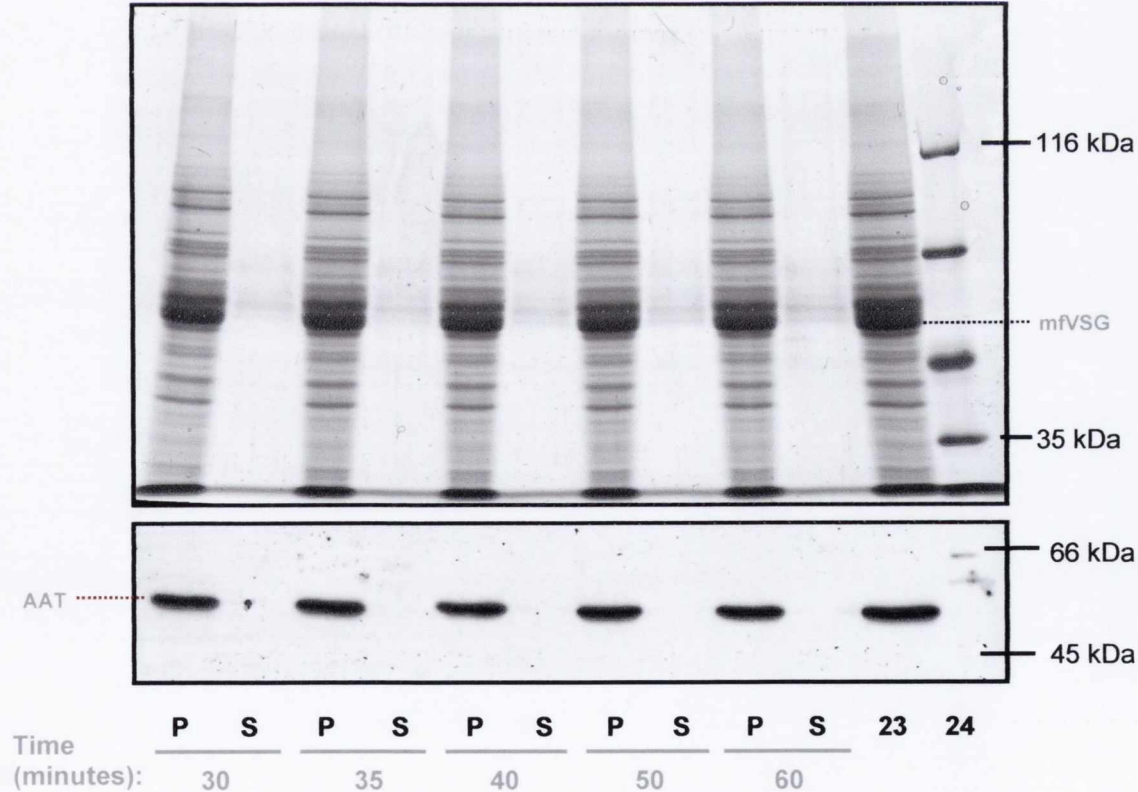
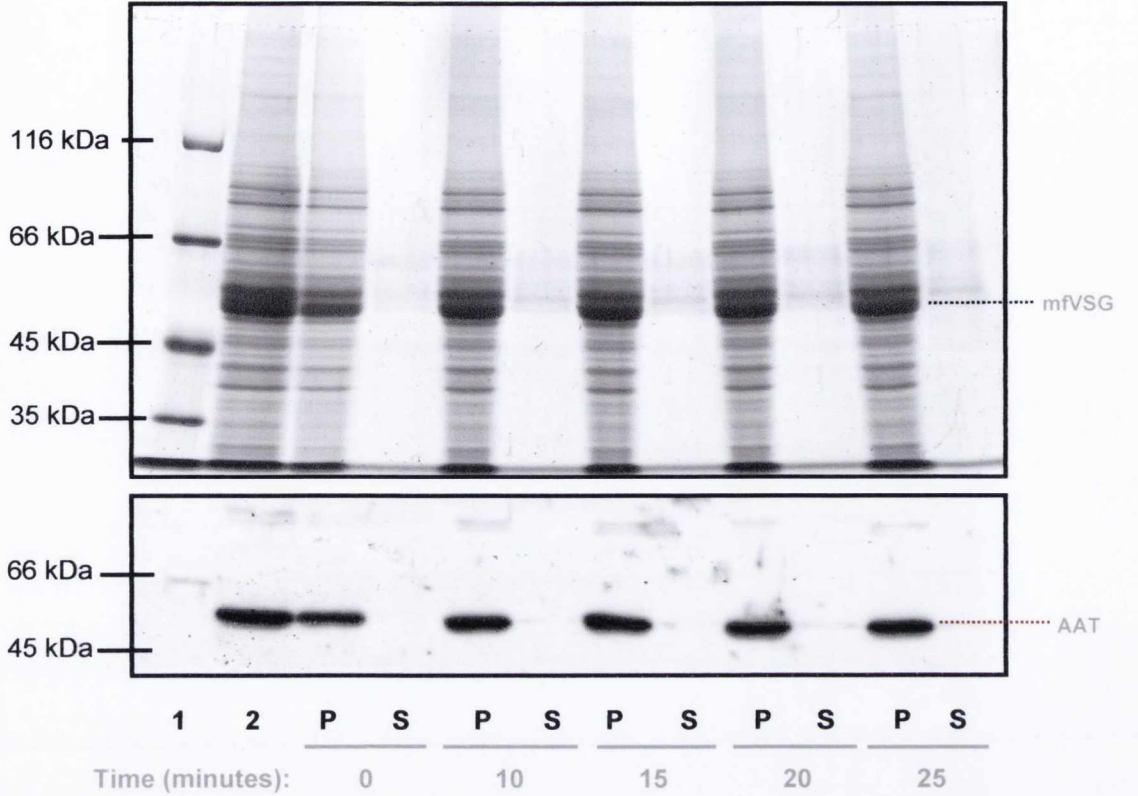
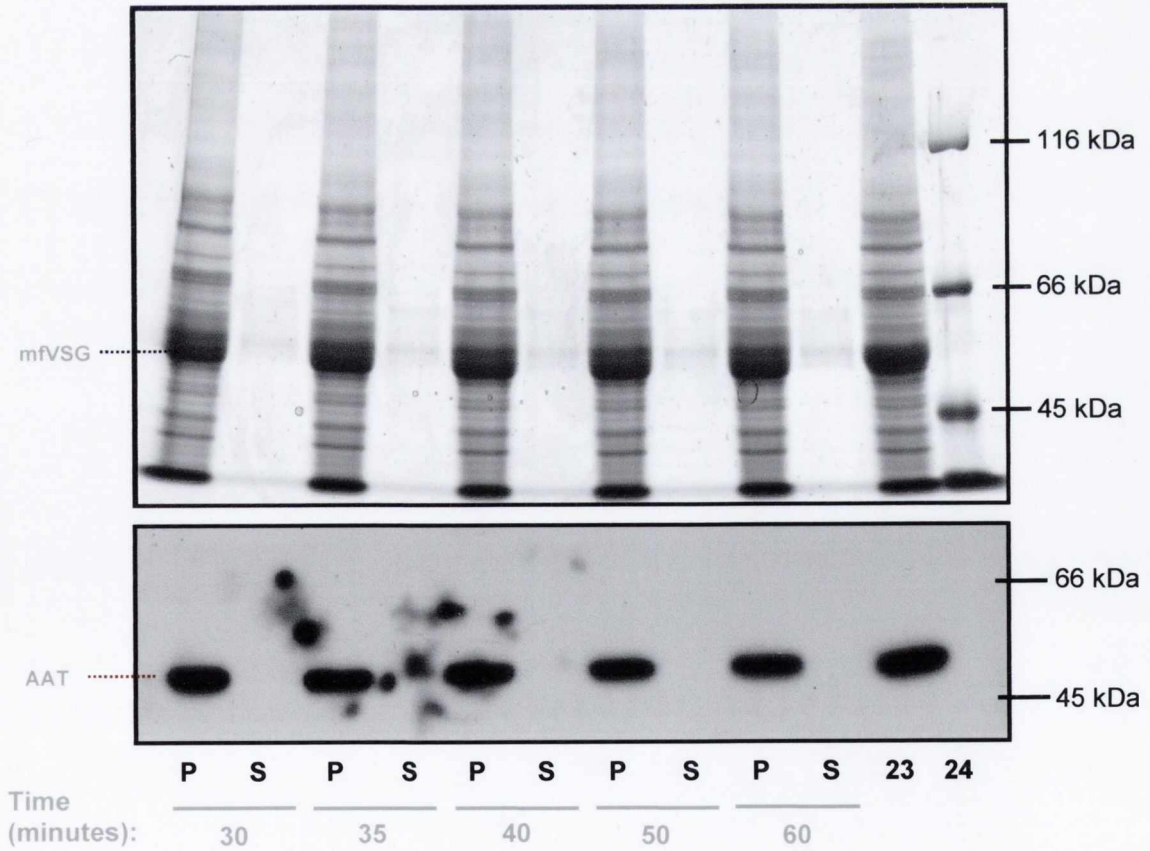
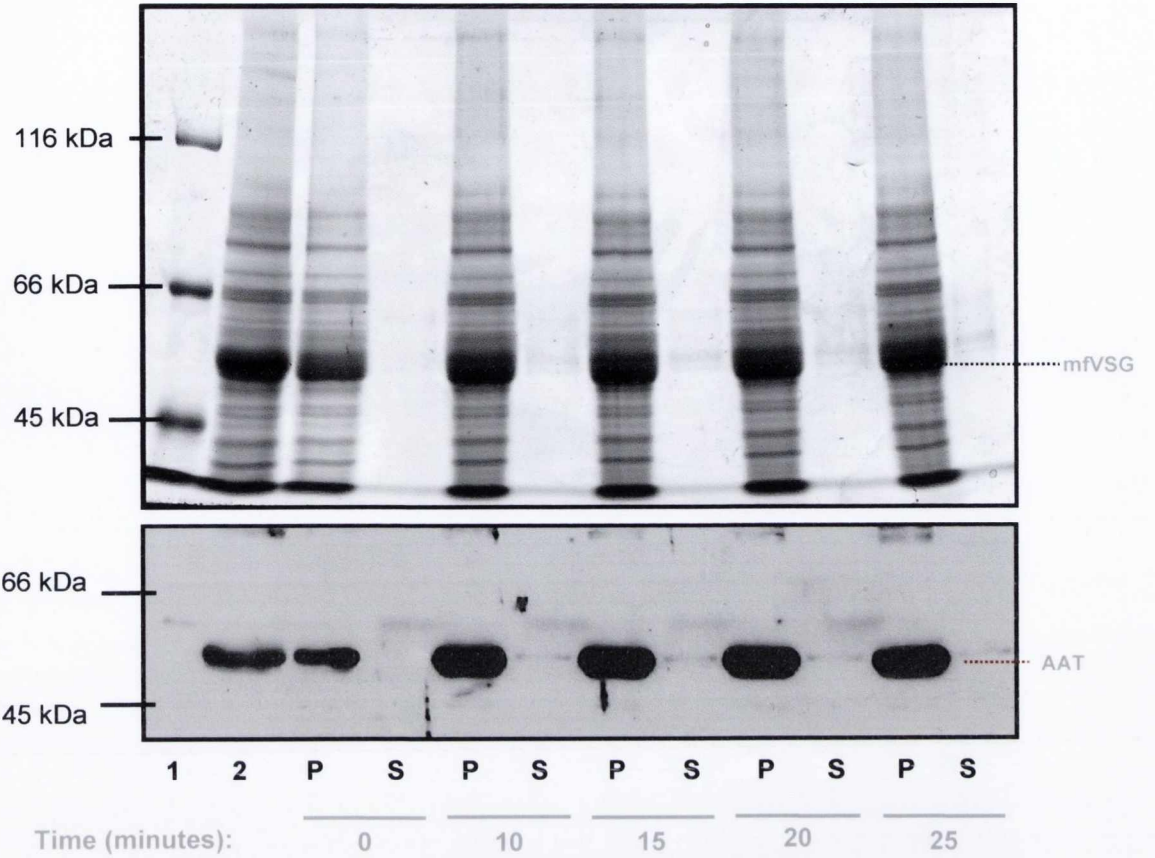


Plate 4.4. Energization of GPI-PLC^{-/-} Bloodstream Form Trypanosomes

GPI-PLC^{-/-} trypanosomes collected from the blood of an infected rat were incubated in Tes buffer containing glucose as described in section 2.6.2. At selected time points, released protein (*i.e.* supernatants following centrifugation of cell sample), and protein remaining bound to the cell (*i.e.* pellets following centrifugation of cell sample), was run on a 10% denaturing gel and stained with Coomassie (2.5 x 10⁶ cell equivalents were loaded per lane). Replica gels were transferred to nitrocellulose membranes and probed with anti-AAT antibody as described in section 2.3.7 (2.0 x 10⁶ cell equivalents were loaded per lane). Detected AAT is labelled. The loading scheme is given below:

- Lane 1: molecular weight standards (Thermo Scientific)
- Lane 2: GPI-PLC^{-/-} whole cell SDS lysate
- P & S Lanes: pellet (P) and supernatant (s) samples taken at the indicated times
- Lane 23: GPI-PLC^{-/-} whole cell SDS lysate
- Lane 24: molecular weight standards (Thermo Scientific)



4.2.6. The Effect of Calcium Acetate on the Release of Cellular Proteins and the VSG

The de-energization of WT and GPI-PLC^{-/-} trypanosomes causes a release of the VSG; the time course of this VSG release correlates with cell rupture. Although the GPI-PLC is not required for cell rupture, the enzyme is required for VSG release into the extracellular medium. To investigate if intracellular Ca²⁺ was required for a more specific release of the VSG, with cells remaining intact during the release process, cells were incubated in hyperosmotic Tes buffer containing 25 mM 2-deoxy-D-glucose and 2 mM calcium acetate in the presence of, or in the absence of, the calcium ionophore A-23187.

When WT bloodstream form trypanosomes were de-energized in the presence of Ca²⁺, or in the presence of Ca²⁺ and A-23187, there was an almost immediate release of cellular material (figure 4.12). The trend of material release is almost identical in GPI-PLC^{-/-} cells (figure 4.13), with perhaps only a slight delay in the onset of release present. In both cases, the release of material is complete by ten minutes, which is in good agreement with earlier results (Voorheis *et al.*, 1982). According to protein assays, the extent of protein release in the presence of calcium is also slightly higher than in the absence of added calcium, with the extracellular protein concentration reaching approximately 2 mg·ml⁻¹ (38% total cellular protein) for WT cells and 2.4 mg·ml⁻¹ (43% total cellular protein) for GPI-PLC^{-/-} cells.

Figure 4.12. De-energization of WT Cells in Tes Buffer in the Presence of Ca^{2+} , with or without the Calcium Ionophore A-23187

WT MITat 1.2 cells were de-energized in hyperosmotic Tes buffer, containing protease inhibitors, 25 mM 2-deoxy-D-glucose and Ca^{2+} (unbroken, dark blue traces), or Ca^{2+} and A-23187 (dashed, light blue traces), for two hours. Two separate experiments were performed. Samples of the cells were taken at the time points shown, cold quenched in five volumes of hyperosmotic Tes buffer, and immediately centrifuged at 18,000 *g* for 45 seconds. The supernatants were further centrifuged at 15,000 *g* for 15 minutes (and at 4 °C) and the absorbencies at 260 nm (shown in 'a') and 280 nm (shown in 'b') were measured. From one experiment, a portion of each supernatant was also kept for determining the protein concentration using the Pierce 660 nm protein assay reagent (shown in 'c'). In 'c', protein concentrations have been corrected to represent the protein concentration released into the extracellular medium (*i.e.* the concentration prior to cold quenching the samples). In each case the individual data points from each experiment are shown, and traces drawn represent the average of the data points.

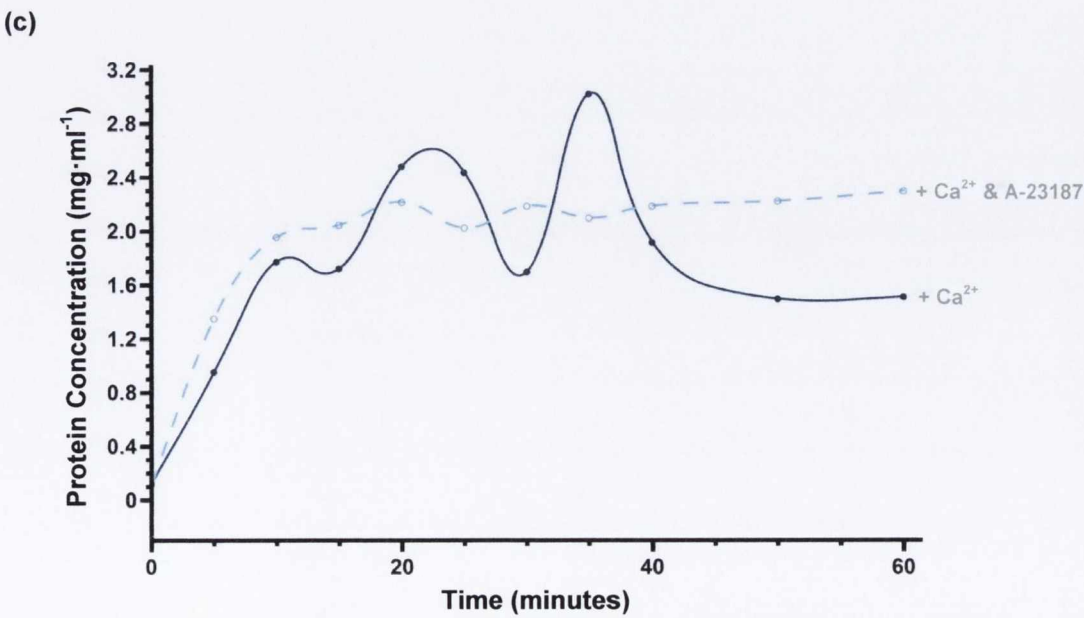
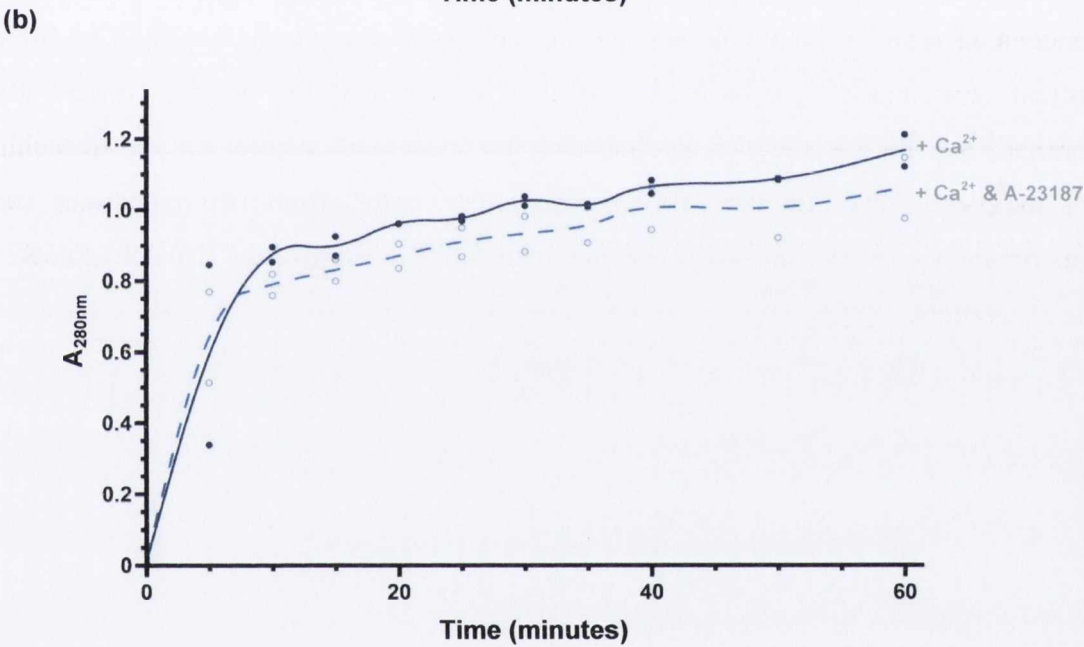
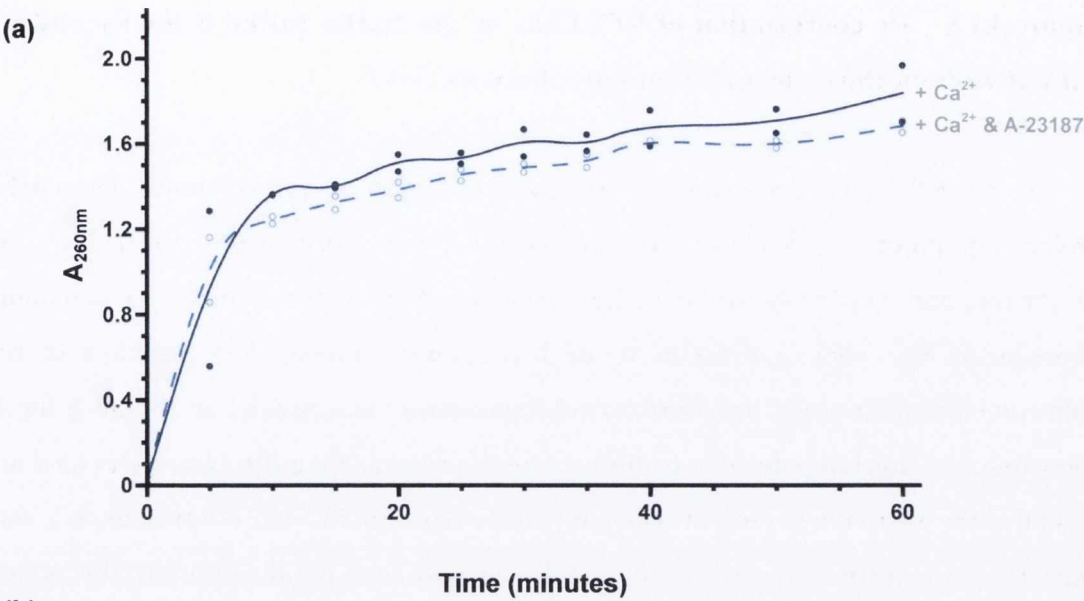
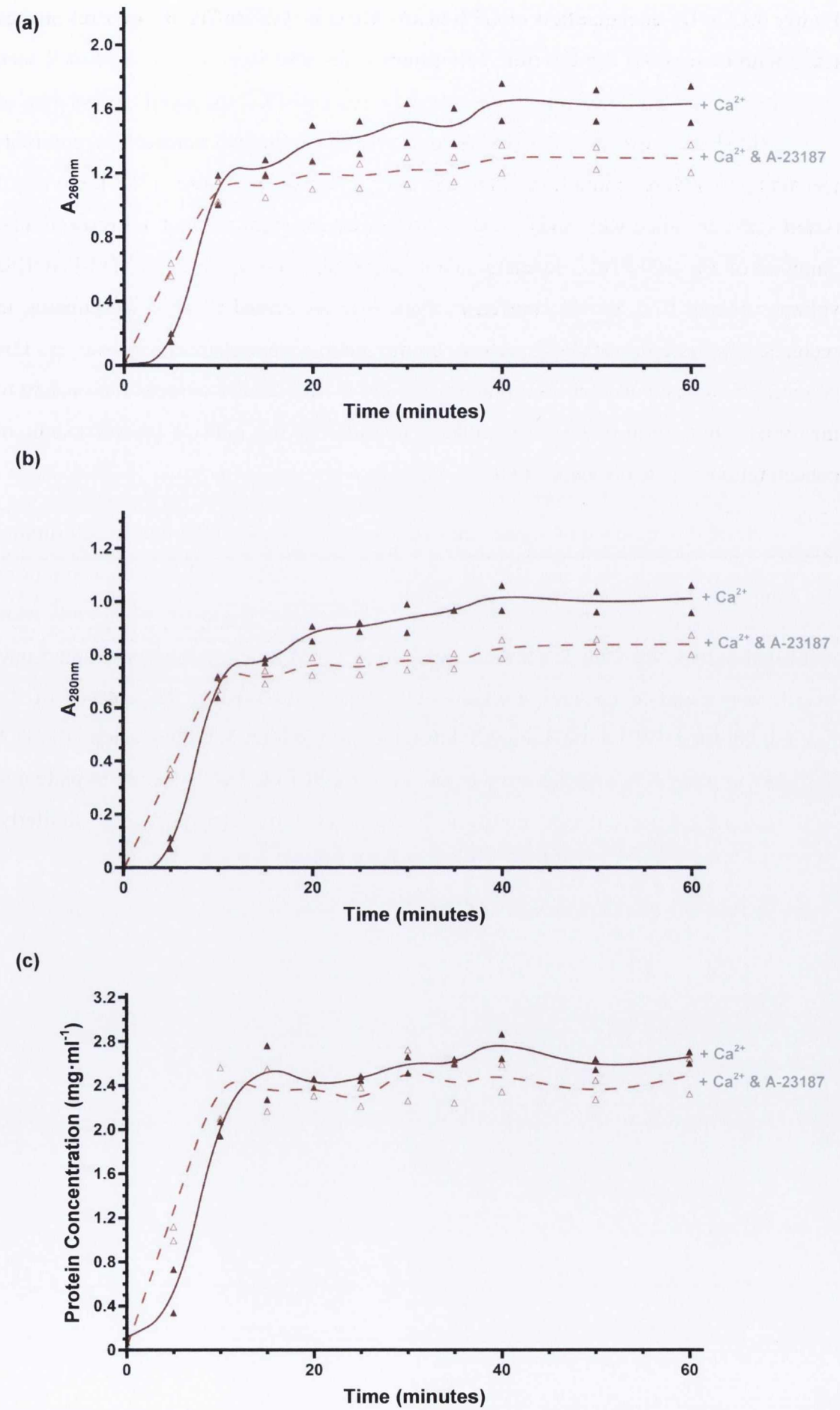


Figure 4.13. De-energization of GPI-PLC^{-/-} Cells in Tes Buffer in the Presence of Ca²⁺, with or without the Calcium Ionophore A-23187

GPI-PLC^{-/-} MITat 1.2 cells were de-energized in hyperosmotic Tes buffer, containing protease inhibitors and 25 mM 2-deoxy-D-glucose containing Ca²⁺ (unbroken, dark red trace) or Ca²⁺ and A-23187 (dashed, light red trace), for two hours. Samples of the cells were taken at the time points shown, cold quenched in five volumes of hyperosmotic Tes buffer, and immediately centrifuged at 18,000 *g* for 45 seconds. The supernatants were further centrifuged at 15,000 *g* for 15 minutes (and at 4 °C) and the absorbencies at 260 nm (shown in 'a') and 280 nm (shown in 'b') were measured. A portion of each supernatant was also kept for determining the protein concentration using the Pierce 660 nm protein assay reagent (shown in 'c'). In 'c', protein concentrations have been corrected to represent the protein concentration released into the extracellular medium (*i.e.* the concentration prior to cold quenching the samples). In each case the individual data points from two repeats of each experiment are shown, and traces drawn represent the average of the two data points.





Crucially, in each of the conditions monitored by SDS-PAGE, general protein release, and release of intracellular AAT follows the same trend of increase that is seen with the absorbance measurements. Again, the GPI-PLC-mediated conversion of mfVSG to sVSG correlates with rupture of the cell. Coomassie stained gels, and blots for AAT, of samples taken during the de-energization of WT cells in the presence of added Ca^{2+} , or added Ca^{2+} and A-23187, are shown in plates 4.5 and 4.6 respectively. Analysis of the GPI-PLC^{-/-} de-energization in the presence of Ca^{2+} , by SDS-PAGE is shown in plate 4.7. In this later case, there is a substantial band, corresponding in molecular weight to mfVSG, present in the sample supernatants. However, this particular band may also represent tubulin, which is very similar in molecular weight to mfVSG. This possibility is not unlikely considering the general greater extent of protein release in the presence of Ca^{2+} .

While the presence of added calcium markedly increased the rate of cell rupture and protein release in WT and GPI-PLC^{-/-} cells, extracellular EGTA at concentrations of 5 mM, did not slow the release process. The release of protein when cells were incubated in hyperosmotic Tes buffer containing 25 mM 2-deoxy-D-glucose and 5 mM EGTA was found to be very similar to the release observed in the absence of the calcium chelator. WT protein release during de-energization in the presence of EGTA is shown in plate 4.8, with the same condition for GPI-PLC^{-/-} cells shown in plate 4.9. EGTA could not prevent cell rupture in bloodstream form trypanosomes. Similarly, EGTA could not prevent GPI-PLC activation in WT cells.

Plate 4.5. De-energization of WT Bloodstream Form Trypanosomes in the presence of Calcium

WT trypanosomes collected from the blood of an infected rat were de-energized in Tes buffer containing 2 mM calcium acetate as described in section 2.6.2. At selected time points, released protein (*i.e.* supernatants following centrifugation of de-energized cell sample), and protein remaining bound to the cell (*i.e.* pellets following centrifugation of de-energized cell sample), was run on a 10% denaturing gel and stained with Coomassie (2.5×10^6 cell equivalents were loaded per lane). Bands corresponding to sVSG and mfVSG are highlighted. Replica gels were transferred to nitrocellulose membranes and probed with anti-AAT antibody as described in section 2.3.7 (2.0×10^6 cell equivalents were loaded per lane). Detected AAT is labelled. The loading scheme is given below:

Lane 1:	molecular weight standards (Thermo Scientific)
Lane 2:	WT whole cell SDS lysate
P & S Lanes:	pellet (P) and supernatant (s) samples taken at the indicated times
Lane 23:	WT whole cell SDS lysate
Lane 24:	molecular weight standards (Thermo Scientific)

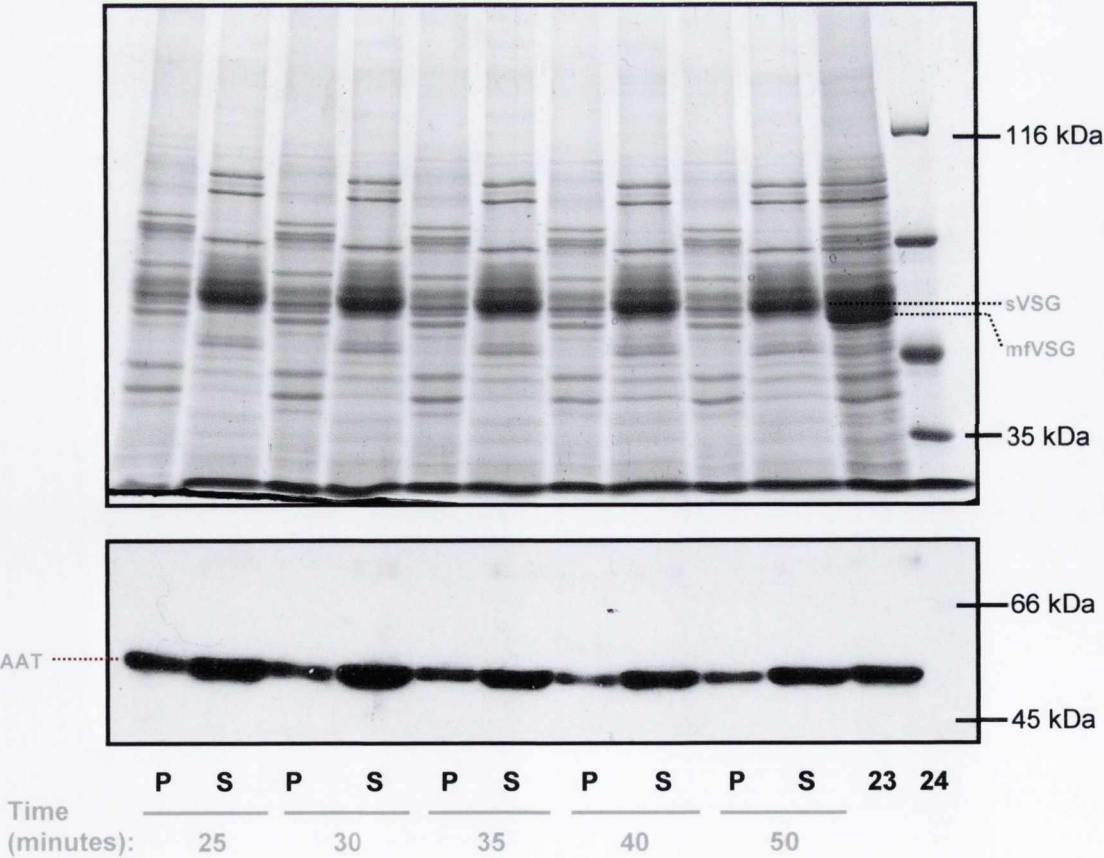
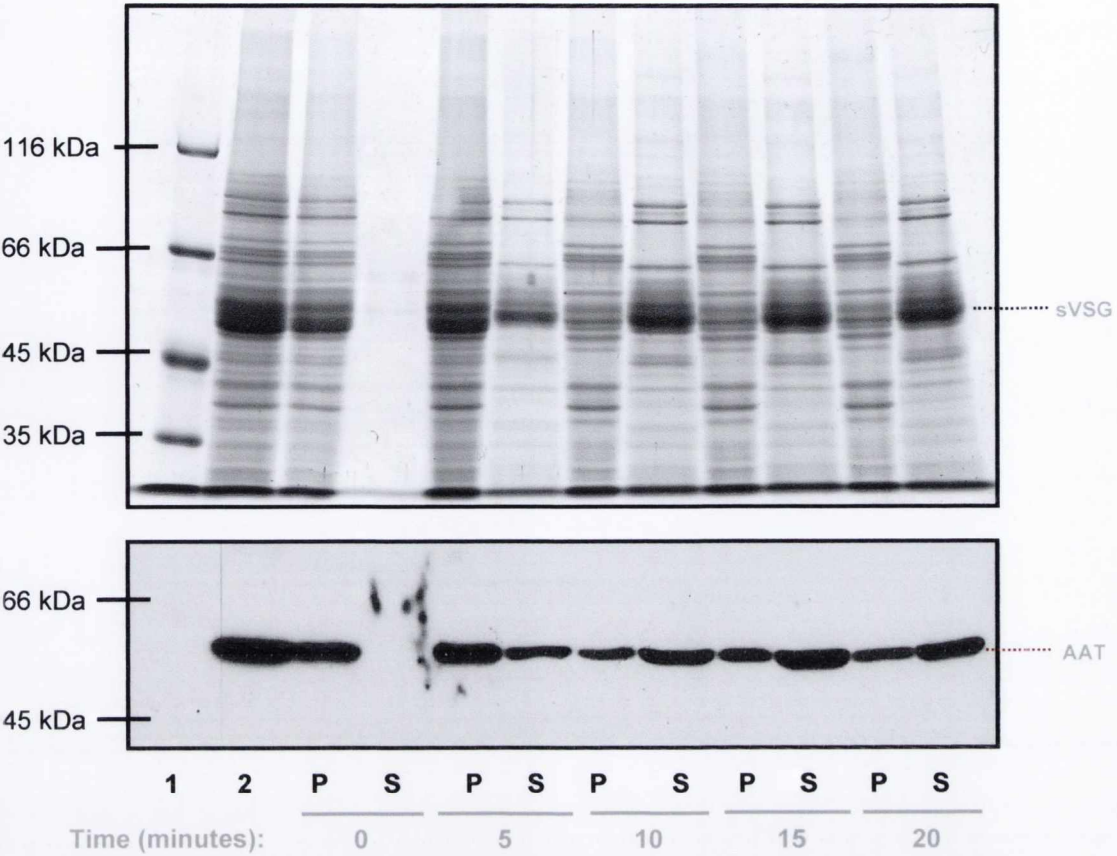


Plate 4.6. De-energization of WT Bloodstream Form Trypanosomes in the presence of A-23187 and Calcium

WT trypanosomes collected from the blood of an infected rat were de-energized in Tes buffer containing 2 mM calcium acetate and 5 $\mu\text{g}\cdot\text{ml}^{-1}$ of the calcium ionophore A-23187, as described in section 2.6.2. At selected time points, released protein (*i.e.* supernatants following centrifugation of de-energized cell sample), and protein remaining bound to the cell (*i.e.* pellets following centrifugation of de-energized cell sample), was run on a 10% denaturing gel and stained with Coomassie (2.5×10^6 cell equivalents were loaded per lane). Bands corresponding to sVSG and mfVSG are highlighted. Replica gels were transferred to nitrocellulose membranes and probed with anti-AAT antibody as described in section 2.3.7 (2.0×10^6 cell equivalents were loaded per lane). Detected AAT is labelled. The loading scheme is given below:

- Lane 1: molecular weight standards (Thermo Scientific)
- Lane 2: WT whole cell SDS lysate
- P & S Lanes: pellet (P) and supernatant (s) samples taken at the indicated times
- Lane 23: WT whole cell SDS lysate
- Lane 24: molecular weight standards (Thermo Scientific)

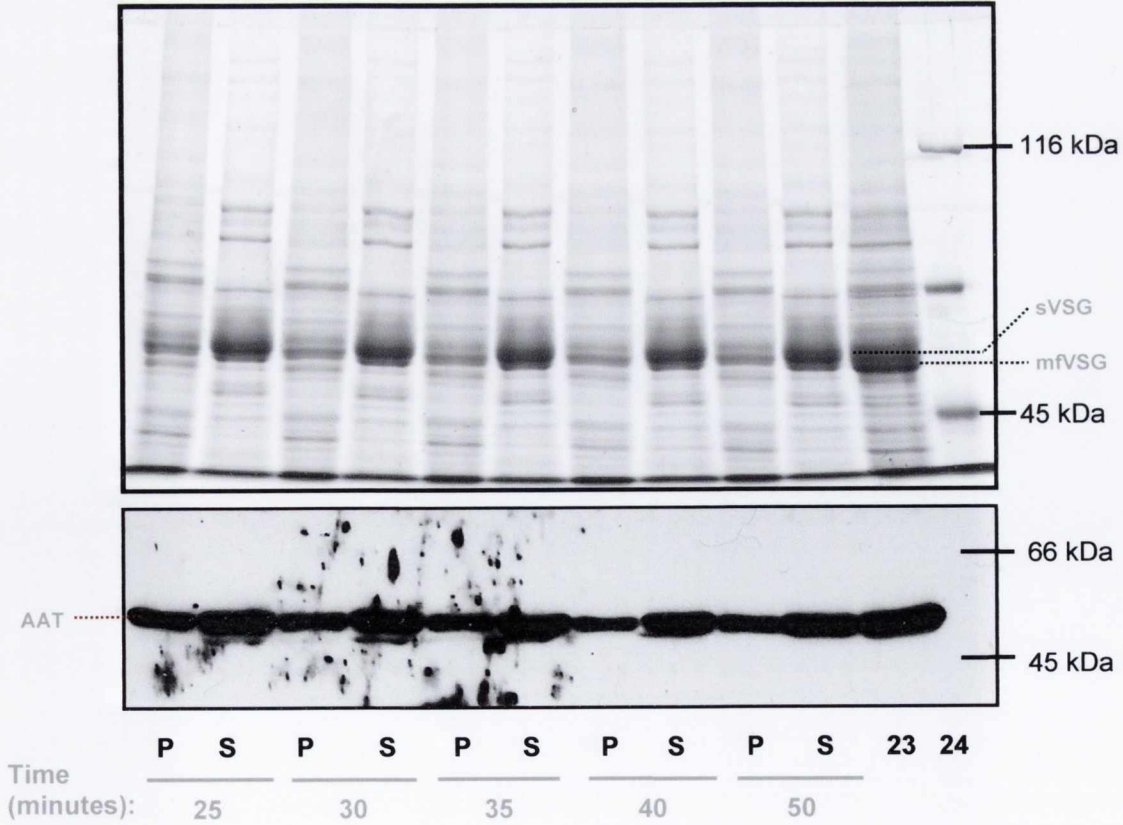


Plate 4.7. De-energization of GPI-PLC^{-/-} Bloodstream Form Trypanosomes in the presence of Calcium

GPI-PLC^{-/-} trypanosomes collected from the blood of an infected rat were de-energized in Tes buffer containing 2 mM calcium acetate as described in section 2.6.2. At selected time points, released protein (*i.e.* supernatants following centrifugation of de-energized cell sample), and protein remaining bound to the cell (*i.e.* pellets following centrifugation of de-energized cell sample), was run on a 10% denaturing gel and stained with Coomassie (2.5×10^6 cell equivalents were loaded per lane). Replica gels were transferred to nitrocellulose membranes and probed with anti-AAT antibody as described in section 2.3.7 (2.0×10^6 cell equivalents were loaded per lane). Detected AAT is labelled. The loading scheme is given below:

- Lane 1: molecular weight standards (Thermo Scientific)
- Lane 2: GPI-PLC^{-/-} whole cell SDS lysate
- P & S Lanes: pellet (P) and supernatant (s) samples taken at the indicated times
- Lane 23: GPI-PLC^{-/-} whole cell SDS lysate
- Lane 24: molecular weight standards (Thermo Scientific)

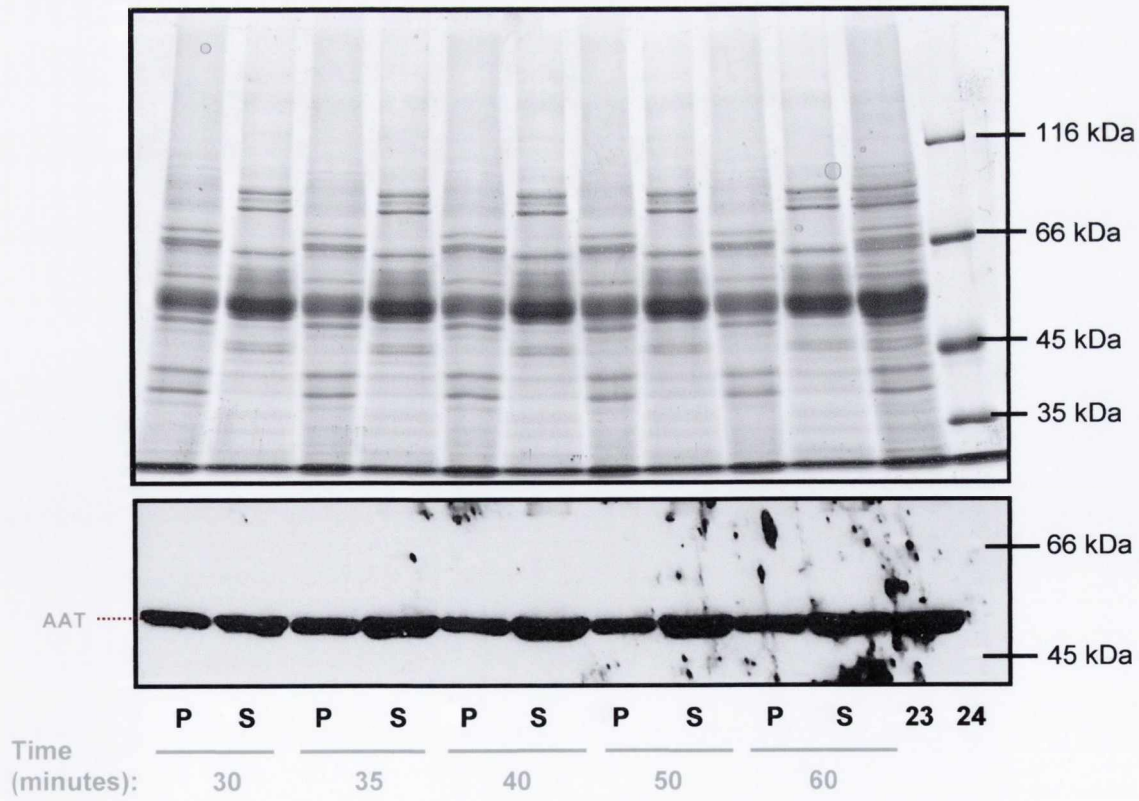
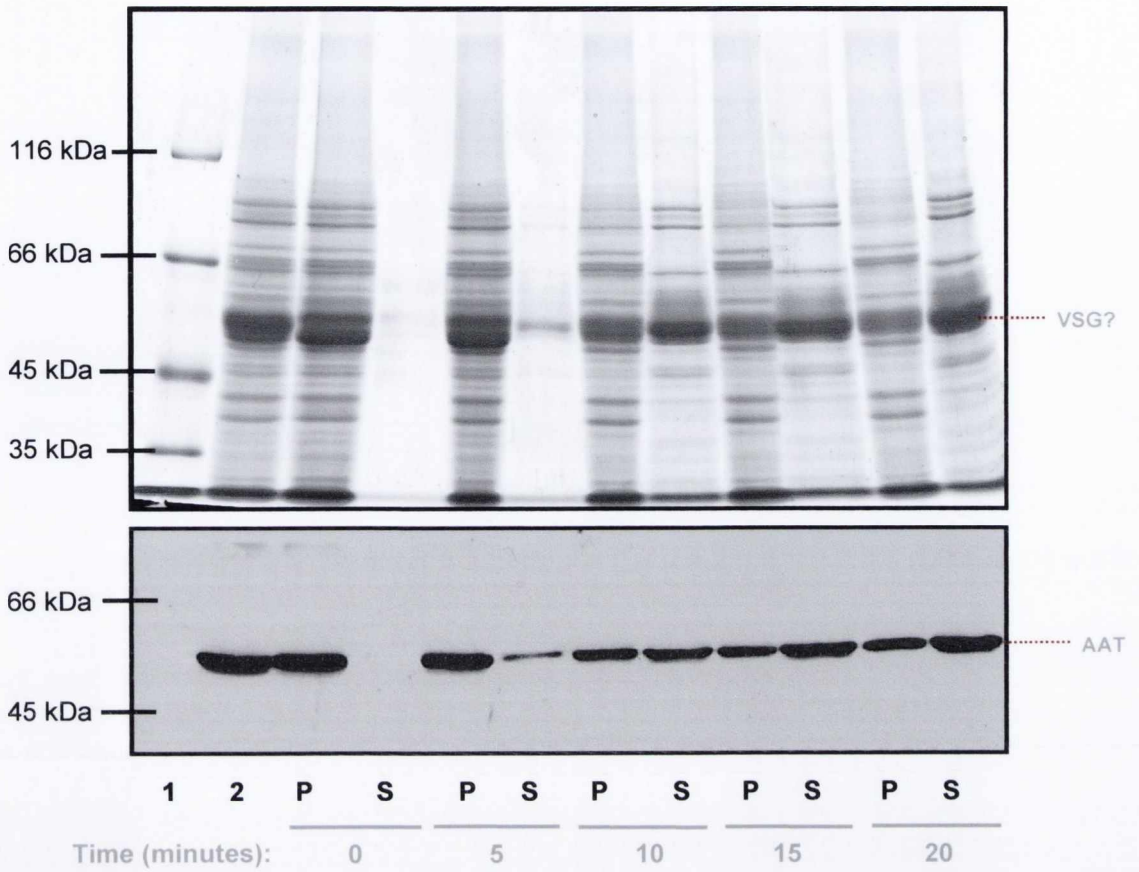


Plate 4.8. De-energization of WT Bloodstream Form Trypanosomes in the presence of EGTA

WT trypanosomes collected from the blood of an infected rat were de-energized in Tes buffer containing 5 mM EGTA as described in section 2.6.2. At selected time points, released protein (*i.e.* supernatants following centrifugation of de-energized cell sample), and protein remaining bound to the cell (*i.e.* pellets following centrifugation of de-energized cell sample), was run on a 10% denaturing gel and stained with Coomassie (2.5×10^6 cell equivalents were loaded per lane). Bands corresponding to sVSG and mfVSG are highlighted. Replica gels were transferred to nitrocellulose membranes and probed with anti-AAT antibody as described in section 2.3.7 (2.0×10^6 cell equivalents were loaded per lane). Detected AAT is labelled. The loading scheme is given below:

- Lane 1: molecular weight standards (Thermo Scientific)
- Lane 2: WT whole cell SDS lysate
- P & S Lanes: pellet (P) and supernatant (s) samples taken at the indicated times
- Lane 23: WT whole cell SDS lysate
- Lane 24: molecular weight standards (Thermo Scientific)

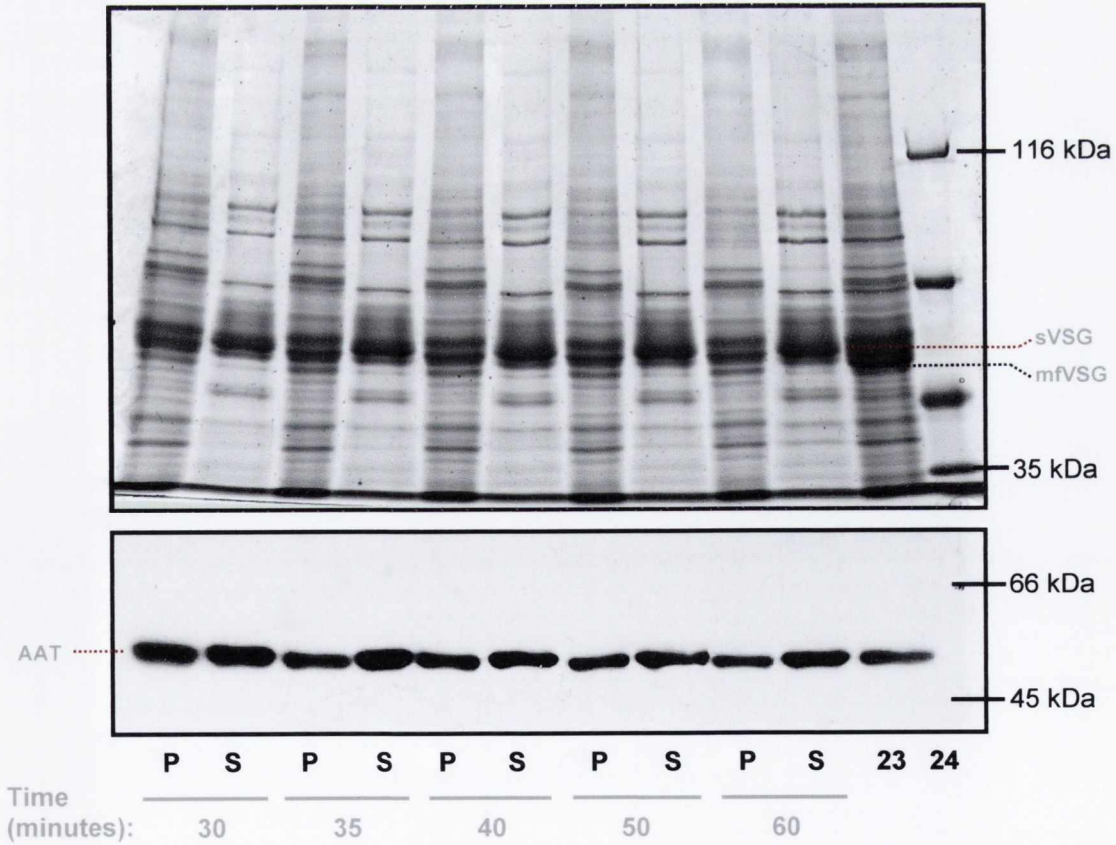
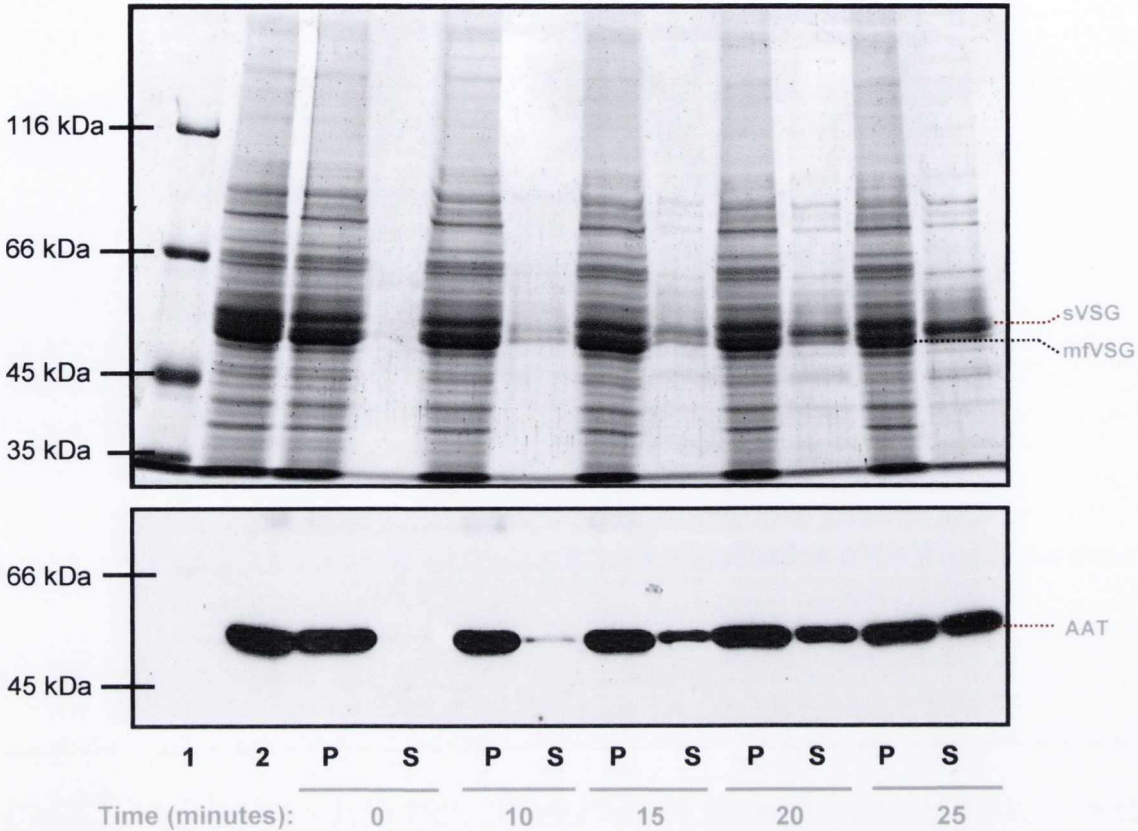
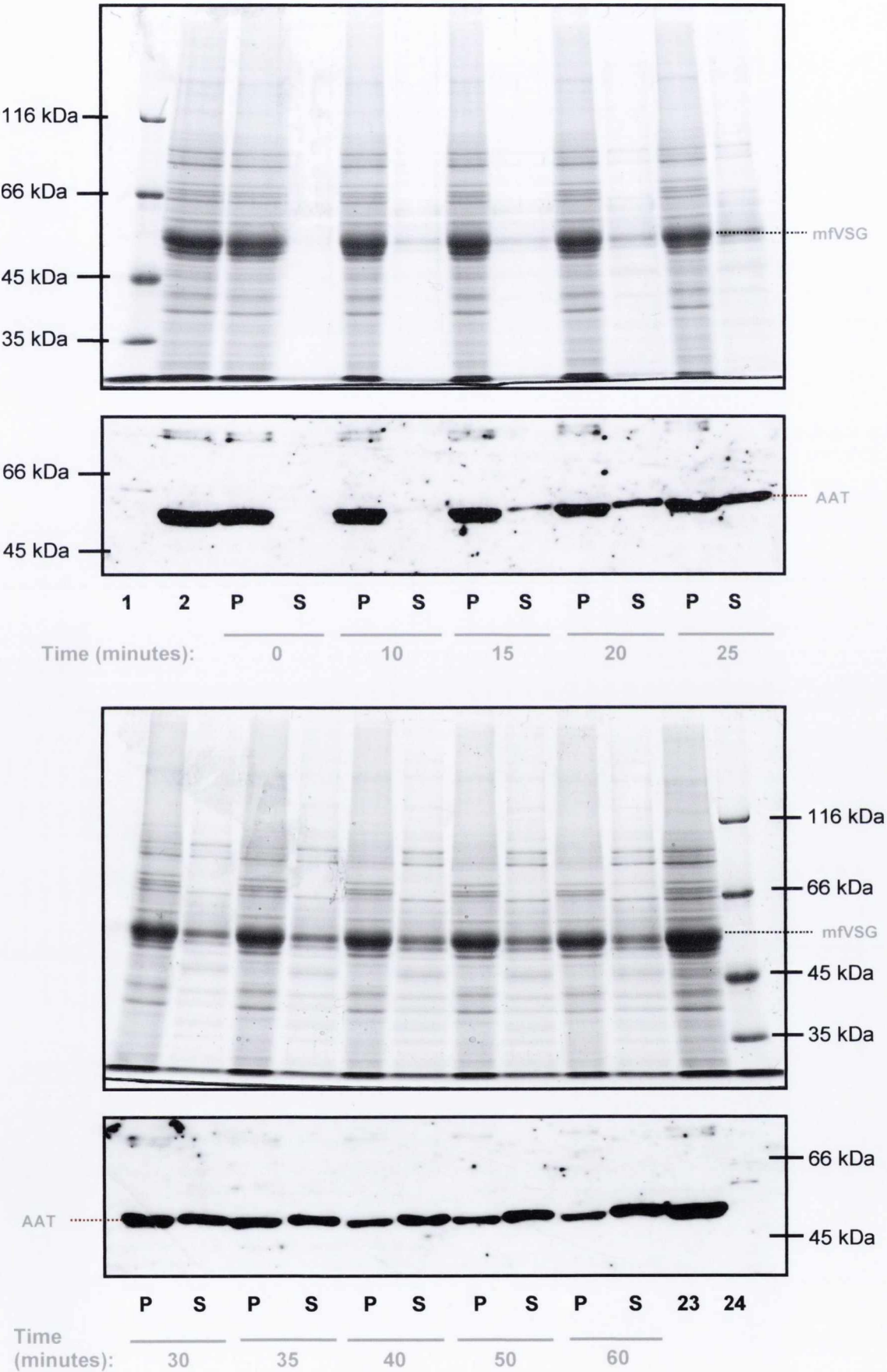


Plate 4.9. De-energization of GPI-PLC^{-/-} Bloodstream Form Trypanosomes in the presence of EGTA

GPI-PLC^{-/-} trypanosomes collected from the blood of an infected rat were de-energized in Tes buffer containing 5 mM EGTA as described in section 2.6.2. At selected time points, released protein (*i.e.* supernatants following centrifugation of de-energized cell sample), and protein remaining bound to the cell (*i.e.* pellets following centrifugation of de-energized cell sample), was run on a 10% denaturing gel and stained with Coomassie (2.5 x 10⁶ cell equivalents were loaded per lane). Replica gels were transferred to nitrocellulose membranes and probed with anti-AAT antibody as described in section 2.3.7 (2.0 x 10⁶ cell equivalents were loaded per lane). Detected AAT is labelled. The loading scheme is given below:

- Lane 1: molecular weight standards (Thermo Scientific)
- Lane 2: GPI-PLC^{-/-} whole cell SDS lysate
- P & S Lanes: pellet (P) and supernatant (s) samples taken at the indicated times
- Lane 23: GPI-PLC^{-/-} whole cell SDS lysate
- Lane 24: molecular weight standards (Thermo Scientific)



4.3. Discussion

Undoubtedly, the accurate and precise characterization of the de-energization of bloodstream form trypanosomes was more challenging than originally anticipated. Reports by other researchers had offered that the de-energization of bloodstream form trypanosomes in hyperosmotic buffer caused a release of the VSG without rupture of the cell (Hanrahan *et al.*, 2009). However, in the publication by Hanrahan *et al.*, no direct evidence was provided that showed trypanosomes remained intact during de-energization. Instead, this conclusion was based on related experimental data from much earlier work originating from the same laboratory; Bowles and Voorheis (1982) demonstrated that incubating bloodstream form trypanosomes in the presence of A-23187 and 1 mM Ca^{2+} caused a release of 80% of the surface coat protein, while much reduced amounts of intracellular proteins were released (*e.g.* 11% of total glucose-6-phosphate dehydrogenase and 34% of total malic enzyme were released). This publication was later used to support the idea that incubating cells in the presence of A-23187, 2 mM Ca^{2+} , and 2-deoxy-D-glucose also failed to rupture trypanosomes (Voorheis *et al.*, 1982). However, in this later report only the release of surface-labelled VSG was followed and the possible leakage of intracellular proteins was not studied.

Preliminary experiments shown in this chapter revealed that incubating trypanosomes in the presence of 25 mM 2-deoxy-D-glucose, in a hyperosmotic buffer, caused a release of protein which, when judged by SDS-PAGE, appeared similar to general cell lysis. It was additionally found throughout this work that increasing the osmotic strength from 350 mOsm to 450 mOsm had no effect on this general protein release (data not shown). Furthermore, regular additions of aliquots of the protease inhibitors PMSF and TLCK failed to prevent cell lysis. In light of previous accounts that reported bloodstream form trypanosomes remained intact during de-energization, particular attention in this chapter was afforded to distinguishing between the release of surface proteins, and the release of intracellular proteins. Initial attempts sought to distinguish between intracellular protein release and surface protein release by differentially labelling proteins with NHS-LC-biotin and sulfo-NHS-LC-biotin. However, these labelling reagents could not adequately differentiate between

intracellular proteins and surface proteins under the conditions attempted here, so their use was abandoned.

Western blotting of released material for the intracellular enzyme AAT was instead adopted as the means for assaying cell rupture. Using this approach it was definitively shown that the de-energization of bloodstream form trypanosomes causes a release of intracellular protein in addition to the VSG. Importantly, the release of VSG always correlated with cell rupture. However, it was not shown that VSG release was a direct result of cell rupture. Added Ca^{2+} caused VSG release to occur much more quickly than the basic de-energization condition, and Ca^{2+} also caused cell rupture and release of the intracellular marker enzyme AAT to occur more quickly than the basic de-energization condition.

With the data presented here it is impossible to discern if cell lysis is necessary for activation of the GPI-PLC. Clearly, activation of the GPI-PLC is not required for cell lysis during the de-energization procedure, as cell rupture occurs in GPI-PLC^{-/-} trypanosomes over the same time course as in WT cells. It is also impossible to tell if intracellular Ca^{2+} acts in one pathway to induce cell rupture (*e.g.* by activation of a calcium dependent protease) and a second separate pathway that leads to activation of the GPI-PLC through some direct protein interaction with the GPI-PLC. Although it is possible that membrane rupture triggers the GPI-PLC-mediated hydrolysis of the mfVSG anchor, without any need for interacting proteins to directly activate the GPI-PLC, it is difficult to predict what specific aspect of this membrane rupture could activate the GPI-PLC. This is particularly true considering the GPI-PLC and VSG are already located in the same cellular compartment.

Although 5 mM EGTA failed to inhibit the VSG release process, and the breakdown of cells, it is possible that equilibrating cells in a cell permeable analogue of EGTA prior to de-energization could finally reveal if intracellular Ca^{2+} was required for general cell rupture, or if intracellular Ca^{2+} could act to specifically activate the GPI-PLC present on the flagellar membrane. It is likely that EGTA added during the experiments presented here could not access the intracellular Ca^{2+} that was inducing cell lysis and protein release, until the trypanosomal membrane had already ruptured and become permeable to large molecules.

Although the regulation of VSG release by intracellular calcium remains an interesting hypothesis warranting further investigation, there is currently no substantial evidence available that shows that the GPI-PLC is regulated directly by calcium, or that the enzyme is regulated through an interaction with a calcium binding protein.

Chapter 5

In Vitro Growth Characteristics of GPI-PLC^{-/-} Trypanosomes

5.1. Introduction

5.1.1. Historical Observations on the Growth of GPI-PLC^{-/-} Cell Lines

The first double knock-out of the GPI-PLC in trypanosomes was achieved by Rolin, and was performed in the AnTat serodeme to study the relationship between GPI-PLC-mediated VSG release and adenylate cyclase activation (Rolin *et al.*, 1996). A second GPI-PLC^{-/-} cell line was developed the following year by the same laboratory, and also prepared in an EATRO 1125-derived cell line. Although it was not known precisely what VSG was expressed by either of these GPI-PLC knock-out cells, the later stock of mutant cells was found to react with anti- AnTat 1.1, AnTat 1.2, AnTat 1.3, and AnTat 1.6 antibody (Webb *et al.*, 1997). Although the GPI-PLC^{-/-} cell line prepared by Webb displayed no “gross” changes in growth rate, there were particular observations which showed that deletion of the GPI-PLC genes had an effect on the growth of trypanosomes.

The first growth phenotype observed was a significantly reduced first peak of parasitemia in mice infected with the mutant GPI-PLC^{-/-} trypanosomes; the cell density of the first peak of parasitemia was at least three times lower than in mice infected with control cells. Indeed a lower overall density of trypanosomes in the bloodstream throughout the time course of infection was measured, and it was postulated that the reduced cell densities may have been at least partly attributable to a reduced growth rate of the GPI-PLC^{-/-} cells (Webb *et al.*, 1997). There was also a significantly increased mean survival time of animals infected with the mutant trypanosomes. The

reduced virulence of the GPI-PLC^{-/-} trypanosomes may have been a result of less vigorous growth, associated with the loss of GPI-PLC activity.

However, another publication has cast doubt on the possibility that there may be any growth phenotype associated with deletion of the GPI-PLC genes in *T. brucei*. A third mutant cell line was been produced by the Donelson laboratory (Grandgenett *et al.*, 2007). Bloodstream form GPI-PLC^{-/-} cells of the MITar serodeme (which reacted with anti-MITat 1.2 antibody) were shown to grow indistinguishably from wild type cells in culture. There was no effect observed on the growth rate of mutant cells, on the maximum density attainable in culture, or on the transformation of bloodstream form cells to procyclic cells. Instead, loss of GPI-PLC activity was found to affect only the transformation of bloodstream form cells to procyclic form cells in cells additionally lacking membrane surface protease B (MSP-B) activity.

The apparent disparity between the results of Grangenett and Webb may be due to differences in the trypanosome stocks (*i.e.* the EATRO 11125 stock *versus* MITar serodeme) used to generate the GPI-PLC^{-/-} mutants in each of the publications, or perhaps the growth differences found *in vivo* are simply not mirrored *in vitro*. In either case, further study is warranted to determine if indeed there is any effect on the growth of bloodstream form trypanosomes following deletion of the GPI-PLC genes. Such a study, which is the focus of this chapter, may be essential in helping to elucidate the true physiological role of the GPI-PLC.

5.1.2. Growth Analysis and the Inhibited Growth Model

The use of nonlinear mathematical models has not been widely adopted for describing trypanosomal growth. While some growth curves allow researchers to observe only considerable differences in the growth of cells, the use of mathematical models allows for cell growth to be rigorously quantified across a variety of parameters. These parameters vary between the available models and it is often useful for researchers to select a model which describes the growth characteristics with parameters intuitive to the subject being studied. The various mathematical models, such as the Gompertz model, the Richards model, and the inhibited growth model, have all been subjected to numerous re-parameterizations (*e.g.* Tjørve and Tjørve, 2010,

Zwietering *et al.*, 1990) in order to aid researchers more intuitively understand the growth of the organism under study.

Two factors led to the adoption of the inhibited growth model for describing the growth of trypanosomes in culture throughout this work. Firstly, in attempting to fit the Gompertz or Richards models to experimental data, overflow or underflow errors frequently occurred. Such errors are the result of the model fitting software (Mathematica versions 7.0.1.0 and 8.0.0.0) encountering numbers which are too large (or too small in the case of underflow errors) for the computer to use during the model fitting procedure. No such overflow or underflow errors were encountered while using the inhibited growth model. Secondly, the parameterization of the inhibited growth model also makes it somewhat more intuitive than other models; the parameters are more easily related to various aspects of trypanosomal cell growth.

The inhibited growth model, also referred to as the logistic model, the logistic differential equation, or as the Verhulst model, models the growth of a population confined to a system that can only support a certain maximum number of individuals. This maximum capacity of the system is termed the carrying capacity (L) and, in relation to the growth of trypanosomes in culture, reflects the maximum density to which the cells can grow under defined conditions. The Verhulst model has the general form:

$$\frac{dy}{dt} = k \left(1 - \frac{y}{L}\right) y \quad (1)$$

Where dy/dt represents the rate of change in population, or cell density, (y) over time (t), with a rate proportional to k (Verhulst 1845). From (1) it can be seen that if the cell density ever exceeds the carrying capacity of the system (*i.e.* if $y/L > 1$) then the growth rate (dy/dt) is a negative value, resulting in a decrease in cell numbers. If the cell density is below the carrying capacity of the system (*i.e.* if $y/L < 1$) then the growth rate is a positive value and the population size increases. If the cell population equals the system's carrying capacity (*i.e.* $y/L = 1$) then the system is in equilibrium – the growth rate is zero so the cell population remains at a constant value.

It should be pointed out that once the system's carrying capacity has been reached, probably due to exhaustion of components required for further growth,

trypanosomes will continue to consume the remaining nutrients available in the system until the environment can no longer support cell growth and survival. Eventual exhaustion of components required for growth therefore results in a deviation from the mathematical models used to describe growth. The subsequent cell death phase is normally not considered in cell growth analysis and is not incorporated by the inhibited growth model (or indeed other commonly used cell growth models).

Integrating and solving (1), introduces the parameter A (defined as $\pm e^{-C}$, where C is the constant of integration and e is the Euler number) and is the form of the equation used throughout this thesis:

$$y = \frac{L}{1 + Ae^{-kt}} \tag{2}$$

This model therefore defines cell growth over time in terms of the maximum density or population of cells achievable (L), and the rate at which this maximum density is achieved (influenced predominantly by k). When the natural logarithm of the cell density is plotted against time, the result is a sigmoidal trace with several important characteristics. Firstly, the maximum growth rate is defined as the slope of the tangent to the curve at the point of inflection. The point at which this tangent intercepts the time axis, or lower asymptote, has been used as a definition of the end of the lag phase (a period of initial slow cell growth) and the beginning of log phase (a period of approximately linear cell growth), (Zwietering *et al.*, 1990). Classically, cells in log phase represent cells which are growing optimally, have overcome initial culture conditions which may not have been perfect for best growth, and are ideally suited for use in experimental work. However, since there is no truly linear region to the inhibited growth model, or indeed other growth models, the selection of a log phase remains somewhat arbitrary and should be clearly defined in each piece of work regarding cell growth analysis.

For this thesis, the beginning of log phase will be as described by Zwietering. The period of time between the start of log phase and the point of maximum growth rate will be the mid-log phase, while the remainder of the log phase continues for an equal amount of time. The end of log phase therefore corresponds to a deviation of the

cell growth from a logarithmically linear increase in cell numbers. The lag phase, mid-log phase, log phase, and system carrying capacity are illustrated in figure 5.1.

5.1.3. Doubling Time

From the inhibited growth model, the rate of proliferation of cells is determined chiefly by the parameter k . However it is not normally intuitive to what extent k affects the cell growth rate. A more useful measure is to describe the growth rate in terms of doubling time – *i.e.* how many hours it would take for the cell population to double. The doubling time at any point on the growth curve can be deduced by first drawing a tangent to the curve at the point in question. The slope, at time point t , (μ_t) of the tangent is given by the usual formula:

$$\mu_t = \frac{\text{Ln}(y_2) - \text{Ln}(y_1)}{t_2 - t_1} \quad (3)$$

For the doubling time, $t_2 - t_1$ can be renamed T_t (*i.e.* the doubling time at time t), and y_2 must equal $2y_1$, in which case (3), once rearranged, becomes:

$$T_t \mu_t = \text{Ln}(2y_1) - \text{Ln}(y_1) \quad (4)$$

Which simplifies as:

$$T_t \mu_t = \text{Ln}\left(\frac{2y_1}{y_1}\right) \quad (5)$$

$$T_t = \frac{\text{Ln}(2)}{\mu_t} \quad (6)$$

So, the doubling time at any point on a logarithmic growth curve can be calculated by dividing $\text{Ln}(2)$ by the slope of the tangent to that point. This is principle is illustrated in figure 5.2.

5.1.4. A Note of Caution

Although logarithmic growth curves provide very valuable information on the growth rates of cells under study, it should be pointed out that the system's carrying capacity is more accurately derived from non-logarithmic growth curves. Calculation of the carrying capacity requires extrapolation of the growth curve data to an upper asymptote, an asymptote that is not precisely observed experimentally. Even minute errors in evaluating the upper asymptote can drastically affect the deduced carrying capacity when the values are converted back to the non-logarithmic cell densities. Therefore, in this chapter both logarithmic and non-logarithmic growth curves are presented; the useful information from each type of growth curve is then rationally compared.

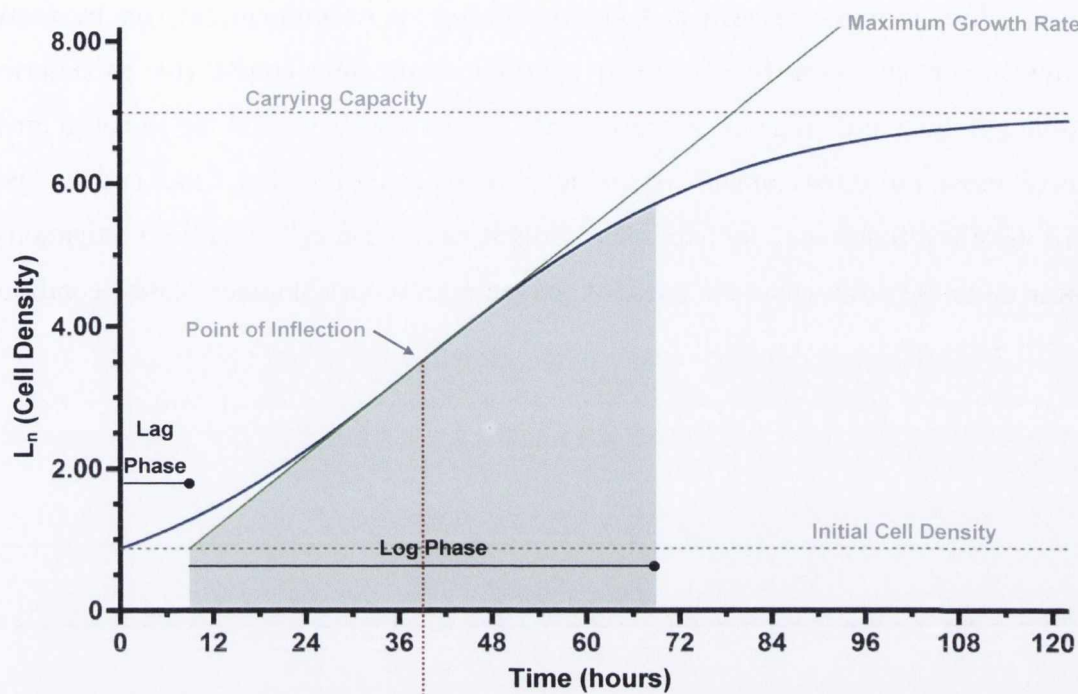
5.1.5. Aims

The true physiological role of the GPI-PLC has remained unknown since the enzyme's discovery. A requirement of the enzyme for optimal growth of the parasite remains unclear as different studies have produced irregular results. This chapter seeks to determine if deletion of the GPI-PLC genes has any effect on the growth of bloodstream form trypanosomes in culture; the effect of deleting the GPI-PLC genes will be rigorously characterized using the inhibited growth model fitted to experimental data.

Figure 5.1. Standard Growth Curve using the Inhibited Growth Model

Shown in (a) is a generalized growth curve, with the log phase of cell growth, and the lag phase, highlighted. The maximum growth rate is defined as a tangent to the curve at the point of inflection (green line); using the inhibited growth model the point of inflection always occurs at half the system's carrying capacity (brown dashed line). The growth rate of the culture can also be plotted as a function of time (b), which more clearly shows the maximum growth rate of the culture. The graph in (b) also illustrates that the growth rate of a cell culture is constantly changing; the growth rate increases until the maximum growth rate is achieved, at which time the growth rate begins to slow as the carrying capacity is reached. The cell death phase is not shown in either (a) or (b). The log phase of cell growth phase is shaded in green; the lag phase and mid-log phase are also labelled.

(a)



(b)

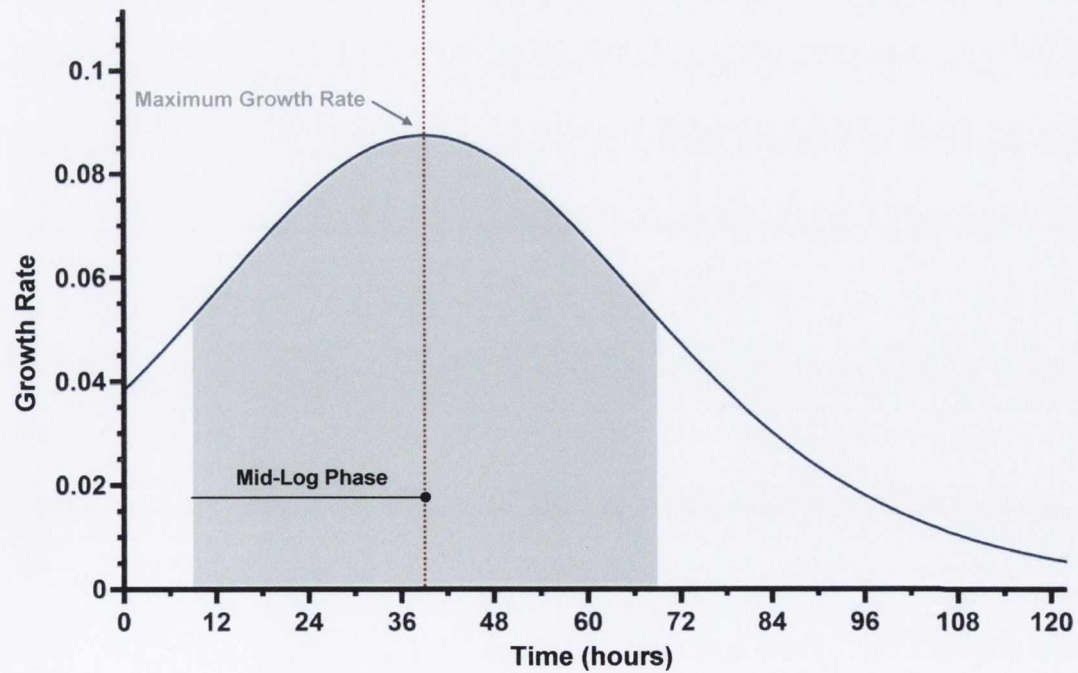
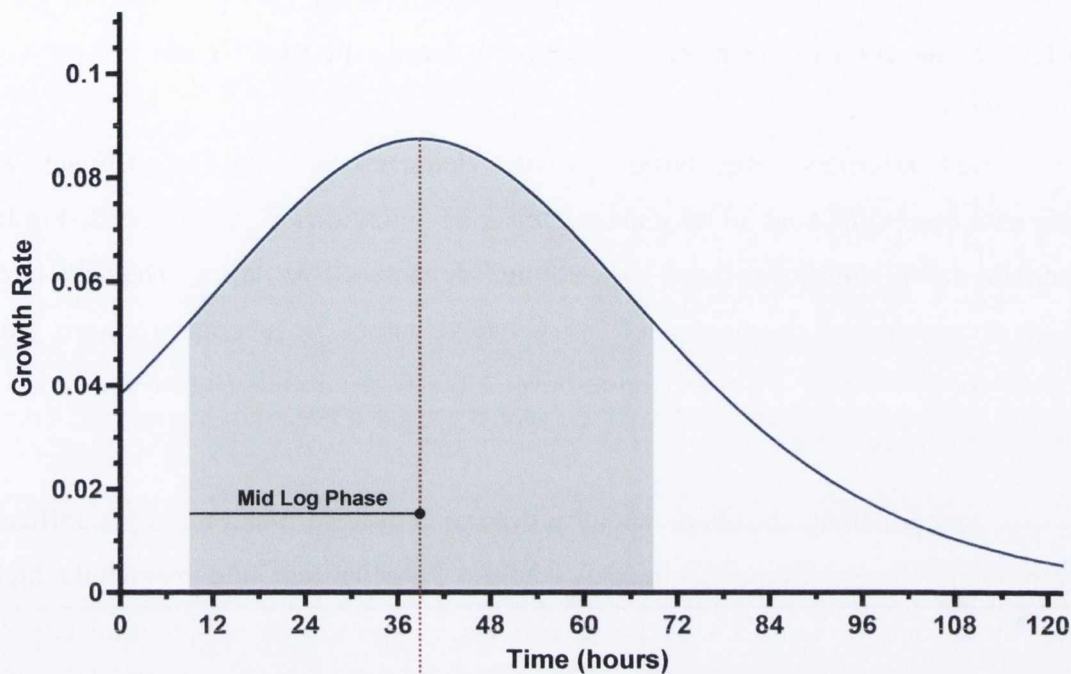


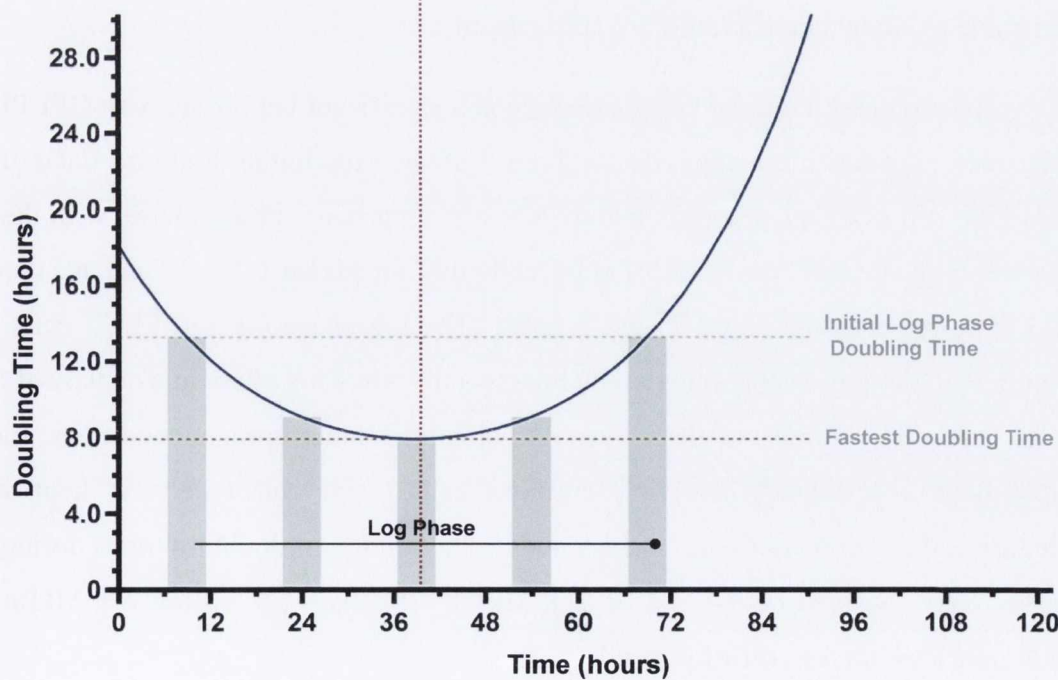
Figure 5.2. Cell Growth Rate Shown as Doubling Time

The growth rate of a cell culture during an experiment (a) can be used to calculate the doubling time of cells over the same time course (b), as described. Although the entire range of doubling times can be readily plotted for an entire growth curve, most useful for comparison are the early log phase doubling times and the fastest cell doubling times (*i.e.* the maximum growth rate of the cells under investigation) as these doubling times cover the range of growth rates observed under healthy conditions.

(a)



(b)



5.2. Results

5.2.1. General Growth Characteristics of WT and GPI-PLC^{-/-} Cells

To determine the basic growth characteristics for bloodstream form trypanosomes, cells kept under standard culturing conditions were resuspended in fresh medium before diluting to 0.5×10^5 cells·ml⁻¹ in each of five flasks. The cells in each flask were counted approximately every twelve hours to generate accurate growth curves for WT MITat 1.2 cells (figure 5.3), WT MITat 1.1 cells (figure 5.4), and GPI-PLC^{-/-} cells (figure 5.5).

The principle characteristics of culture growth were found to vary significantly between the three cell types studied. WT MITat 1.2 cells were able to reach the highest density of each of the three cell types, with a carrying capacity of approximately 7.9×10^6 cells·ml⁻¹ obtained (note that using the carrying capacity from the logarithmic growth curve of WT MITat 1.2 cells yields a value of 2.5×10^7 cells·ml⁻¹). Identical culture conditions were able to support less than half this number of GPI-PLC^{-/-} cells, which reached a maximum density of only 3.4×10^6 cells·ml⁻¹. WT MITat 1.1 cells were able to attain a density of 6.5×10^6 cells·ml⁻¹.

In each case cultured cells experienced a significant lag phase, with GPI-PLC^{-/-} cells even experiencing a slight death phase following the initial dilution. Each of the wild-type cell lines spent approximately seventy eight hours in log phase, with the log phase ending at a cell density of 6.7×10^6 cells·ml⁻¹ for MITat 1.2 cells, and at a density of 2.95×10^6 cells·ml⁻¹ for MITat 1.1 cells. The log phase for GPI-PLC^{-/-} cells was significantly longer, lasting ninety nine hours; cells exited log phase at a density of 2.48×10^6 cells·ml⁻¹. To ensure all cells were maintained under optimal conditions, for all subsequent experiments involving cultured cells, each cell type was kept from exceeding these maximum log phase values. The minimum doubling times during log phases were found to be 9.4 hrs for WT MITat 1.2 cells, 11.6 hrs for WT MITat 1.1 cells, and 12.4 hrs for GPI-PLC^{-/-} cells.

Analysis of the proportion of cells in each cell cycle stage revealed no obvious cell cycle defect, although there were found to be more errors of cell division in GPI-

PLC^{-/-} cells (figure 5.6). Compared to WT cells for example, 7.6% of GPI-PLC^{-/-} cells were multi-nucleated (*i.e.* the cells had more than two nuclei), had no nuclei, or had numerous kinetoplasts (*i.e.* more than two). This figure for WT cells was a more modest 2.8%. The percentages of cells in the '2K1N' stage of the cell cycle were 8.9% and 10.1% for WT and GPI-PLC^{-/-} cells respectively; in the '2K2N' stage the percentages were 7.1% and 8.4% for WT and GPI-PLC^{-/-} cells respectively, while the remainder of the cell populations were at the '1K1N' stage. Microscopic images of cultured trypanosomes at each of these cell cycle stages is shown in plate 5.1.

Figure 5.3. Growth Curve for WT MITat 1.2 Bloodstream Form Trypanosomes

WT MITat 1.2 cells were resuspended in fresh medium before diluting to 0.5×10^5 cells·ml⁻¹ in each of five flasks. The cell density in each flask was determined approximately every twelve hours. The mean of each determination is plotted in (a), with error bars showing the standard error of the mean; where error bars are not visible, the error is smaller than the representation of the time point. The cell densities were converted to the natural logarithm of the cell densities in (b) allowing for the log phase of cell growth to be determined as described in section 5.1.2. The log phase extends from 1×10^4 cells·ml⁻¹ to 6.7×10^6 cells·ml⁻¹ as represented in (c), and is shaded green in (a), (b) and (c). The solutions to the inhibited growth model for each of (a) and (b) are given below:

$$\text{Graph (a): } \text{Cell Density} = \frac{7.8598}{1+52,347e^{-0.1402t}}$$

(correlation coefficient = 0.9902)

$$\text{Graph (b): } \ln(\text{Cell Density}) = \frac{7.8437}{1+6.6785e^{-0.0375t}}$$

(correlation coefficient = 0.9981)

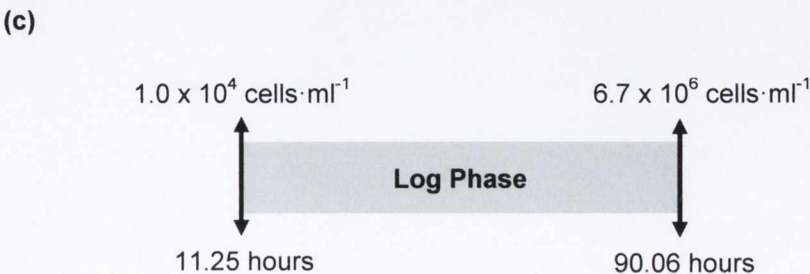
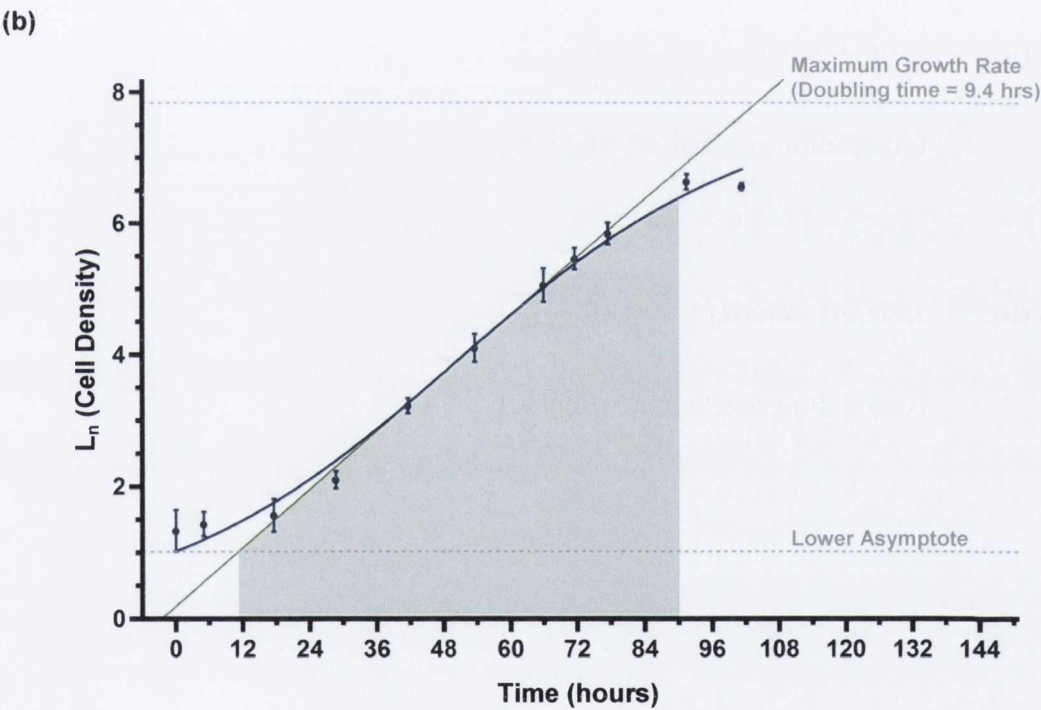
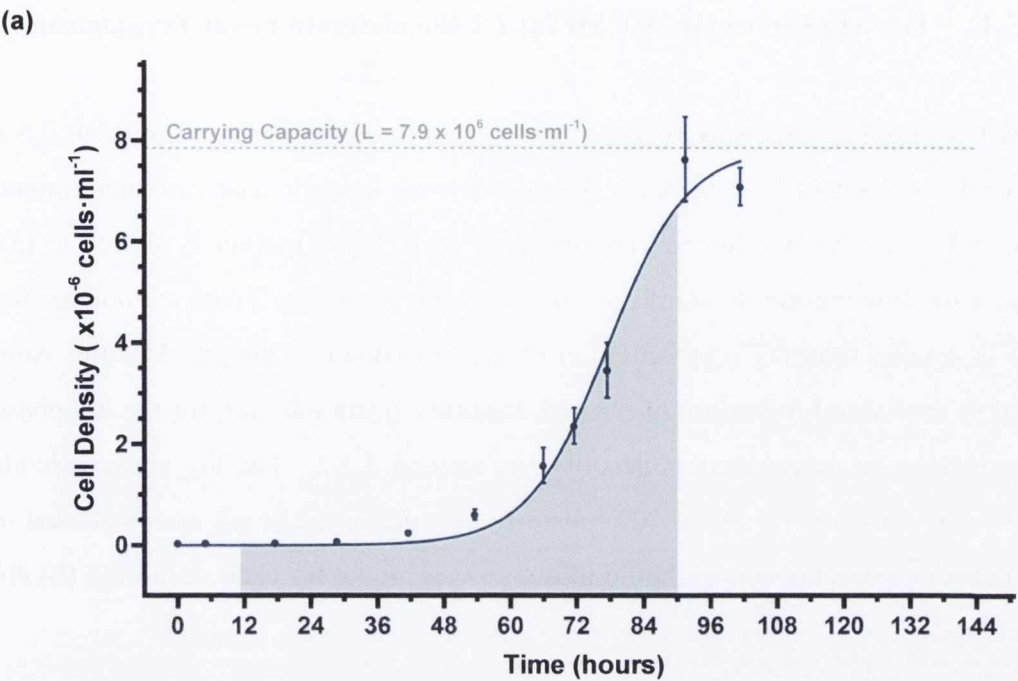


Figure 5.4. Growth Curve for WT MITat 1.1 Bloodstream Form Trypanosomes

WT MITat 1.1 cells were resuspended in fresh medium before diluting to 0.5×10^5 cells·ml⁻¹ in each of five flasks. The cell density in each flask was determined approximately every twelve hours. The mean of each determination is plotted in (a), with error bars showing the standard error of the mean; where error bars are not visible, the error is smaller than the representation of the time point. The cell densities were converted to the natural logarithm of the cell densities in (b) allowing for the log phase of cell growth to be determined as described in section 5.1.2. The log phase extends from 1.9×10^4 cells·ml⁻¹ to 2.95×10^6 cells·ml⁻¹ as represented in (c), and is shaded in (a), (b) and (c). The solutions to the inhibited growth model for each of (a) and (b) are given below:

Graph (a):
$$Cell\ Density = \frac{6.5232}{1+608.6e^{-0.0691t}}$$

(correlation coefficient = 0.9996)

Graph (b):
$$Ln(Cell\ Density) = \frac{6.7396}{1+5.5e^{-0.0353t}}$$

(correlation coefficient = 0.9930)

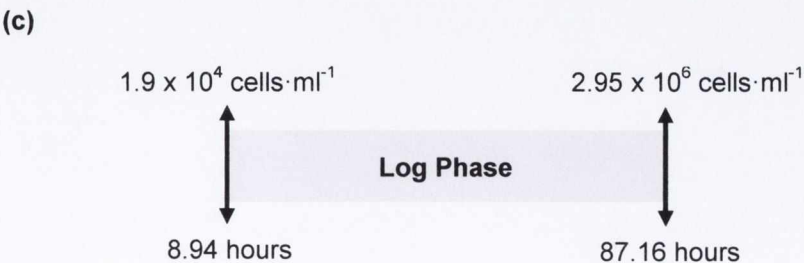
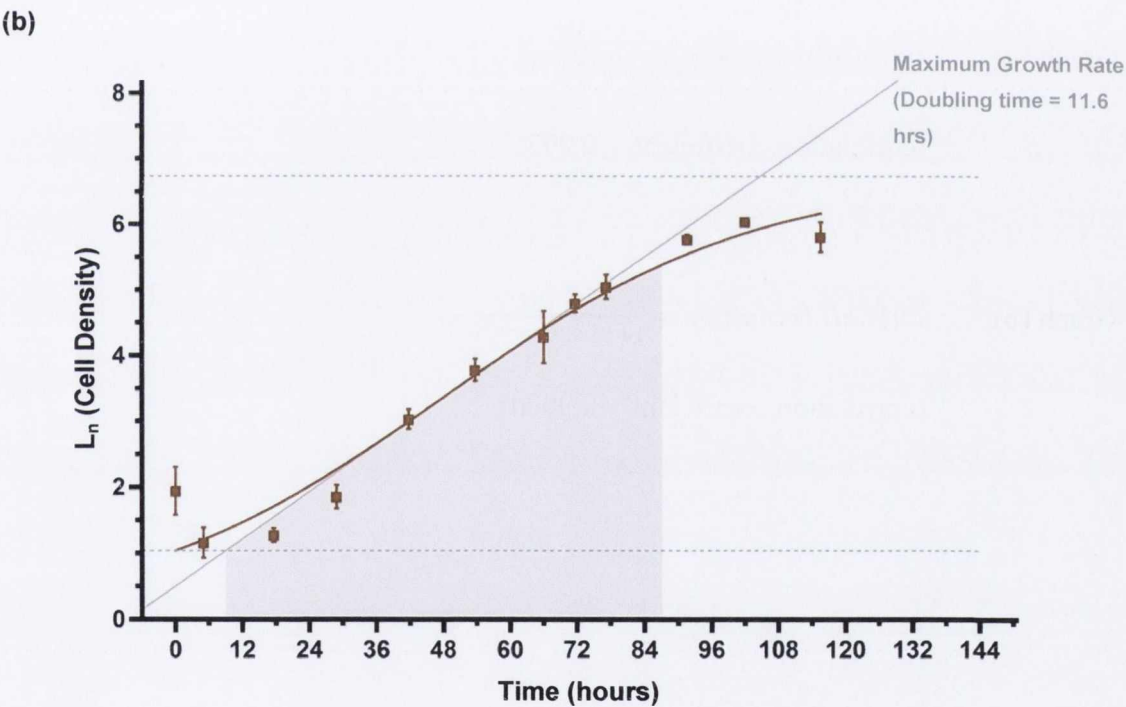
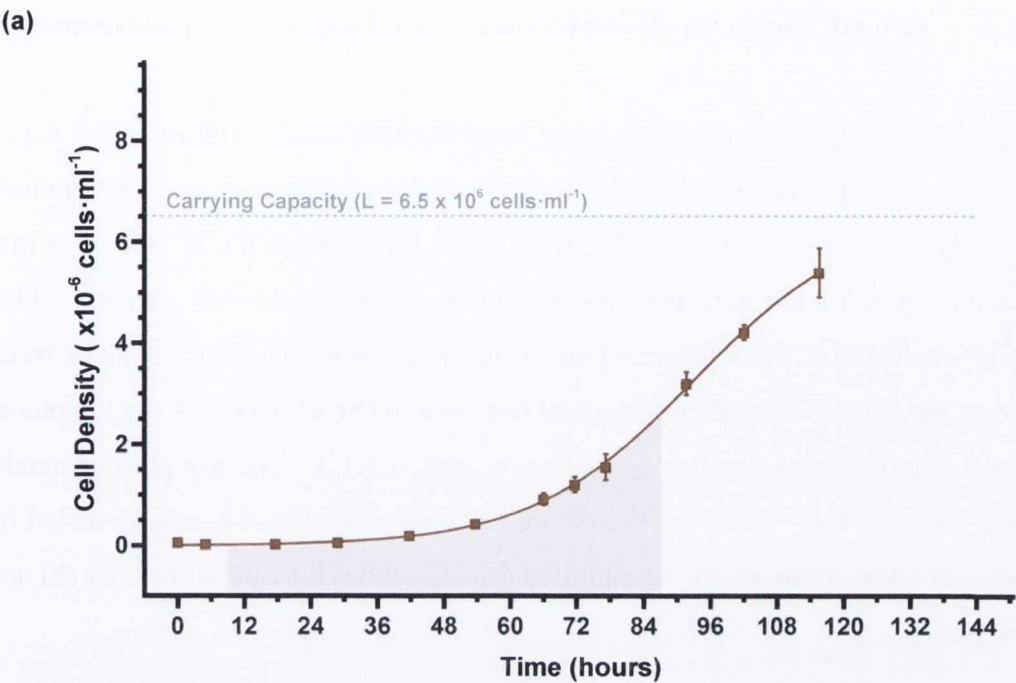


Figure 5.5. Growth Curve for Bloodstream Form GPI-PLC^{-/-} Trypanosomes

GPI-PLC^{-/-} cells were resuspended in fresh medium before diluting to 0.5×10^5 cells·ml⁻¹ in each of five flasks. The cell density in each flask was determined approximately every twelve hours. The mean of each determination is plotted in (a), with error bars showing the standard error of the mean; where error bars are not visible, the error is smaller than the representation of the time point. The cell densities were converted to the natural logarithm of the cell densities in (b) allowing for the log phase of cell growth to be determined as described in section 5.1.2. The log phase extends from 2.8×10^4 cells·ml⁻¹ to 2.5×10^6 cells·ml⁻¹ as represented in (c), and is shaded in (a), (b) and (c). The solutions to the inhibited growth model for each of (a) and (b) are given below:

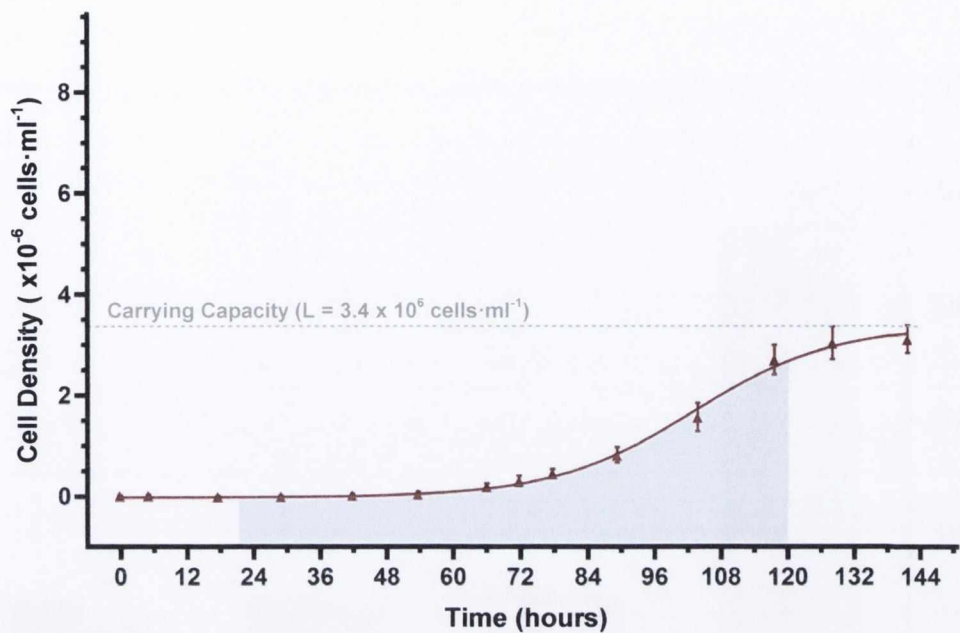
$$\text{Graph (a): } \text{Cell Density} = \frac{3.3763}{1+3,545e^{-0.0794t}}$$

(correlation coefficient = 0.9975)

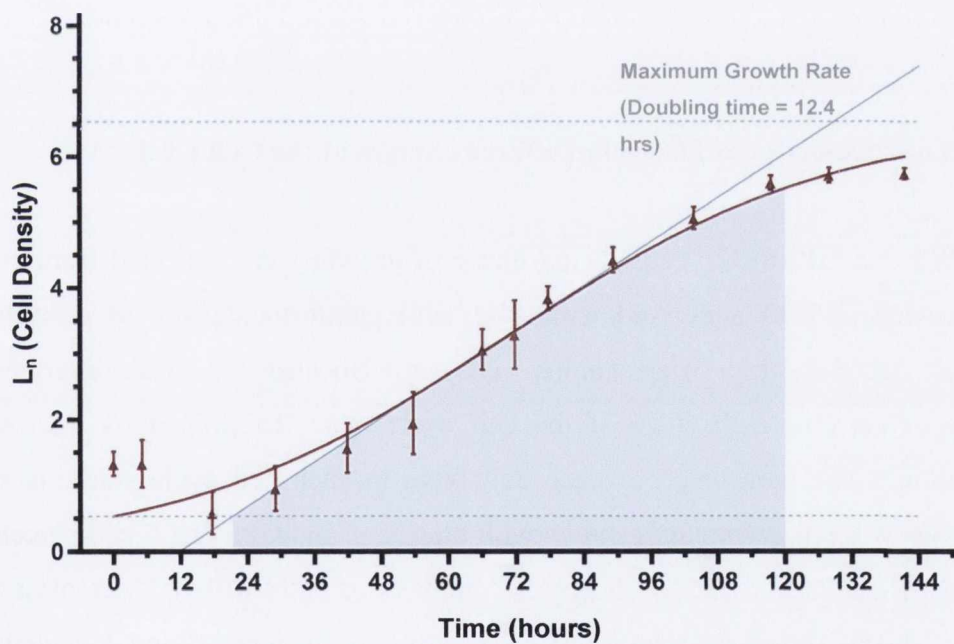
$$\text{Graph (b): } \ln(\text{Cell Density}) = \frac{6.5391}{1+11.1308e^{-0.0314t}}$$

(correlation coefficient = 0.9909)

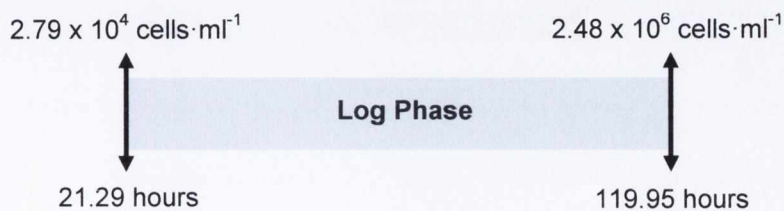
(a)



(b)



(c)



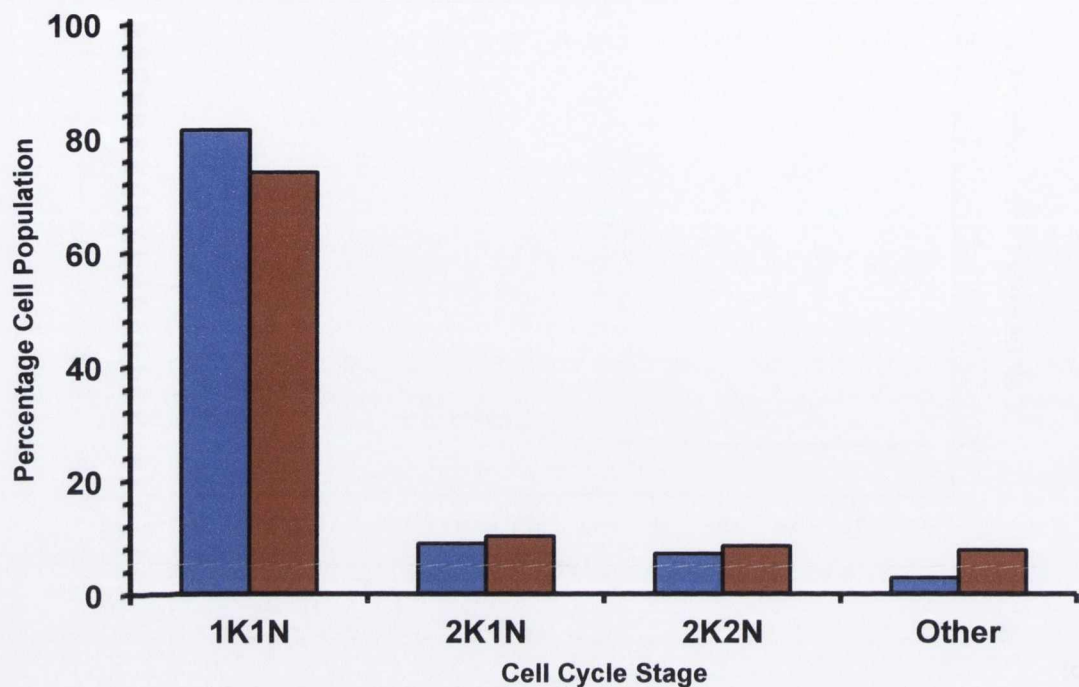


Figure 5.6. Proportions of Cells in Different Stages of the Cell Cycle

WT and GPI-PLC^{-/-} cells in log phase of growth were collected from culture, washed twice in PBS and fixed with 6% (w/v) paraformaldehyde as described in Materials and Methods. After staining cells with Hoechst 33342, as described, the numbers of cells in each stage of the cell cycle were determined by microscopic examination. The percentage of each cell type, in each cell cycle stage, is shown above, with WT cell percentages shown with blue bars, and GPI-PLC^{-/-} cell percentages shown with red bars. ‘1K1N’ shows the numbers of cells with 1 kinetoplast and 1 nucleus, ‘2K1N’ shows the number of cells with two kinetoplasts and 1 nucleus, and ‘2K2N’ shows the number of cells with two kinetoplasts and two nuclei. ‘Other’ refers to cell bodies with no nuclei (*i.e.* “zoids”), or multi-nucleated cells. In total, 496 WT cells and 386 GPI-PLC^{-/-} cells were counted.

Plate 5.1. Stages of the cell cycle in bloodstream form *Trypanosoma brucei*

Bloodstream form trypanosomes, collected from culture during the log growth phase, are shown opposite. An entire field of view, through a 63X oil immersion objective, showing a phase contrast image, Hoechst 33342 staining, and a merge of both these images, are presented in panels (a), (b), and (c) respectively. Examples of trypanosomes during the cell cycle phases 1K1N, 2K1N and 2K2N, are shown in panels (d, e, f), (g, h, i) and (j, k, l) respectively. The kinetoplast is labelled with a dashed arrow and the nucleus with a filled arrow in (e). All scale bars are 10µm.

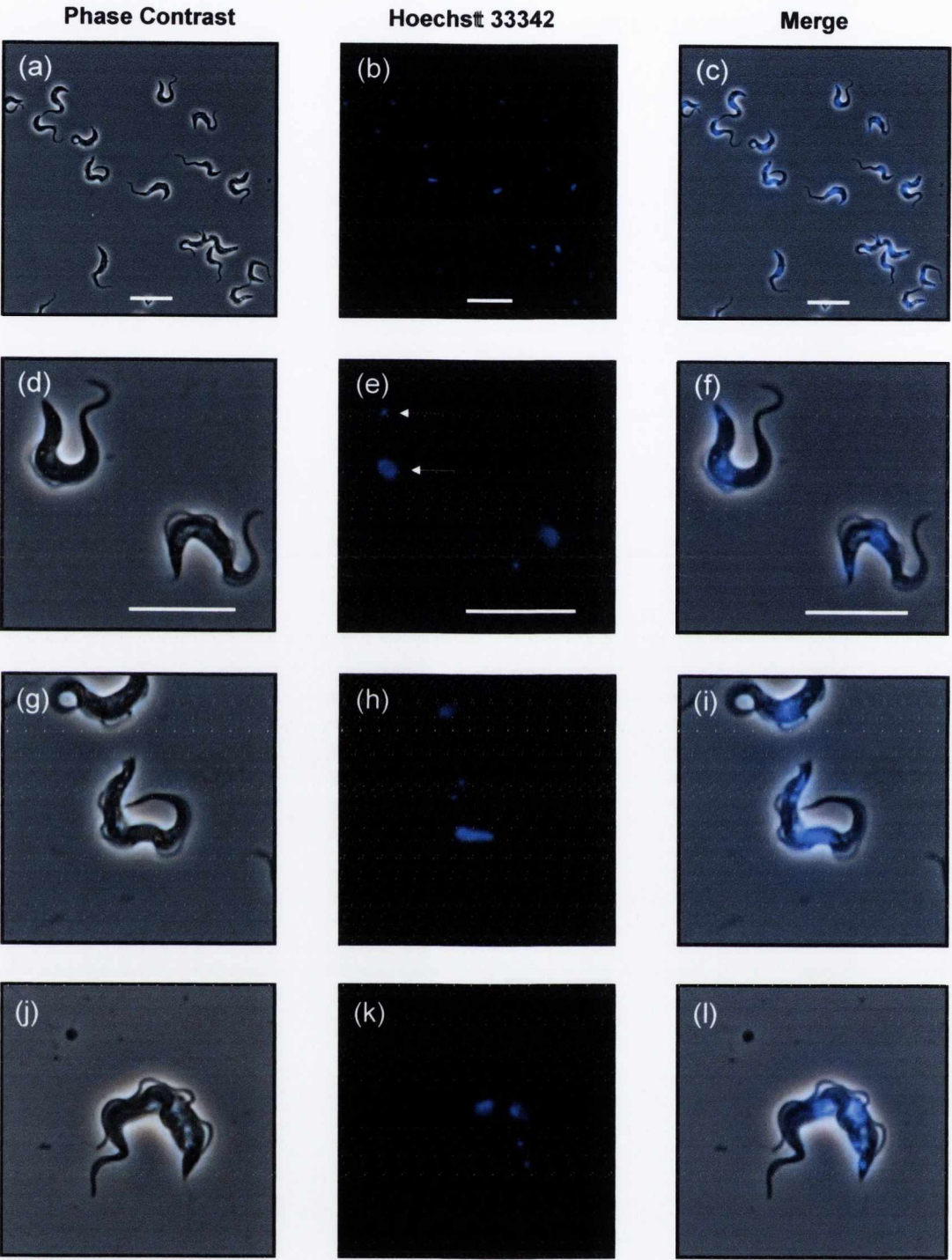
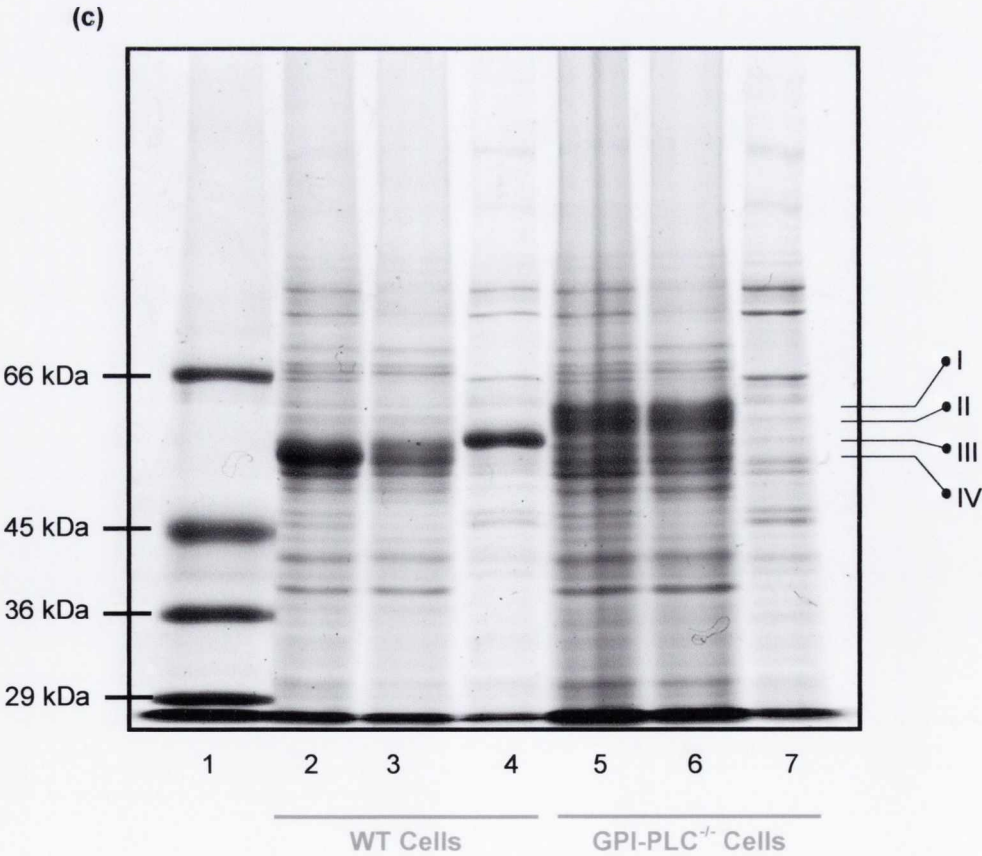
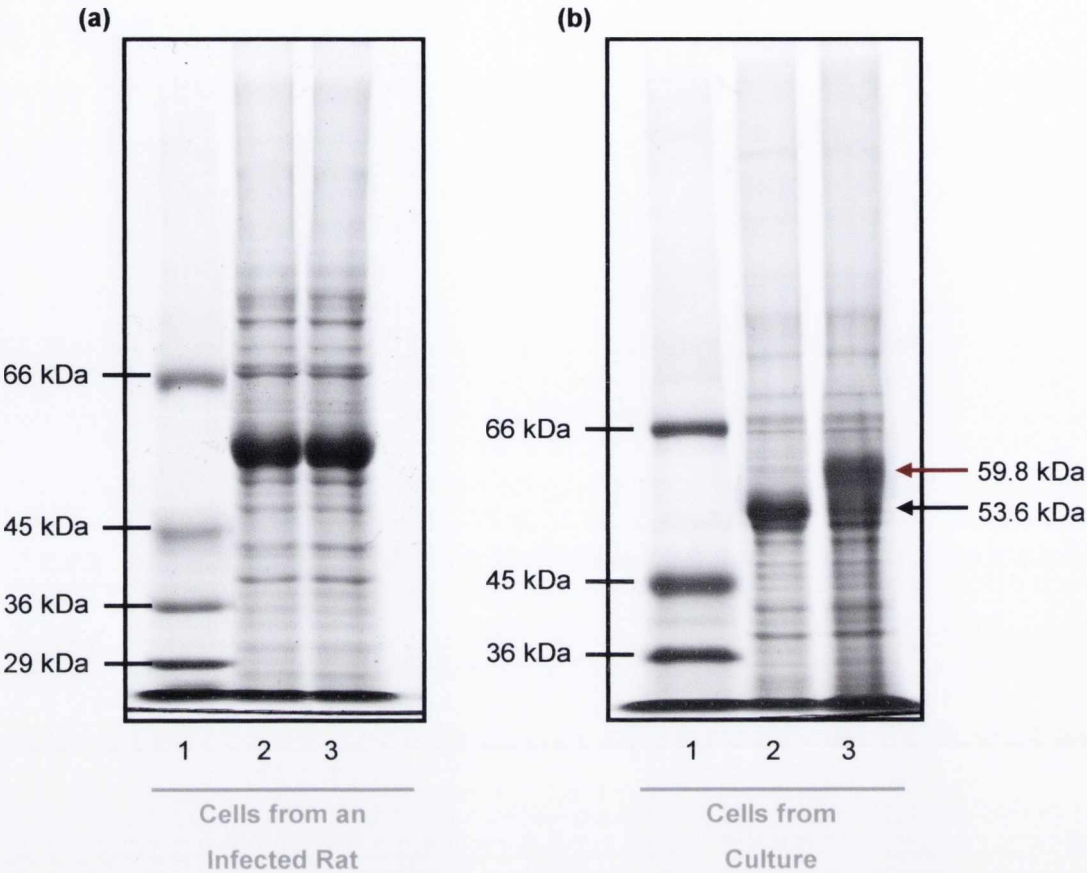


Plate 5.2. VSG Expression is Unusual in Cultured GPI-PLC^{-/-} Trypanosomes

Trypanosomes purified from the blood of an infected rat were prepared for SDS-PAGE as described in ‘materials and methods’ and run on a 10% gel, shown opposite in (a). Trypanosomes harvested from culture were similarly prepared and also run on a 10% gel, shown in (b). In gel (b) MITat 1.2 VSG is highlighted by a black arrow, while the unknown bands are highlighted by a red arrow. Trypanosomes collected from culture were additionally lysed in water to facilitate GPI-PLC activation and subsequent release of the VSG. The resulting gel of VSG release is shown in (c). The unknown bands in lane 6 of gel (c), labelled I – IV, were excised from the gel for MALDI-ToF analysis. The BSA standard (lane 1), mfVSG from WT cells (lane 2), and sVSG from WT cells (lane 3) were also taken for mass spectrometric analysis. In each gel, the equivalent to 2.5×10^6 cells was loaded per lane. The loading scheme for each gel is given below:

Gel (a):	Lane 1:	molecular weight standards (Sigma)
	Lane 2:	WT trypanosomes
	Lane 3:	GPI-PLC ^{-/-} trypanosomes
Gel (b):	Lane 1:	molecular weight standards (Sigma)
	Lane 2:	WT trypanosomes
	Lane 3:	GPI-PLC ^{-/-} trypanosomes
Gel (c):	Lane 1:	molecular weight standards (Sigma)
	Lane 2:	WT trypanosomes
	Lane 3:	pellet following lysis of WT cells in water
	Lane 4:	supernatant following lysis of WT cells in water
	Lane 5:	GPI-PLC ^{-/-} trypanosomes
	Lane 6:	pellet following lysis of GPI-PLC ^{-/-} cells in water
	Lane 7:	supernatant following lysis of GPI-PLC ^{-/-} cells in water



5.2.2. GPI-PLC^{-/-} Cells Expressed an Unexpected VSG Variant

The growth curves in the previous section show that wild-type trypanosomes may grow at different rates in culture if they express different VSG variants. To ensure GPI-PLC^{-/-} cells also expressed MITat 1.2 VSG a sample of cells was prepared for SDS-PAGE (plate 5.2).

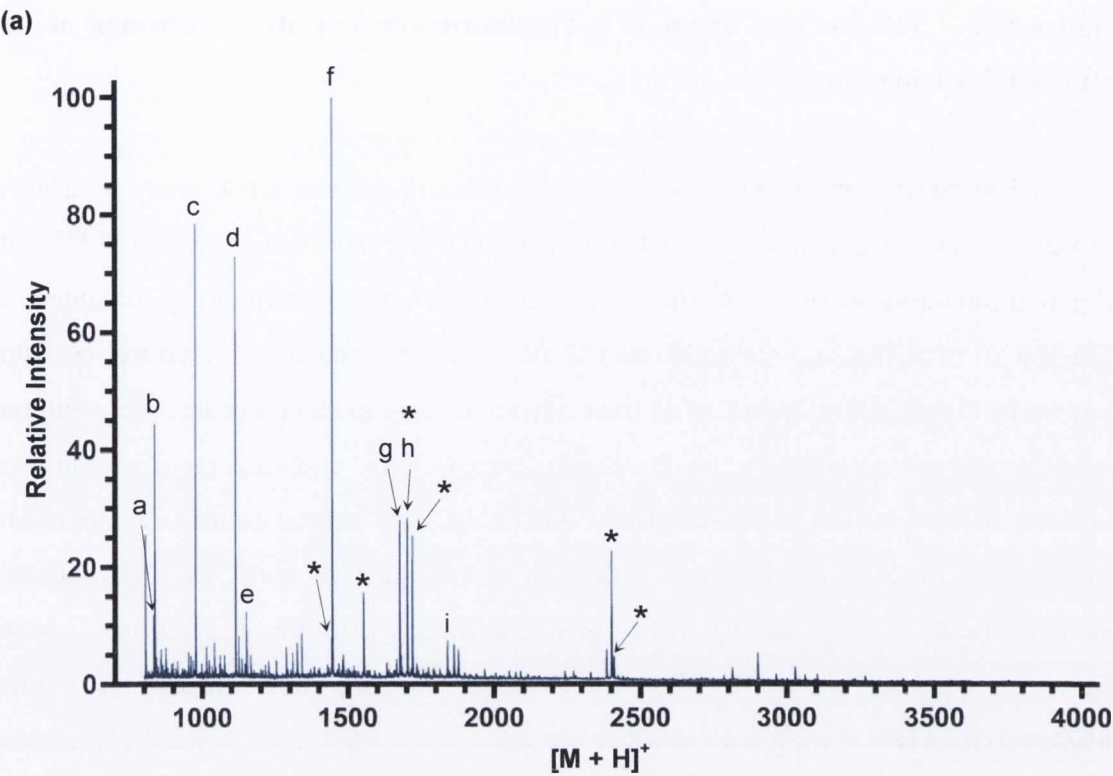
WT and GPI-PLC^{-/-} cells collected from the blood of an infected rat were found to express identical VSGs, with one major band at 54.7 kDa visible in both cell types. However, in cells collected from culture there was a noticeable difference in VSG expression. WT cells showed one prominent band at 53.6 kDa, consistent with MITat 1.2 VSG, while VSG from GPI-PLC^{-/-} cells presented as a smear of protein between molecular weights of approximately 53 kDa and 60 kDa. This smear of protein was not consistent with the MITat 1.2 VSG variant. Stimulation of GPI-PLC activation by osmotic shock confirmed that the GPI-PLC^{-/-} cells contained no GPI-PLC activity, and had therefore not been contaminated with other cell stocks during their history of culture in the lab.

There were two possibilities that could have caused the unusual banding pattern seen with cultured GPI-PLC^{-/-} cells. Firstly, deletion of GPI-PLC activity may have resulted in abnormal VSG processing, causing an apparent increase in molecular weight during SDS-PAGE. Secondly, the cultured GPI-PLC cell stocks could express a different VSG than previously thought, and different to the VSG expressed by GPI-PLC^{-/-} cell stocks used for infecting rats. To test both possibilities, four selected 'bands' from the GPI-PLC^{-/-} VSG smear were subjected to MALDI-ToF examination, and the resulting spectra were compared to MITat 1.2 mfVSG and MITat 1.2 sVSG. The BSA standard was also subjected to examination as an in-gel control, and was correctly identified (not shown).

From the gel shown in plate 5.2 (c), the band corresponding to mfVSG from WT cells was identified as MITat 1.2 VSG (accession number P26332), despite contamination of the mass spectrum with peaks corresponding to trypsin-digested tubulin, (figure 5.7). Similarly, the band corresponding to sVSG from WT cells was identified as the MITat 1.2 variant, this time with no tubulin contamination detectable (figure 5.8).

Figure 5.7. The Membrane-bound Form of the VSG from WT Trypanosomes was Confirmed as the MITat 1.2 Variant

The band corresponding to mfVSG in plate 4.1 (c), was cut from a replica gel, de-stained, and subjected to trypsin digestion and subsequent MALDI-ToF MS identification as described in section 2.4. A mass spectrum was obtained as detailed in ‘materials and methods’, and is shown in (a), opposite. The entire spectrum was de-isotoped and all peaks of at least 5% intensity, *i.e.* twenty three peaks, were entered into the MS-Fit application of ProteinProspector. No constant modifications, or variable modifications, were selected. Searching was limited to the *Trypanosoma* taxonomy in the NCBItr database, with an error tolerance set to 0.2 Da. Nine detected particles, peaks a - i, were matched to peptides from trypsin-digested MITat 1.2 VSG (accession P26332), shown in (b), yielding 20.8% sequence coverage with an average error of - 0.0246 Da. Six detected particles were matched to the alpha chain of trypsin-digested tubulin and are marked with ‘*’.



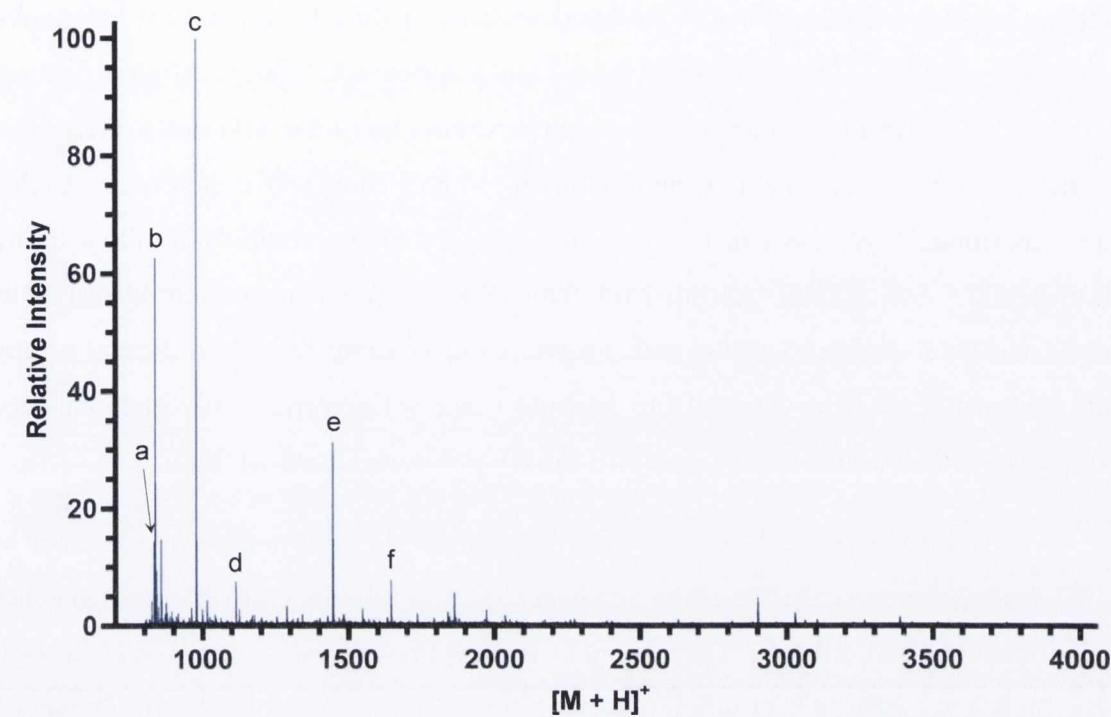
(b)

Peak Label	Measured Mass (mono-isotopic)	Predicted Mass (mono-isotopic)	Sequence
a	832.4129	832.4458	(K)MRDAALR(A)
b	836.4453	836.4625	(K)AGYVGLTR(Q)
c	976.5057	976.5574	(K)RPAATYLGK(A)
d	1113.5977	1113.6303	(K)LTILAYYR(M)
e	1151.4526	1151.4820	(K)DQNECKSPCK(W)
f	1445.6858	1445.7132	(R)ASIYAEINHGTNR(A)
g	1675.8952	1675.9377	(K)GRIDEYLNLLLQTK(E)
h	1701.8619	1701.8013	(K)WHNDAENKKCTLDK(E)
i	1838.7861	1838.8344	(K)YFSGHENEQWTAIEK(L)

Figure 5.8. Soluble VSG from WT Trypanosomes was also Confirmed as the MITat 1.2 Variant

The band corresponding to WT cell sVSG in plate 4.1 (c), was cut from a replica gel, de-stained, and subjected to trypsin digestion and subsequent MALDI-ToF MS identification as described in section 2.4. A mass spectrum was obtained as detailed in 'materials and methods', and is shown in (a), opposite. The entire spectrum was de-isotoped and all peaks of at least 5% intensity, *i.e.* eleven peaks, were entered into the MS-Fit application of ProteinProspector. No constant modifications, or variable modifications, were selected. Searching was limited to the *Trypanosoma* taxonomy in the NCBI database, with an error tolerance set to 0.1 Da. Six detected particles, peaks a - f, were matched to peptides from trypsin-digested MITat 1.2 VSG (accession P26332), shown in (b), yielding 10.1% sequence coverage with an average error of 0.0153 Da.

(a)



(b)

Peak Label	Measured Mass (mono-isotopic)	Predicted Mass (mono-isotopic)	Sequence
a	832.5053	832.4458	(K)MRDAALR(A)
b	836.5435	836.4625	(K)AGYVGLTR(Q)
c	976.6205	976.5574	(K)RPAATYLGK(A)
d	1113.7267	1113.6303	(K)LTILAYYR(M)
e	1445.8434	1445.7132	(R)ASIYAEINHG TNR(A)
f	1644.9937	1644.8452	(R)ASIYAEINHG TNR AK(A)

No bands taken from the GPI-PLC^{-/-} VSG smear could be matched to MITat 1.2 VSG. Instead, two bands, with molecular weights of 60.2 kDa and 57.9 kDa, were identified as the MITat 1.3 variant (accession number AAX36025), (figure 5.9 and figure 5.10). Tubulin contamination was detected in one of the MITat 1.3 VSG mass spectra, which is unsurprising as tubulin alpha and beta chains have molecular weights of approximately 50 kDa and are therefore expected to run similarly to VSG during SDS-PAGE. The remaining bands from the GPI-PLC^{-/-} VSG smear were identified as alpha and beta tubulin, with no peak masses corresponding to trypsin digests of any VSG variant present in the NCBItr database (data not shown). The total sequence coverages obtained for MITat 1.2 and MITat 1.3 VSGs are shown in figure 5.11.

It should be pointed out that while contaminating peak masses in a number of VSG spectra were matched to alpha and beta chains of tubulin, typically these matches were made to tubulin from *Trypanosoma evansi*, and not *Trypanosoma brucei* (in fact, no matches to alpha or beta tubulin chains from *T. brucei* were observed). However, since the peptides matched to *T. evansi* tubulin detected during MALDI-ToF are identical in sequence to peptides from *T. brucei* (figure 5.12), this ‘incorrect’ identification is not unexpected.

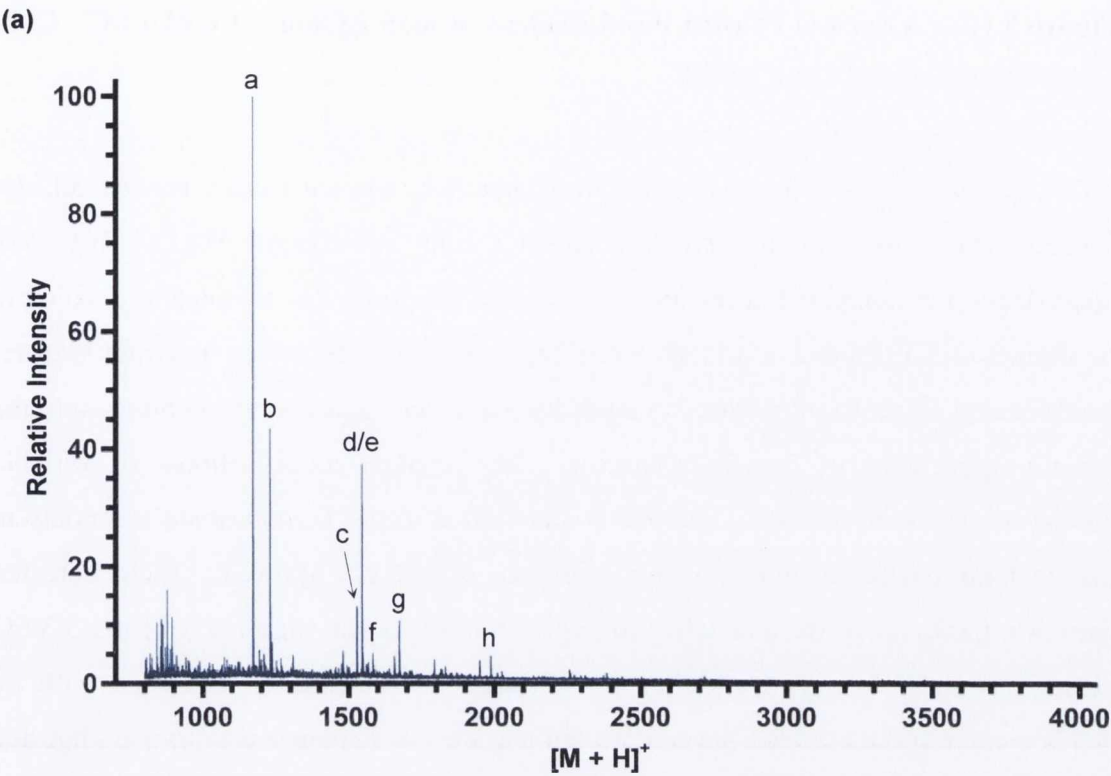
5.2.3. Monitoring the Change in GPI-PLC^{-/-} Cell VSG Expression

Since WT cells expressing different VSG coats were shown to exhibit different growth characteristics, it was crucial that both WT and GPI-PLC^{-/-} cell lines expressed the same VSG variant during growth curves. With a large degree of uncertainty surrounding cultured GPI-PLC^{-/-} cell stabilates (one batch of GPI-PLC^{-/-} cells was further shown, by MALDI-ToF mass spectrometry, to express MITat 1.5 VSG [data not shown]) it was decided to use stabilates normally used for infecting animals to inoculate fresh cultures. Once these cells were established in cultures, the cell densities were kept within log phase until use in experiments. Additionally, samples of cultured cells were routinely tested for VSG expression by SDS-PAGE.

GPI-PLC^{-/-} cells established in culture by inoculating fresh HMI-9 medium with infected rat blood were shown to be able to maintain MITat 1.2 VSG expression for at least twenty days after inoculation. However GPI-PLC^{-/-} cells did eventually switch to the expression of a higher molecular weight VSG variant (similar to MITat 1.3 VSG) at some point after twenty days and before thirty seven days in culture (figure 5.13). Although it remained uncertain as to what was inducing a change in VSG expression in GPI-PLC^{-/-} cells, the ability to establish GPI-PLC^{-/-} cells which expressed MITat 1.2 VSG for this length of time was encouraging in allowing the growth characteristics to be fairly compared to WT cells.

Figure 5.9. GPI-PLC^{-/-} Cells were Found to Express MITat 1.3 VSG

The band labelled 'I' in plate 4.1 (c), was cut from a replica gel, de-stained, and subjected to trypsin digestion and subsequent MALDI-ToF MS identification as described in 'materials and methods'. A mass spectrum was obtained as detailed in 'materials and methods', and is shown in (a), opposite. The entire spectrum was de-isotoped and all peaks of at least 5% intensity, *i.e.* seventeen peaks, were entered into the MS-Fit application of ProteinProspector. No constant modifications, or variable modifications, were selected. Searching was limited to the *Trypanosoma* taxonomy in the NCBI nr database, with an error tolerance of 0.2 Da allowed. Eight detected particles, peaks a - h, were matched to peptides from trypsin-digested MITat 1.3 VSG (accession AAX36025), listed in (b), yielding 18.7% sequence coverage with an average error of 0.0310 Da.

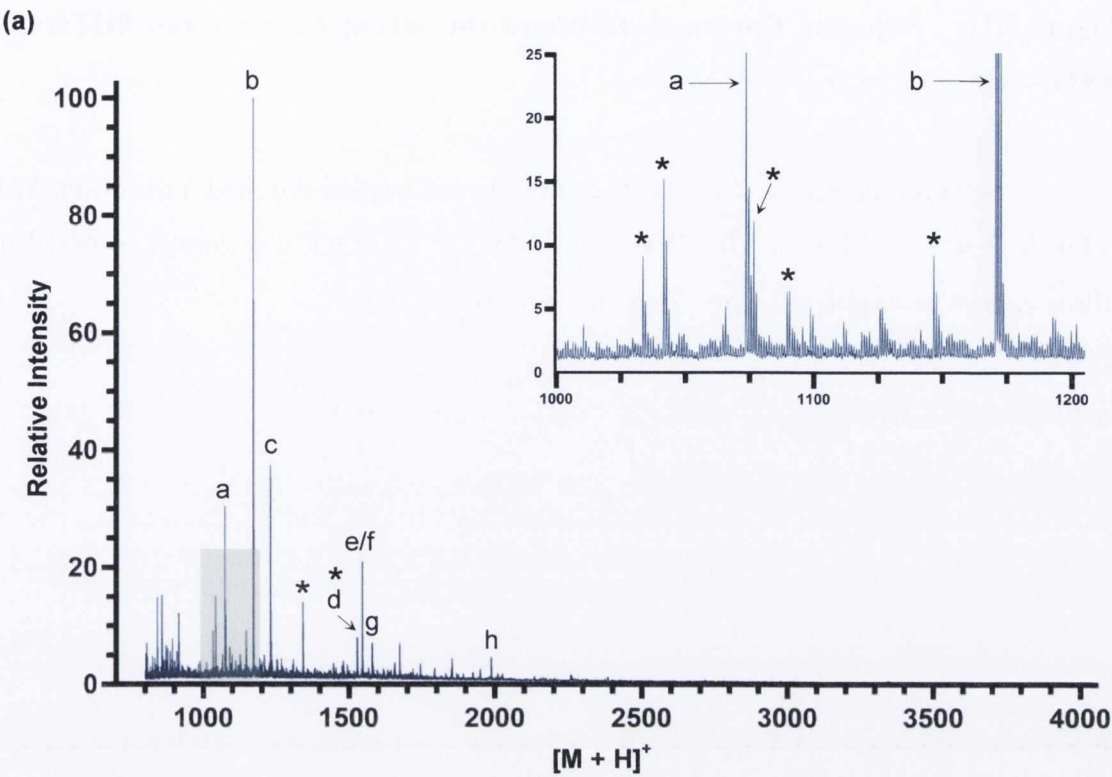


(b)

Peak Label	Measured Mass (mono-isotopic)	Predicted Mass (mono-isotopic)	Sequence
a	1170.6746	1170.6113	(R)AGLTNAPEELR(N)
b	1229.7347	1229.6848	(K)EISNVAEALKR(T)
c	1527.7424	1527.6315	(K)SECKDS DGCKWNR(T)
d	1544.7670	1544.7128	(K)QPPEGFQSDPTWR(K)
e	1544.7670	1544.8287	(K)ATTVAATIVCVCHKK(N)
f	1582.8088	1582.9104	(R)KQWPIWVTAAAALK(A)
g	1672.8789	1672.8078	(K)QPPEGFQSDPTWRK(Q)
h	1987.0962	1987.0342	(R)LSEIQKQNE DTTPTAIK(A)

Figure 5.10. A Second Protein Band Present in SDS Lysates of GPI-PLC^{-/-} Cells was Found to be MITat 1.3 VSG

The band labelled 'II' in panel c of plate 4.1, was cut from a replica gel, de-stained, and subjected to trypsin digestion and subsequent MALDI-ToF MS identification as described in section 2.4. A mass spectrum was obtained as detailed in 'materials and methods', and is shown in (a), opposite. The entire spectrum was de-isotoped and all peaks of at least 5% intensity, *i.e.* nineteen peaks, were entered into the MS-Fit application of ProteinProspector. No constant modifications, or variable modifications, were selected. Searching was limited to the *Trypanosoma* taxonomy in the NCBIInr database, with an error tolerance of 0.2 Da allowed. Eight detected particles, peaks a - h, were matched to peptides from trypsin-digested MITat 1.3 VSG (accession AAX36025), listed in (b), yielding 18.9% sequence coverage with an average error of 0.0421 Da. Seven detected particles were matched to trypsin-digested beta tubulin and are marked with '*'. For clarity identifying some peaks, the green region in (a) is shown inset, and enlarged.



(b)

Peak Label	Measured Mass (mono-isotopic)	Predicted Mass (mono-isotopic)	Sequence
a	1073.6362	1073.5837	(K)EISNVAEALK(R)
b	1170.6811	1170.6113	(R)AGLTNAPEELR(N)
c	1229.7358	1229.6848	(K)EISNVAEALKR(T)
d	1527.7386	1527.6315	(K)SECKDSGCKWNR(T)
e	1544.7575	1544.7128	(K)QPPEGFQSDPTWR(K)
f	1544.7575	1544.8287	(K)ATTVAATIVCVCHKK(N)
g	1578.9553	1578.9326	(K)LIKVGDSKAVPPAQR(Q)
h	1987.0947	1987.0342	(R)LSEIQKQNEDTTPTAIK(A)

Figure 5.11. Sequence Coverages Obtained for MITat 1.2 VSG and MITat 1.3 VSG

Peptides detected in the mass spectra of in-gel trypsin-digested VSGs from WT cells (MITat 1.2 VSG) and GPI-PLC^{-/-} cells (MITat 1.3 VSG) are shown, coloured in blue, aligned with each sequence in (a) and (b) respectively.

(a)

MPSNQEARLFLAVLVLAQVLPILVDSAAEKGFQAFWQPLCQVSEELDDQPKGALFTLQAAASKIQKMRDAALRASIIY
MRDAALRASIIY

AEINHGTNRAKAAVIVANHYAMKADSGLEALKQTLSSQEVTTATATASYLKGRIDEYLNLLLQTKESGTS GCMMDTSGT
AEINHGTNRAK GRIDEYLNLLLQTK

NTVTKAGGTIGGVPCKLQLSPIQPKRPAATYLGKAGYVGLTRQADAANNFHDNDAECRLASGHNTNGLGKSGQLSAAV
RPAATYLGKAGYVGLTR

TMAAGYVTVANSQTAVTVQALDALQEASGAHQFWIDAWKAKKALTGAETAEFRNETAGIAGKTGVTKLVEEALLKKK

DSEASEIQTELKKYFSGHENEQWTAIEKLISEQPVAQNLVGDNQPTKLGELEGNAKLTTILAYYRMETAGKFEVLTQK
YFSGHENEQWTAIEK LTTILAYYR

HKPAESQQQAAETEGSCNKKDQNECKSPCKWHNDAENKKCTLDKEEAKKVADETA KDGTGNTNTTGSSNSFVISKTP
DQNECKSPCKWHNDAENKKCTLDK

LWLAVLLF

(b)

MQAAALLLLVLRITSIEAAADDVNPDDNKEDFAVLCALAAALANLQTTVPSIDTSGLAAYDNLQQLNLSLSSKEWKSL

FNKAADSNGSPKQPPEGFQSDPTWRKQWPIWVTAAAALKAENKEAAVLARAGLTNAPEELRNRRALALIPLLAQAEQI
QPPEGFQSDPTWRKQWPIWVTAAAALK AGLTNAPEELR

RDRLSEIQKQNEDTTPTAIAKALNKAVYGQDKETGAVYNSADCFSGNVADSTQNSCKAGNQASKATTVAATIVCVCHK
LSEIQKQNEDTTPTAIAK ATTVAATIVCVCHK

KNGGNDAAACGRLINHQSDAGANLATASSDFGDI IATCAARPPKPLTAAYLDSALAAVSARIRFKNGNGYLKFKFAT
K

GCTGSASEGLCVEYTALTAATMQNFYKIPWVKEISNVAEALKRTEKDAAESTLLSTWLKASENQNSVAQKLIKVGDS
EISNVAEALKR

KAVPPAQRQTQNKPGSNCNKNLKKSECKDSGCKWNRTEETEGDFCKPKETGTENPAAGTGEAAGANTETKKCSDDK
SECKDSGCKWNR

TEGDCKDGCKWDGKECKDSSILATKKFALTTVSAAFVALLF

Figure 5.12. Online Peptide Mass Fingerprinting Databases Cannot Distinguish Between Tubulin from Different Species of Trypanosomes

The amino acids sequences of beta tubulin from five species of trypanosome were obtained from the NCBI protein database and aligned using the online ClustalW2 sequence alignment program (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The alignment of *T. evansi* (a), *T. brucei* (b, shown in bold face), *T. equiperdum* (c), *T. cruzi* (d) and *T. grayi* (e) beta tubulin sequences is shown opposite, with the regions detected by mass spectrometric analysis coloured red. The peptides of *T. brucei* beta tubulin detected by mass spectrometry are identical in sequence to the same regions of beta tubulin from *T. evansi* and *T. equiperdum*.

(a) MREIVCVQAGQCGNQIGSKFWEVISDEHGVDPGTGYQGSDQLQLERINVYFDEATGGRYV 60
(b) **MREIVCVQAGQCGNQIGSKFWEVISDEHGVDPGTGYQGSDQLQLERINVYFDEATGGRYV** 60
(c) MREIVCVQAGQCGNQIGSKFWEVISDEHGVDPGTGYQGSDQLQLERINVYFDEATGGRYV 60
(d) MREIVCVQAGQCGNQIGSKFWEVISDEHGVDPGTGYQGSDQLQLERINVYFDEATGGRYV 60
(e) MREIVCVQAGQCGNQIGSKFWEVISDEHGVDPGTGYQGSDQLQLERINVYFDEATGGRYV 60

(a) PRSVLIDLEPGTMDSVRAGPYGQIFRPDNFIFGQSGAGNNWAKGHYTEGAELIDSVLDVC 120
(b) **PRSVLIDLEPGTMDSVRAGPYGQIFRPDNFIFGQSGAGNNWAKGHYTEGAELIDSVLDVC** 120
(c) PRSVLIDLEPGTMDSVRAGPYGQIFRPDNFIFGQSGAGNNWAKGHYTEGAELIDSVLDVC 120
(d) PRAVLIDLEPGTMDSVRAGPYGQIFRPDNFIFGQSGAGNNWAKGHYTEGAELIDSVLDVC 120
(e) PRSVLIDLEPGTMDSVRAGPYQLFRPDNFIFGQSGAGNNWAKGHYTEGAELIDSVLDVC 120
.*:***.*:*****

(a) CKEAESCDCLQGQFQICHSLGGGTGSGMGTLLISK**LRQYPDR**IMMTFSIIPSPKVSATVV 180
(b) **CKEAESCDCLQGQFQICHSLGGGTGSGMGTLLISKLRQYPDR**IMMTFSIIPSPKVSATVV 180
(c) CKEAESCDCLQGQFQICHSLGGGTGSGMGTLLISK**LRQYPDR**IMMTFSIIPSPKVSATVV 180
(d) RKEAESCDCLQGQFQICHSLGGGTGSGMGTLLISK**LRQYPDR**IMMTFSIIPSPKVSATVV 180
(e) RKEAESCDCLQGQFQICHSLGGGTGSGMGTLLISK**LRQYPDR**IMMTFSIIPSPKVSATVV 180
*****.*:*****.*:*****

(a) EPYNTTLSVHQLVENSDESMCIDNEALYDICFRTLKLTPTFGDLNHLVSAVVSGVTCCL 240
(b) **EPYNTTLSVHQLVENSDESMCIDNEALYDICFRTLKLTPTFGDLNHLVSAVVSGVTCCL** 240
(c) EPYNTTLSVHQLVENSDESMCIDNEALYDICFRTLKLTPTFGDLNHLVSAVVSGVTCCL 240
(d) EPYNTTLSVHQLVENSDESMCIDNEALYDICFRTLKLTPTFGDLNHLVSAVVSGVTCCL 240
(e) EPYNTTLSVHQLVENSDESMCIDNEALYDICFRTLKLTPTFGDLNHLVSAVMGVTCCCL 240
*****.*:*****

(a) RFPQGQLNSDLRKLAVALNLPFPRLLHFFMMGFAPLTSRGSQQYRGLSVPELTQQMFDAKNMM 300
(b) **RFPQGQLNSDLRKLAVALNLPFPRLLHFFMMGFAPLTSRGSQQYRGLSVPELTQQMFDAKNMM** 300
(c) RFPQGQLNSDLRKLAVALNLPFPRLLHFFMMGFAPLTSRGSQQYRGLSVPELTQQMFDAKNMM 300
(d) RFPQGQLNSDLRKLAVALNLPFPRLLHFFMMGFAPLTSRGSQQYRGLSVPELTQQMFDAKNMM 300
(e) RFPQGQLNSDLRKLAVALNLPFPRLLHFFMMGFAPLTSRGSQQYRGLSVPELTQQMFDAKNMM 300

(a) QAADPRHGRYLTASALFRGRMSTKEVDEQMLNVQNKNSYFIEWIPNNIKSSVCDIPPKG 360
(b) **QAADPRHGRYLTASALFRGRMSTKEVDEQMLNVQNKNSYFIEWIPNNIKSSVCDIPPKG** 360
(c) QAADPRHGRYLTASALFRGRMSTKEVDEQMLNVQNKNSYFIEWIPNNIKSSVCDIPPKG 360
(d) QAADPRHGRYLTASALFRGRMSTKEVDEQMLNVQNKNSYFIEWIPNNIKSSICDIPPKG 360
(e) QAADPRXGRYLTASALFRGRMSTKEVDEQMLNVLNKNSYFIEWIPNNIKSSICDIPPKG 360
***** *****.*:*****

(a) LKMAVTFIGNNTCIQEMFRRVGEQFTLMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVS 420
(b) **LKMAVTFIGNNTCIQEMFRRVGEQFTLMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVS** 420
(c) LKMAVTFIGNNTCIQEMFRRVGEQFTLMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVS 420
(d) LKMAVTFIGNNTCIQEMFRRVGEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVS 420
(e) LKMAVTFIGNNTCIQEMFRRVGEQF----- 385
*****.*:*****

(a) EYQQYQDATIEEEGEFDEEEQY 442
(b) **EYQQYQDATIEEEGEFDEEEQY** 442
(c) EYQQYQDATIEEEGEFDEEEQY 442
(d) EYQQYQDATIEEEGEFDEEEQY 442
(e) -----

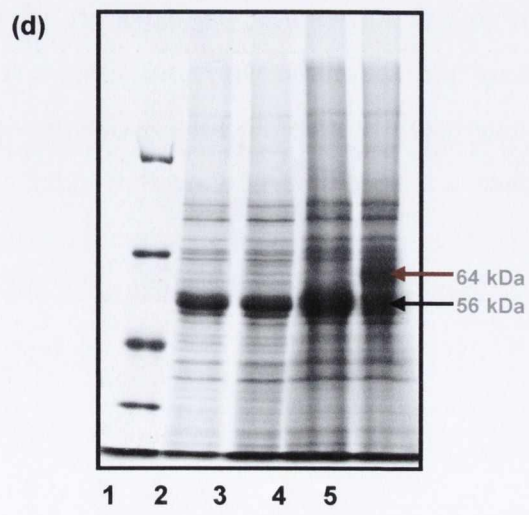
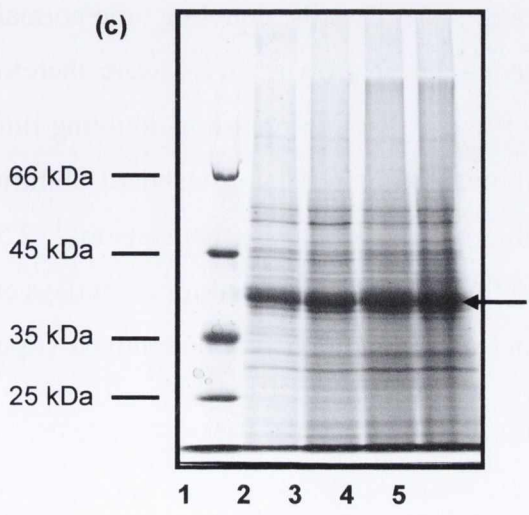
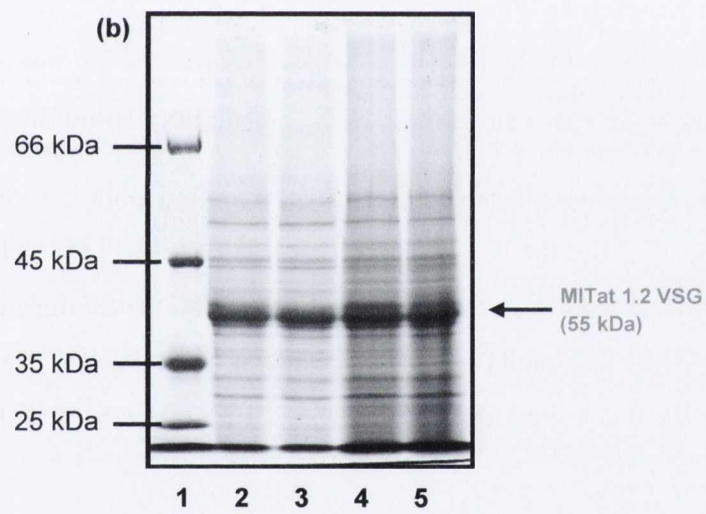
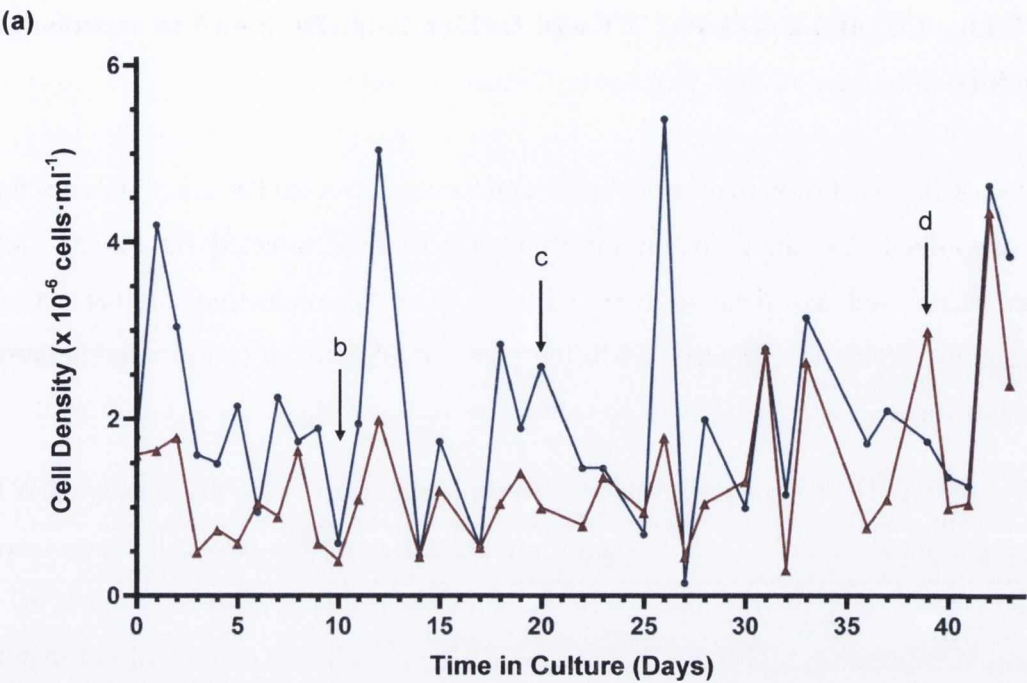
Figure 5.13. MITat 1.2 GPI-PLC^{-/-} Cells Purified from the Blood of an Infected Rat can Switch to a New VSG Variant in Culture

GPI-PLC^{-/-} cells, known to express MITat 1.2 VSG, were purified from the blood of an infected rat and transferred to standard culturing conditions in the presence of antibiotics as described in section 2.2.8. WT MITat 1.2 trypanosomes were established in culture in precisely the same way. After one week, antibiotics were omitted from cell medium and the cell densities were recorded daily over forty four days, shown opposite in (a); the blue trace shows WT cell density (—●—), while the red trace (—▲—) shows GPI-PLC^{-/-} cell density. On days 10 (arrow for gel shown in ‘b’), 20 (arrow for gel shown in ‘c’), and 37 (arrow for gel shown in ‘d’) cells from culture were prepared for SDS-PAGE as described in ‘materials and methods’. The SDS lysates of cultured WT and GPI-PLC^{-/-} cells were run on 10% gels alongside WT and GPI-PLC^{-/-} cells purified from infected blood. In each gel, a black arrow points to MITat 1.2 VSG while a red arrow is used to highlight an unknown VSG band in (d). The loading scheme for both gels is shown below:

Gel in (b):	Lane 1:	molecular weight standards (Thermo Scientific)
	Lane 2:	WT cells from infected blood
	Lane 3:	GPI-PLC ^{-/-} cells from infected blood
	Lane 4:	WT cells after 10 days in culture
	Lane 5:	GPI-PLC ^{-/-} cells after 10 days in culture

Gel in (c):	Lane 1:	molecular weight standards (Thermo Scientific)
	Lane 2:	WT cells from infected blood
	Lane 3:	GPI-PLC ^{-/-} cells from infected blood
	Lane 4:	WT cells after 29 days in culture
	Lane 5:	GPI-PLC ^{-/-} cells after 29 days in culture

Gel in (d):	Lane 1:	molecular weight standards (Thermo Scientific)
	Lane 2:	WT cells from infected blood
	Lane 3:	GPI-PLC ^{-/-} cells from infected blood
	Lane 4:	WT cells after 47 days in culture
	Lane 5:	GPI-PLC ^{-/-} cells after 47 days in culture



5.2.4. Growth Characteristics of WT and GPI-PLC^{-/-} Cells known to express the Same VSG

Since it was shown that GPI-PLC^{-/-} could maintain a MITat 1.2 VSG coat for some time after establishing in culture from stabilates of infected rat blood, such stabilates were used to inoculate fresh cultures prior to conducting another set of growth curves. Cells were maintained in log phase at all times prior to initiating these new growth curves.

As before, HMI-9 medium was able to support almost twice the density of WT cells compared to GPI-PLC^{-/-} cells; culturing capacities of the two cell types were found to be 7.5×10^6 cells·ml⁻¹ and 4×10^6 cells·ml⁻¹ respectively, shown in figure 5.14 (a). Despite expressing MITat 1.2 VSG, the GPI-PLC^{-/-} cell line could still not match the minimum doubling time of WT cells. Tangents to the points of maximum growth rate showed a minimum doubling time for WT cells of 6.5 hrs, and 9.6 hrs for GPI-PLC^{-/-} cells as shown in figure 5.14 (b). At the onset of log phase WT cells showed a doubling time of 8.9 hrs, while GPI-PLC^{-/-} cells showed a doubling time of 12 hrs.

Both WT and GPI-PLC^{-/-} cell lines exhibited only a short lag phase in these growth curves (2.7 hrs for WT cells and 1.9 hrs for GPI-PLC^{-/-} cells). However, this is difficult to interpret as the reasons cells display lag phases during growth is still only speculated. GPI-PLC^{-/-} cells exited lag phase at 2.3×10^6 cells·ml⁻¹, similar to before, while WT cells also exited lag phase at 2.3×10^6 cells·ml⁻¹, which is a much less than previously.

The doubling times reported above represent the growth rates at a single point of growth during these particular growth curves, rather than the doubling time normally seen during routine work in culture. A second set of growth curves were therefore undertaken to more accurately determine WT and GPI-PLC^{-/-} cell line doubling times over a longer time period, and under conditions better reflecting standard culturing practices. WT and GPI-PLC^{-/-} cells were sub-cultured daily to 1×10^5 cells·ml⁻¹, 2.5×10^5 cells·ml⁻¹ and 5×10^5 cells·ml⁻¹. These cultures were maintained for eight days and the average doubling time of cells kept under each condition was determined (figure 5.15) over this time.

Under each condition tested, WT cells doubled at a faster rate than GPI-PLC^{-/-} cells. Both cell types grew optimally when sub-cultured daily to 1×10^5 cells·ml⁻¹, with WT MITat 1.2 cells doubling every 6.8 hrs, and GPI-PLC^{-/-} MITat 1.2 cells doubling every 8.1 hrs. As cells were sub-cultured to higher densities doubling times increased, likely as the cells more frequently approached the carrying capacity of the medium. Over the three conditions tested, GPI-PLC^{-/-} cells showed an average increase in doubling time of 2.6 hrs over their WT counterparts.

SDS-PAGE of cells immediately prior to initiating growth curves for this section confirmed that both cell types expressed the same VSG, consistent with MITat 1.2 VSG, shown in figure 5.16 (a). Significantly, SDS-PAGE of cells maintained by sub-culturing, every 24 hrs, to 1×10^5 cells·ml⁻¹, 2.5×10^5 cells·ml⁻¹, and 5×10^5 cells·ml⁻¹, showed that none of the cells had switched VSG by the end of these growth curves, even when maintained at higher than normal cell densities, shown in figure 5.16 (b). This later result suggests that high cell density alone is not likely to induce VSG switching in cultured GPI-PLC^{-/-} cells over this time period.

Figure 5.14. Growth Curves for MITat 1.2 WT Cells and MITat 1.2 GPI-PLC^{-/-} Cells

WT (blue trace, $\bullet$) and GPI-PLC^{-/-} cells (red trace, $\blacktriangle$), both expressing MITat 1.2 VSG, were resuspended in fresh medium before diluting to 0.5×10^5 cells·ml⁻¹ in each of five flasks. The cell density in each flask was determined approximately every twelve hours. The mean of each determination is plotted in (a), with error bars showing the standard error of the mean; where error bars are not visible, the error is smaller than the representation of the time point. The cell densities were converted to the natural logarithm of the cell densities in (b). Maximum growth rates are shown in (b) with dashed lines, and correspond to doubling times of 6.5 hrs for WT cells, and 9.6 hrs for GPI-PLC^{-/-} cells. The solutions to the inhibited growth models in (a) and (b) are given below:

Graph (a): WT Cells: $Cell\ Density = \frac{7.4922}{1 + 147.741e^{-0.1033t}}$

(correlation coefficient = 0.9948)

GPI-PLC^{-/-} Cells: $Cell\ Density = \frac{3.9587}{1+310.488e^{-0.1318t}}$

(correlation coefficient = 0.9969)

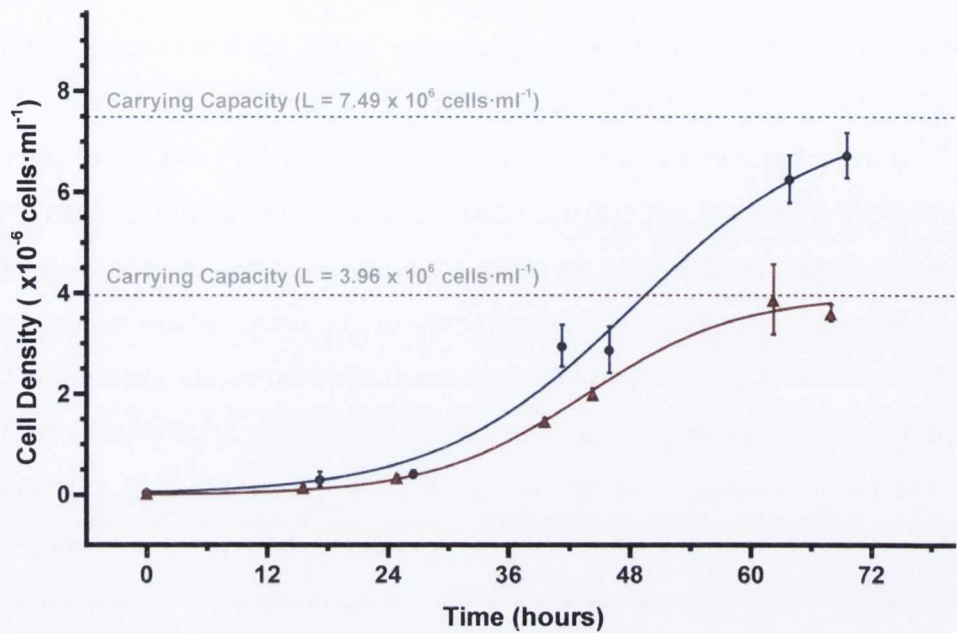
Graph (b): WT Cells: $Ln(Cell\ Density) = \frac{6.9095}{1+3.7707e^{-0.0617t}}$

(correlation coefficient = 0.9986)

GPI-PLC^{-/-} Cells: $Ln(\text{Cell Density}) = \frac{6.6253}{1+2.8357e^{-0.0434t}}$

(correlation coefficient = 0.9984)

(a)



(b)

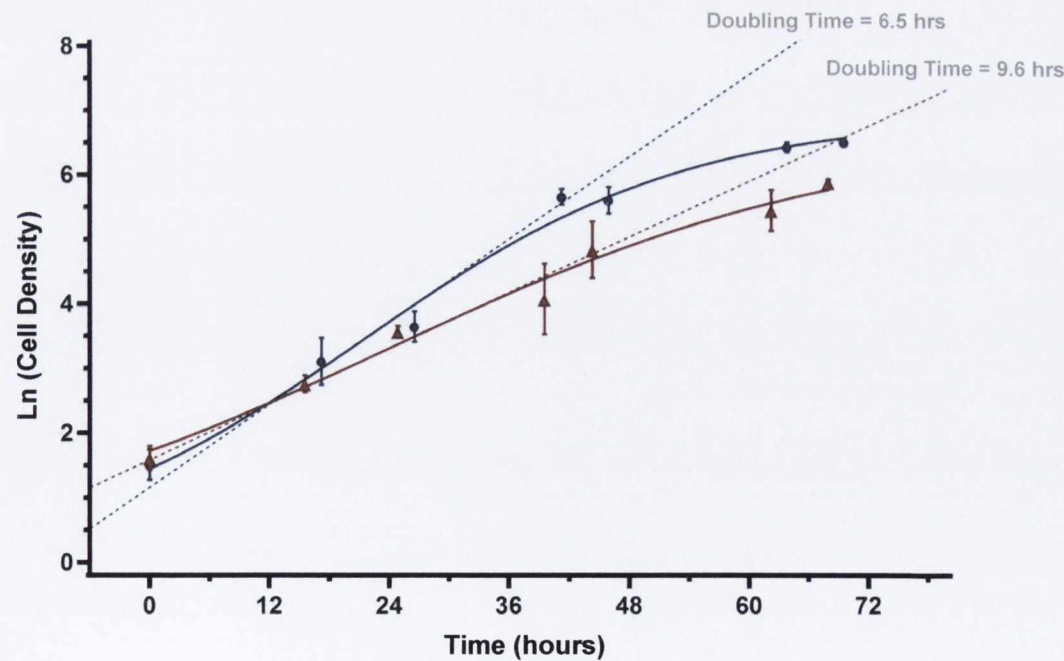


Figure 5.15. Comparison of the Doubling Times of Cells Sub-Cultured Daily to Different Cell Densities

WT and GPI-PLC^{-/-} cells known to express MITat 1.2 VSG were sub-cultured daily to each of three different cell densities. In (a), cells were sub-cultured to 1×10^5 cells·ml⁻¹, in (b) cells were sub-cultured to 2.5×10^5 cells·ml⁻¹, and in (c) cells were sub-cultured to 5×10^5 cells·ml⁻¹. In each case, data points represent the mean count in each of three flasks multiplied by the previous dilution factors used to maintain the cultures. Error bars represent the standard error of the mean; where no error bar is visible then the error is smaller than the representation of the point. Data for WT cells are shown by blue traces (—●—), while data for GPI-PLC^{-/-} cells are shown by red traces (—▲—). The mean doubling time of each condition for each cell type, calculated by dividing $\ln(2)$ by the slope of each trace, is shown next to the appropriate trace.

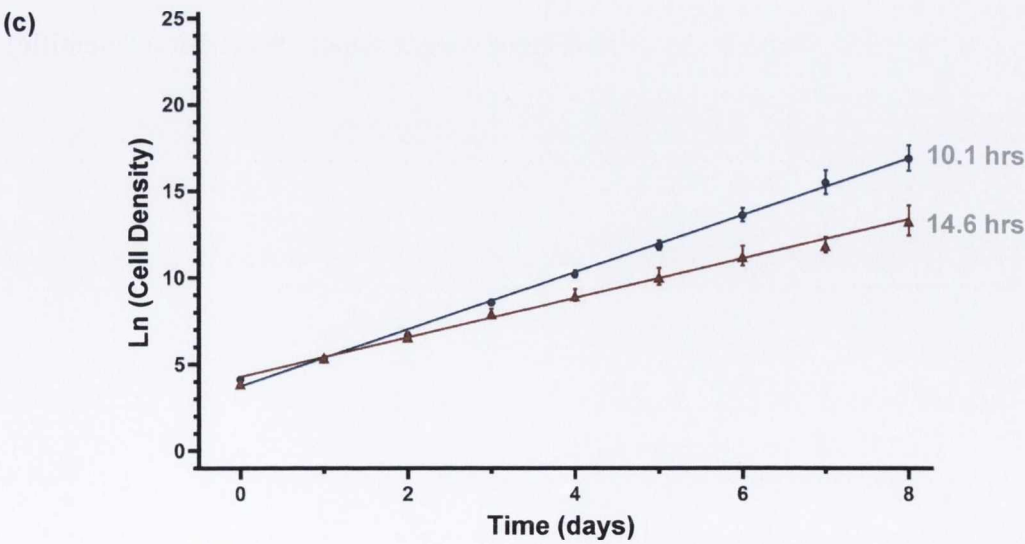
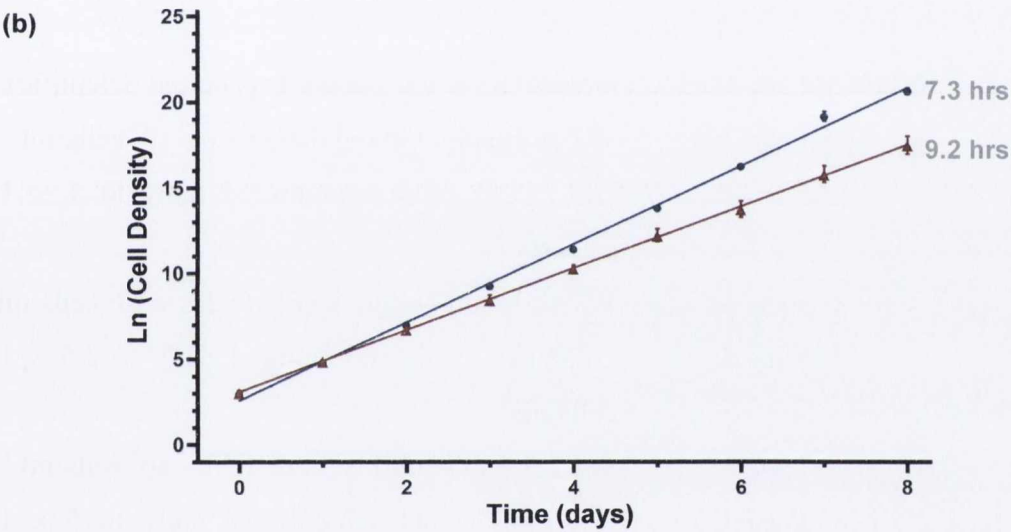
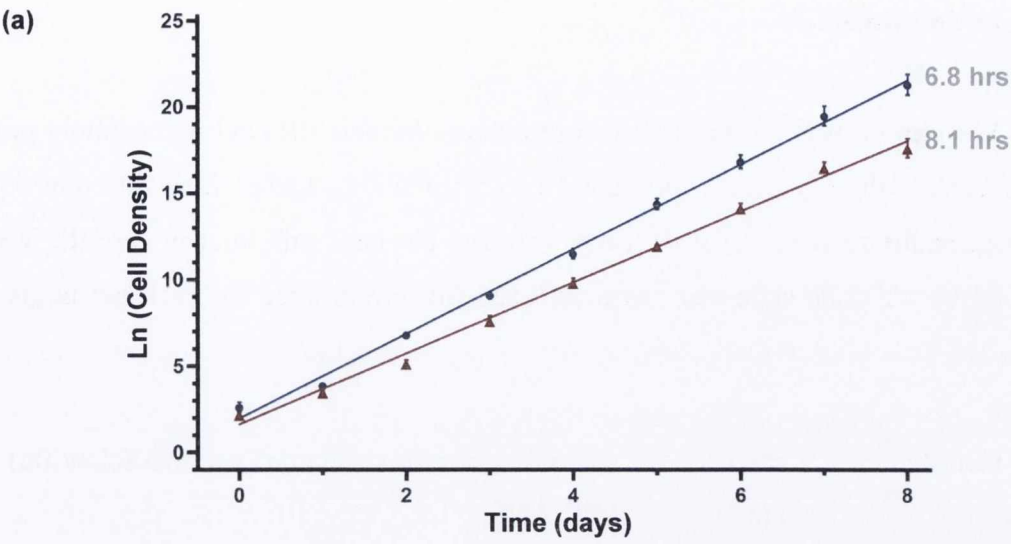
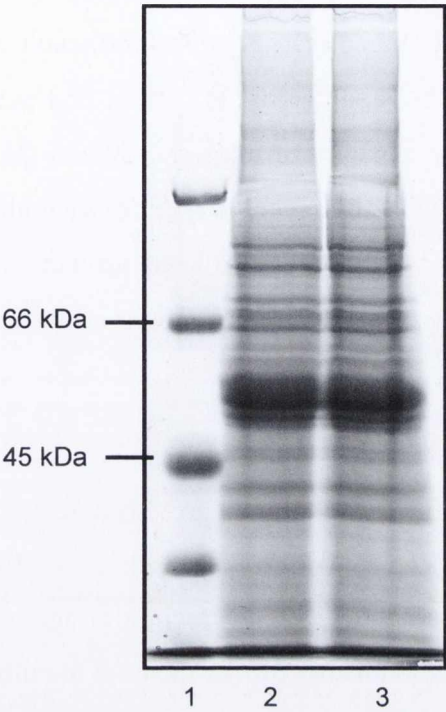


Figure 5.16. WT and GPI-PLC^{-/-} Cells did not Switch VSG whilst Maintained at High Cell Densities

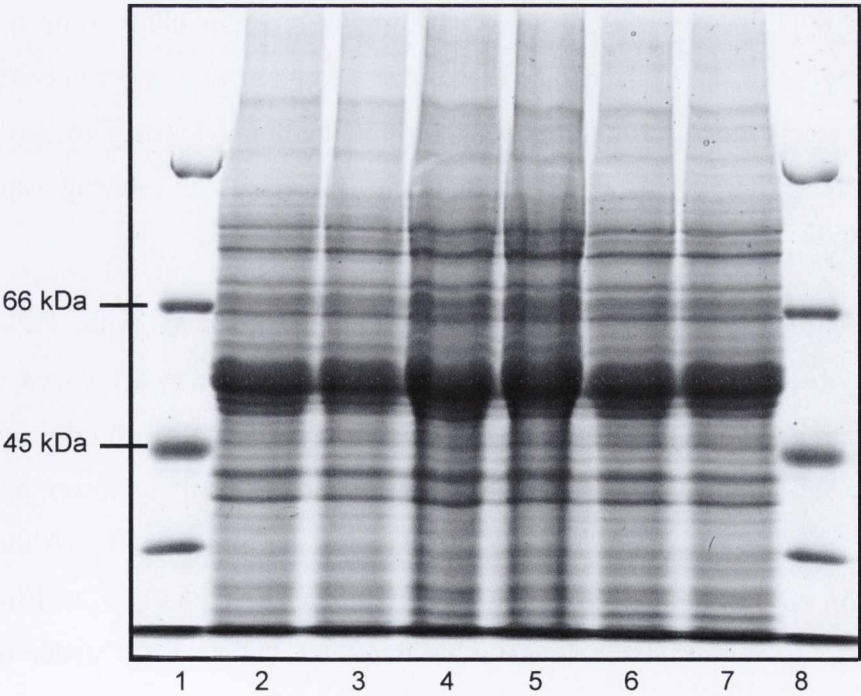
Samples of WT and GPI-PLC^{-/-} cells were run on a 10% gel immediately prior to starting growth curves (a), and again after maintaining under three different sub-culturing conditions (b). 2.5×10^6 cells were run per lane, and in each case the VSG expressed by the cells appeared identical. The loading scheme for each gel is given below:

Gel (a):	Lane 1:	molecular weight standards (Thermo Scientific)
	Lane 2:	WT cells
	Lane 3:	GPI-PLC ^{-/-} cells
Gel (b):	Lane 1:	molecular weight standards (Thermo Scientific)
	Lane 2:	WT cells sub-cultured daily to 1×10^5 cells·ml ⁻¹
	Lane 3:	GPI-PLC ^{-/-} cells sub-cultured daily to 1×10^5 cells·ml ⁻¹
	Lane 4:	WT cells sub-cultured daily to 2.5×10^5 cells·ml ⁻¹
	Lane 5:	GPI-PLC ^{-/-} cells sub-cultured daily to 2.5×10^5 cells·ml ⁻¹
	Lane 6:	WT cells sub-cultured daily to 5×10^5 cells·ml ⁻¹
	Lane 7:	GPI-PLC ^{-/-} cells sub-cultured daily to 5×10^5 cells·ml ⁻¹
	Lane 8:	molecular weight standards (Thermo Scientific)

(a)



(b)



5.3. Discussion

In this chapter, bloodstream form trypanosomes lacking GPI-PLC activity were shown to display three unusual characteristics in culture, each of which significantly distinguishes them from their WT counterparts. Firstly, and perhaps most strikingly, standard HMI-9 medium had the capacity to support only half the maximum number of GPI-PLC^{-/-} cells compared to WT cells. Secondly, under a number of different growth conditions, GPI-PLC^{-/-} exhibited a markedly reduced growth rate. Finally, the GPI-PLC^{-/-} cell line was found to switch to different VSG variants, a trait which was not seen here with WT cells.

Altogether, the results presented in this chapter broadly support the findings of Webb *et al.*, (1997), who demonstrated similar growth defects of GPI-PLC^{-/-} cells *in vivo*. In animals infected with trypanosomes lacking the GPI-PLC, a lower overall level of parasitemia was found in the bloodstream throughout the time course of infection. This scenario appears to closely mirror the case in culture, where GPI-PLC^{-/-} cells cannot reach the same densities achieved by WT cells. In none of the growth curves presented here did the carrying capacity for GPI-PLC^{-/-} cells exceed 4×10^6 cells·ml⁻¹, while WT cells frequently reached densities that exceeded 7×10^6 cells·ml⁻¹. While no data has been presented to explain the difference in carrying capacities for the two cell types, it is possible that a component of the medium plays some role. A reduced ability to 'scavenge' a necessary growth factor, or a greater, more uncontrolled, rate of uptake of a necessary component in the medium (leading to an earlier exhaustion of that component) could explain the difference in carrying capacities observed.

The reduced level of parasitemia in animals infected with GPI-PLC^{-/-} trypanosomes, observed by Webb, may be partially attributable to a reduced growth rate of the mutant cell line. In an *in vivo* environment, a reduced growth rate combined with normal host immune response would be expected to result in lower peaks of parasitemia, or a lower observed carrying capacity for the mutant cells. Although a reduced growth rate was not directly shown *in vivo*, here the GPI-PLC^{-/-} cell line was shown to consistently multiply at lower rates than WT cells. Even under optimal conditions (*i.e.* daily sub-culturing to 1×10^5 cells·ml⁻¹), GPI-PLC^{-/-} trypanosomes took

an extra 1.3 hrs to double, and this reduced growth rate became even more pronounced under less ideal conditions.

The ability of the GPI-PLC^{-/-} cell line to switch to a different VSG variant in culture may explain the apparent discrepancies between the work in this chapter and the growth characteristics of GPI-PLC^{-/-} cells observed by Grandgenett *et al.*, (2007). Since the growth rate of, and the carrying capacity for, bloodstream form trypanosomes is dependent on which VSG variant is expressed, it is possible that the GPI-PLC^{-/-} cells used by Grandgenett *et al.*, unsuspectingly switched to a VSG variant which allowed the GPI-PLC^{-/-} cells to multiply with characteristics identical to WT MITat 1.2 cells. For this thesis, great care was taken to ensure that GPI-PLC^{-/-} and WT cells expressed the same VSG during growth analysis, and samples were taken regularly for analysis of VSG expression and switching.

The ability of bloodstream form trypanosomes to switch to a new VSG variant in culture, although perhaps uncommon, has already been shown; cloned WT cells have been shown to switch to different VSG variants in culture in a similar time period to that observed with GPI-PLC^{-/-} cells during this current work (Doyle *et al.*, 1980). However, there are no reports of WT, bloodstream form, trypanosomes undergoing antigenic variation in HMI-9 medium, nor was any instance of VSG switching in WT MITat 1.2 cells observed during this thesis. That the GPI-PLC is not required for antigenic variation is clear, (Webb *et al.*, 1997), but the possibility that GPI-PLC^{-/-} cells may undergo antigenic variation with greater frequency than WT cells is an attractive topic for future study.

Chapter 6

General Discussion

6.1. A Review; the GPI-PLC and Interacting Proteins

The GPI-PLC, in bloodstream forms of *T. brucei*, is localized to the flagellar membrane as a patchy array along the flagellar attachment zone (Hanrahan *et al.*, 2009). While acylation of the GPI-PLC might be responsible for the enzyme's association with the flagellar membrane, acylation alone does not account for a localization along a such specific region of the flagellum. The calcium binding protein located in the flagellum of *Trypanosoma cruzi* (FCaBP), for example, has been shown to specifically localize to the inner leaflet of the flagellar membrane only when it is dually acylated and bound to Ca^{2+} (Engman *et al.*, 1989; Godsel and Engman 1999). Similarly, PI-PLC in *T. cruzi* is localized to the outer leaflet of the plasma membrane in an acylation-dependent manner (de Paulo Martins *et al.*, 2010). However neither *T. cruzi* PI-PLC or FCaBP are restricted within their membrane domains to the same remarkable extent that is seen with *T. brucei* GPI-PLC, so there is currently no precedent for even an acylated GPI-PLC to be restricted to the linear array along the FAZ in the absence of a partner protein that anchors it in place.

Further evidence for the interaction of the GPI-PLC with a partner protein comes from the previous evidence that calcium could be involved in a putative GPI-PLC activation pathway; incubating cells in the presence of calcium and a calcium ionophore was shown to cause a release of surface proteins (Bowles and Voorheis, 1982; Voorheis *et al.*, 1982), and cause a conversion mfVSG to sVSG (Jackson 1983). If the introduction of intracellular calcium caused the GPI-PLC to become active, then there must be some means to transduce an internal calcium signal to an activation signal for a protein, with no recognized calcium binding domain, on the exterior of the flagellar membrane.

Therefore, two separate strategies were adopted to search for proteins involved in regulating the localization and activity of the GPI-PLC. The first strategy aimed to directly co-immunoprecipitate possible partner proteins with the GPI-PLC, and then identify these proteins by peptide mass fingerprinting following MALDI-ToF mass spectrometric analysis. Although excellent protocols were established and validated for MALDI-ToF mediated identification of tryptic and chymotryptic protein digests, ultimately no firm evidence was found that the GPI-PLC could specifically co-immunoprecipitate with any other proteins under the conditions tested here. One immunoprecipitation condition, however, may be worthwhile investigating further; immunoprecipitating the GPI-PLC from 1.5×10^9 bloodstream form trypanosomes, lysed in the presence of n-octylglucoside, showed approximately four low molecular weight (*i.e.* less than 35 kDa) protein bands that did not appear in immunoprecipitations from GPI-PLC^{-/-} cells. Since most of these bands were located in the smear representing the IgG light chain, it is necessary to repeat the immunoprecipitation experiment in the absence of reducing agents. This precaution would not only clarify whether the protein bands represent specific trypanosomal proteins, but would also significantly reduce contamination of subsequent mass spectra with peptides arising from proteolytic digestion of the antibody light chain. A second necessary precaution is to test if protein bands that co-immunoprecipitated from WT cell lysates also co-immunoprecipitate in GPI-PLC^{-/-} lysates in the presence of a recombinant GPI-PLC. It seems especially prudent to conduct this control considering the amount of proteins that non-specifically co-immunoprecipitated with anti-GPI-PLC-conjugated Protein A agarose beads, particularly when n-octylglucoside had been used.

The second strategy adopted for studying what proteins might interact with the GPI-PLC involved following the means by which the GPI-PLC is activated during the de-energization of bloodstream form trypanosomes. In particular, the role of calcium in this process was monitored. Further information on how the GPI-PLC becomes activated could also have provided information on what conditions were more likely to be successful in later immunoprecipitation experiments, or indeed in other approaches used to find candidate partner proteins for the GPI-PLC. The trigger for inducing GPI-PLC-mediated hydrolysis of the VSG anchor during de-energization was unknown, although one possibility was that starving cells of glucose deprived them of an energy-dependent ability to maintain a low cytoplasmic Ca^{2+} concentration. A resulting

increase in intracellular Ca^{2+} during de-energization could then subsequently activate the GPI-PLC through some unknown signalling process. In favour of this hypothesis was the observation that cells that were de-energized in addition to being treated with A-23187 and Ca^+ , released their surface coat more quickly than cells that had been just treated with A-23187 and Ca^{2+} (Voorheis *et al.*, 1982).

However, two initial pieces of evidence quickly established that the de-energization of bloodstream form trypanosomes was not as mild a condition for stimulating VSG release as previously thought. Since the time course of protein release (followed by monitoring the absorbance of released material at 280 nm) was almost identical in WT trypanosomes and in mutant trypanosomes lacking the means to hydrolyse the mfVSG GPI anchor, the release of VSG could not account for the majority of protein release observed. Similarly, analysis by SDS-PAGE showed that many proteins were being released into the extracellular medium during the de-energization process, certainly more protein was being released than could be attributed to a small percentage of cell death over the time course of the experiment. Further investigation showed conclusively that there was a complete loss of cellular integrity when bloodstream form trypanosomes were de-energized. This loss of cellular integrity was followed by a decrease in AAT present in cells during the de-energization time course, and an increase in AAT in the extracellular medium. Release of AAT followed precisely the time course of VSG release, and the time course of general protein release observed in WT cells and in GPI-PLC^{-/-} cells. The addition of extra Ca^{2+} stimulated rupture of the trypanosomes under the conditions used for this thesis.

While it is difficult to accurately quantify the relative levels of intracellular protein release and VSG release observed here, there is a likely explanation for the much greater extent of surface protein release, compared to intracellular enzyme release, which was observed earlier by Bowles and Voorheis (1982). As cells begin to rupture due to de-energization for example, intracellular proteins such as malic enzyme and AAT escape out of leaks in the trypanosomal membrane in a process that is ultimately dependent on the extent of damage to the membrane. The VSG however, is released by a catalytic process mediated by the GPI-PLC; once sufficient GPI-PLC has been activated then the entire VSG coat can be quickly released. Bowles and Voorheis (1982) found that, of the cytoplasmic enzymes tested, malic enzyme was released to the

greatest extent with approximately 34% of the total cellular activity being released following a ten minute incubation in Tes buffer containing calcium and A-23187. This extent of release corresponds to estimates here, using the Pierce 660 nm protein assay reagent, that 29% of the total cellular protein is released during trypanosomal de-energization (taking the amount of protein in trypanosomes to be 5.6 mg per 10^9 cells; Voorheis and Martin, 1980). A second reason that Bowles and Voorheis may have seen such a low release of some assayed intracellular enzymes is the possibility that rupture of the cell membrane is mediated, at least partly, by a proteolytic event. In such a circumstance it is entirely possible that many enzymes released during the incubation of trypanosomes with A-23187 and Ca^{2+} could have been proteolytically inactivated.

Clearly, incubating trypanosomes under de-energizing conditions causes both rupture of the plasma membrane, and GPI-PLC-mediated release of the VSG as sVSG. The presence of additional calcium stimulates both processes, although it is currently unclear whether GPI-PLC activation in the presence of additional Ca^{2+} is a result of cell rupture, or whether intracellular calcium can, independently of cell rupture, activate the GPI-PLC. One possible way to answer this question would be to equilibrate trypanosomes in a cell-permeable analogue of EGTA, such as EGTA-tetra(acetoxymethyl ester), (EGTA-AM), prior to de-energizing. If trypanosomes then ruptured during a subsequent de-energization incubation but failed to convert mfVSG to sVSG, then intracellular calcium would have been identified as a necessary precursor to GPI-PLC activation, implicating calcium binding proteins in a GPI-PLC activating signalling pathway. If trypanosomes failed to rupture but still hydrolysed mfVSG to sVSG, then high intracellular calcium levels must be responsible for cell rupture, while some other aspect of the de-energization process must be responsible for GPI-PLC activation. Similarly, detergent lysis of cells, or detergent-free lysis of cells (*e.g.* osmotic shock), that have been pre-treated with EGTA-AM would also clarify whether the GPI-PLC can be activated by cell rupture in the absence of any intracellular calcium leakage. Clarifying whether intracellular Ca^{2+} leakage is a necessary precursor to the GPI-PLC-mediated conversion of mfVSG to sVSG is a vital step in elucidating what proteins might be involved in regulating, and interacting with the GPI-PLC.

6.2. Growth Characteristics of GPI-PLC^{-/-} Trypanosomes

During the initial immunoprecipitation studies carried out for this thesis, it was observed that it was much more difficult to obtain 1×10^9 GPI-PLC^{-/-} cells, in culture, than it was to obtain 1×10^9 WT MITat 1.2 cells. As this difficulty became a more consistent challenge, it was decided to characterize the growth of GPI-PLC^{-/-} cells in culture and compare their growth with cultured WT cells. The *in vivo* growth differences in GPI-PLC^{-/-} cells reported by Webb *et al.* (1997), supported the initial belief here that deletion of the GPI-PLC genes had a physiological affect on the growth of the bloodstream form parasites *in vitro*, despite a separate study to the contrary (Grandgenett *et al.*, 2007).

Initial suspicions were soon confirmed experimentally after it was shown that GPI-PLC^{-/-} cells could only grow to half the maximally density obtainable by WT cells. It was additionally shown that GPI-PLC^{-/-} exhibited longer doubling times in culture compared to their WT counterparts. The shortest doubling time observed WT MITat 1.2 cells, maintained for eight days under optimal culturing conditions, was found to be 6.8 hrs while for GPI-PLC^{-/-} cells the minimum doubling time was found to be 8.1 hrs. When cells were diluted to 0.5×10^5 cells·ml⁻¹ and growth followed until the system's carrying capacity had been reached then the maximum growth rate of WT cells corresponded to a doubling time 6.5 hrs, while the equivalent doubling time for GPI-PLC^{-/-} cells was found to be a more modest 9.6 hrs.

However, before it can be categorically stated that deletion of the GPI-PLC genes results in a specific, significant reduction in the growth rate of bloodstream form trypanosomes, it should be confirmed that the reduced growth rates seen throughout this work are specific to the bloodstream stage of the trypanosome and are not seen after bloodstream forms of the parasite have been transformed to procyclic forms. Indeed transformation of WT and GPI-PLC^{-/-} bloodstream form trypanosomes to procyclic form trypanosomes was tried multiple times during this work. Cultures of WT and GPI-PLC^{-/-} cells resuspended in a 50:50 mix of HMI-9:SDM-79, with added citrate and *cis*-aconitate (3 mM and 5 mM concentrations of both compounds were tried) and then incubated at 27 °C (Brun and Schönenburger, 1981) failed to survive the transformation process (data not shown). Attempts to include hemin (7.5 µg·ml⁻¹),

glycerol (4 mM), and even using freshly recrystallized *cis*-aconitate failed to improve differentiation (data not shown). These difficulties however were later attributed to a faulty calibration of the CO₂ sensor in the laboratory's 27 °C incubator, as it was determined that the CO₂ readings were off by 50% (this failure had been initially ruled out as large changes in the CO₂ concentrations were expected to cause a noticeable change in the medium's colour). Transformation of WT and GPI-PLC^{-/-} cells has not been attempted again since resolving this issue, and this experiment remains a priority in confirming that growth defects observed following deletion of the GPI-PLC genes are limited to the trypanosome's bloodstream stage.

Throughout this work, WT cells consistently reached higher densities in culture compared to GPI-PLC^{-/-} cells. It seems quite possible that the GPI-PLC^{-/-} cells are less efficient at extracting one, or more, necessary components from the culture medium and tracking down what this component is could highlight a novel potential physiological role for the GPI-PLC. Alternatively, GPI-PLC^{-/-} trypanosomes could exhaust key components of the medium more quickly than their WT counterparts. An approach systemically testing the various components in HMI-9 medium could yield some clarifying information, however such an approach would be quite laborious and would undoubtedly require some account to be taken of batch to batch variation in FBS. In this respect it would be necessary to test and compare the effects of a fully defined medium on the growth characteristics of WT, and GPI-PLC^{-/-}, MITat 1.2 bloodstream form trypanosomes. Hirumi *et al.*, (1997) have devised such a serum-free, fully defined, medium that could be used, namely HMI-244.

The observation that GPI-PLC^{-/-} cells switched to different variants of the surface glycoprotein in culture, an event that was not detected in WT cells cultured throughout this work, was not investigated any further than documented in this thesis (although one further batch of laboratory GPI-PLC^{-/-} cells was found to express only MITat 1.5 VSG; data not shown). While VSG switching has been observed before, in cloned WT trypanosomes maintained in culture (Doyle *et al.*, 1980), the frequency of VSG switching in GPI-PLC trypanosomes was intriguing and this should be a topic for future studies. The ability of GPI-PLC^{-/-} cells to switch, with unexpected frequency, to a different VSG also offers a possible reason for the difference in observed growth characteristics reported here, and those described elsewhere. It remains entirely possible that the GPI-PLC^{-/-} cells of Grandgennet *et al.*, (2007) had unknowingly

switched to a different VSG variant, which allowed the GPI-PLC cells to grow with identical characteristics to WT cells.

6.3. Conclusions

The physiological role of the GPI-PLC in bloodstream form trypanosomes remains unknown. While various lines of evidence suggest that the GPI-PLC must interact with other trypanosomal proteins, immunoprecipitation experiments carried out for this work failed to identify any possible candidate proteins. Previous suggestions that the GPI-PLC may be activated by intracellular calcium, introduced using a calcium ionophore or through de-energizing cells, must now be re-examined as these conditions were shown here to additionally cause cell rupture. The regulation of the GPI-PLC undoubtedly remains ambiguous, however it is now clear that the GPI-PLC is required for vigorous growth of bloodstream form trypanosomes. Data presented here showed a reduced growth rate, and a reduced maximum density, of GPI-PLC^{-/-} cells in culture, and these results bear a striking similarity with *in vivo* studies performed by other researchers. This raises the likely possibility that the GPI-PLC plays an important, albeit non-essential, role in the growth of bloodstream form trypanosomes. However what this precise role is will remain speculative until the growth defects of GPI-PLC^{-/-} trypanosomes can be rationally justified.

Appendix A

Purification of a Recombinant MBP-GPI-PLC Fusion Protein

A.1. Introduction

Although the GPI-PLC can be purified from trypanosomal cell extracts, the procedure requires substantial numbers of trypanosomes to produce practical amounts of protein. This appendix presents a protocol for purifying a soluble, recombinant form of *T. brucei* GPI-PLC. Hanrahan (2006) successfully expressed the GPI-PLC using a pMALTM expression vector to produce a maltose-binding protein (MBP) fusion with the GPI-PLC (MBP-GPI-PLC) in the BL21 strain of *Escherichia Coli* (*E. coli*). However, this initial expression, and subsequent purification, of recombinant MBP-GPI-PLC resulted in the co-purification of the bacterial chaperone GroEL. A procedure developed by Rohman and Harrison-Lavoie (2000) was later adapted by Hanrahan, and applied to the MBP-GPI-PLC purification strategy, ultimately leading to the purification of the recombinant fusion protein in the absence of any contaminating GroEL (Hanrahan, personal communication). The procedure was further changed for this work, where the MBP-GPI-PLC was initially to be used as a probe for proteins that may interact with the GPI-PLC. The AD494 strain of *E. coli* expressing MBP-GPI-PLC used here was also generated by Hanrahan.

A.2. Methods

A.2.1. Preparation of Denatured Bacterial Lysates

Denatured bacterial lysate was prepared as described by Rohman and Harrison-Lavoie (2000). Briefly, *E. coli* were grown to a density of $A_{600} \approx 0.8$ before harvesting

the cells by centrifuging at 4,000 *g* for fifteen minutes, at 4 °C. The resulting pellet was resuspended in 1 ml of PBS (see table 2.1.1., and additionally containing 0.1% (w/v) Triton[®] X-100) for every 20 ml of original bacterial culture. Bacterial cells were then lysed by sonicating with four pulses, each pulse lasting one minute. After lysis, the bacterial suspension was centrifuged at 13,000 *g* for twenty minutes, at 4 °C, and the supernatant collected and freeze-dried overnight. Lyophilized material was finally resuspended in the minimum amount of 8 M urea required (approximately one tenth the volume of the lysed bacterial suspension), before aliquoting samples and storing at -20 °C until use. Note that immediately prior to use, and after thawing, the bacterial lysates were incubated for twenty minutes, at 37 °C, in the presence of the protease inhibitors PMSF, TLCK, pepstatin A, and leupeptin, at the usual concentrations.

A.2.2. Induction and Release of MBP-GPI-PLC

A scraping of *E. coli* AD494, previously transformed with the DNA for a MBP-GPI-PLC fusion protein (Hanrahan, 2006), was used to inoculate 40 ml of sterile LB broth (50 µg.ml⁻¹ kanamycin, 100 µg.ml⁻¹ ampicillin). Following an overnight incubation, at 37 °C with shaking, 10 ml of the resulting culture was added to 500 ml of sterile LB broth (50 µg.ml⁻¹ kanamycin, 100 µg.ml⁻¹ ampicillin). This culture was incubated at 37 °C, with shaking, and the bacterial growth monitored by measuring the absorbance, at 600 nm, of 1 ml samples taken at various time points. Once the absorbance had reached approximately 0.5, the cultures were induced by adding 25 µl of 1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG) and lowering the incubation temperature to 18 °C. The culture was incubated overnight with agitation before centrifuging a 4,000 *g*, for twenty minutes at 4 °C. The resulting bacterial pellet was washed once in 50 ml column buffer (20mM Tris, pH 7.4, 200 mM NaCl) and before leaving overnight at -20 °C.

After the overnight freezing, the bacterial pellet was resuspended in 10 ml column buffer containing 10 mM ATP, 20 mM MgCl₂, 50 mM KCl and protease inhibitors. Lysoszyme was added to the bacterial suspension, to yield a final lysozyme concentration of 1 mg.ml⁻¹, before incubating the bacteria on ice for five minutes and

subsequently sonicating in twenty second pulses for a total of three minutes. The crude lysate was then centrifuged for thirty minutes at 9,000 *g*, at 4 °C. An aliquot, 340 µl, of previously prepared, denatured bacterial lysate was added to the resulting supernatant, which was then incubated at 37 °C for ten minutes. Finally, the sample was centrifuged for one hour at 10,000 *g* and at 4 °C. The supernatant was collected for purification of the recombinant MBP-GPI-PLC.

Samples of pre-, and post-, induction *E. coli* were subjected to SDS-PAGE and are shown in plate A.1.

A.2.3. Affinity Chromatography on Amylose Resin

E. coli extract, containing recombinant MBP-GPI-PLC, was applied to an amylose resin column (10 ml, at 4°C) and allowed to completely enter the resin by gravity flow. The column was successively washed with 50 ml column buffer (also containing 10 mM ATP, 20 mM MgCl₂, 50 mM KCl), 50 ml column buffer (5 mM ATP, 20 mM MgCl₂, 50 mM KCl) and finally with column buffer containing no additional ingredients. This later wash was continued until all ATP (monitored at 260 nm) was completely eluted from the column. MBP-GPI-PLC was eluted from the amylose resin by washing the column through with column buffer containing 10 mM maltose. Elution of bound protein was followed at 280 nm; samples eluates containing suitable amounts of protein were run on a denaturing gel (figure A.1) before combining appropriate fractions and further purifying by size exclusion chromatography.

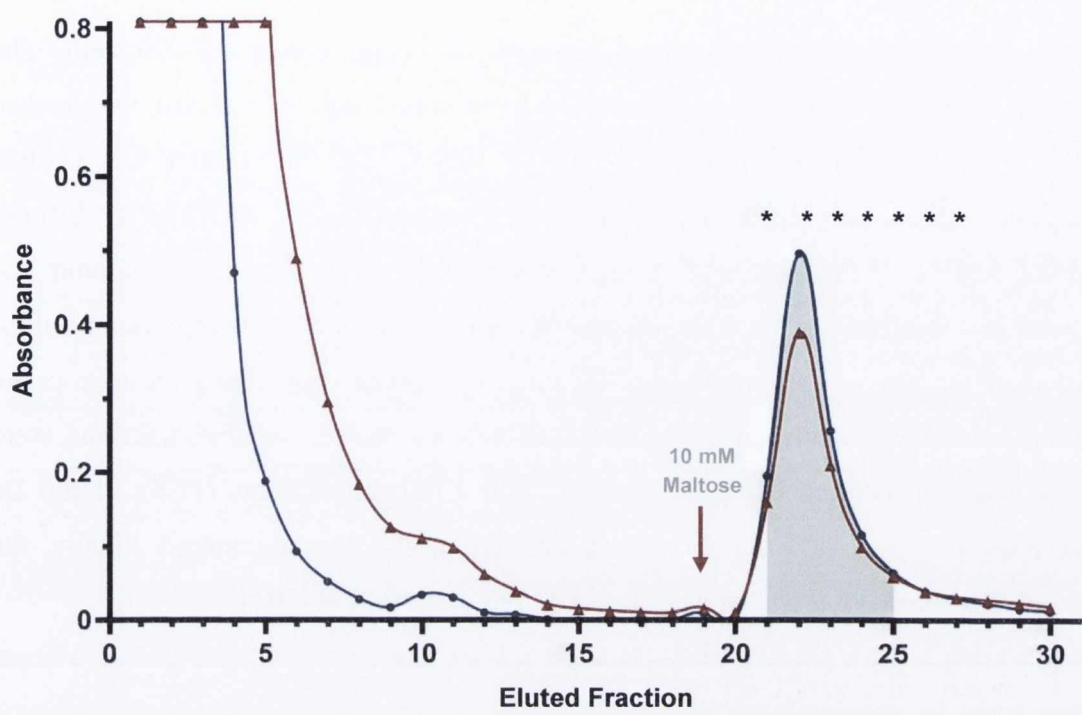
Following use, the amylose column was always regenerated by washing successively with water (30 ml), 0.1% (w/v) SDS (30 ml), water (10 ml) and column buffer (50 ml), as directed by the manufacturers. When not in use, the resin was stored at 4 °C in column buffer containing 0.02% (w/v) NaN₃.

Figure A.1. Purification of Recombinant MBP-GPI-PLC from *E. coli* Lysate by Affinity Chromatography

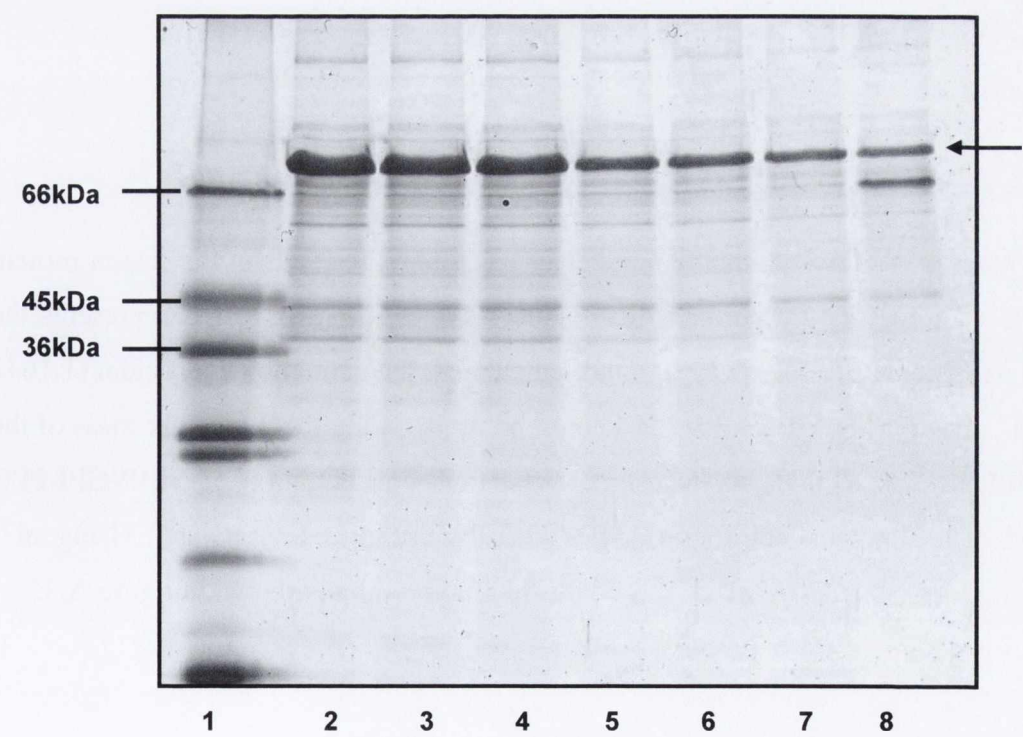
The lysate from *E. coli* cultures was applied to an amylose column as described in section A.2.3. The column was washed while monitoring the A₂₈₀ (blue trace) and A₂₆₀ (red trace) of one in two dilutions of the eluents (each approximately 1 ml), shown in (a). Bound fusion protein was eluted from the column with 10 mM maltose, in column buffer, which was applied once the column had been washed free of ATP (shown with a red arrow). Samples of fractions marked (*) were run on a 15% polyacrylamide gel, after boiling in the presence of 5X sample buffer, shown opposite in (b), with the MBP-GPI-PLC marked (black arrow). The gel loading scheme is given below. Fractions shaded green in (a) correspond to lanes 2 to 6 in (b), and were pooled before applying to an S-200 size exclusion column.

Lane 1:	Molecular weight markers (Sigma-Aldrich)
Lane 2:	20 µl fraction 21
Lane 3:	20 µl fraction 22
Lane 4:	20 µl fraction 23
Lane 5:	20 µl fraction 24
Lane 6:	20 µl fraction 25
Lane 7:	20 µl fraction 26
Lane 8:	20 µl fraction 27

(a)



(b)



A.2.4. Size Exclusion Chromatography

Size exclusion chromatography was achieved using Sephacryl S-200 resin. The column, 100 ml in volume (*i.e.* a diameter of 1 cm and a length of 100 cm), was packed and calibrated previously (Hanrahan, 2006). Before use, the column was washed overnight with column buffer under gravity flow (approximately 9.3 ml.hr⁻¹). Affinity purified MBP-GPI-PLC (section A.2.3.) was applied to the top of the column and allowed to completely enter the resin before washing the through with column buffer. Elution of protein was monitored at 280 nm, with eluates containing an appropriate quantity of protein analysed by SDS-PAGE (figure A.2). Suitable fractions were pooled and concentrated using a Vivaspinn, with a polyethersulfone (PES) 10,000 Da molecular weight cut-off membrane, as directed by the manufacturers. Finally, the purified MBP-GPI-PLC was aliquoted and stored, until use, at -20 °C.

A.2.5. Estimation of Protein Concentration

The concentration of MBP-GPI-PLC was estimated as described by Gill and von Hippell (1989), solving for the extinction coefficient of the fusion protein using the formula:

$$\varepsilon = 5550(W) + 1340(Y) + 150(C)$$

Where, W corresponds the number of tryptophan residues in the fusion protein (*i.e.* 14), Y represents the number of tyrosine residues (*i.e.* 28), and C represents the number of cysteine residues (*i.e.* 8). Once calculated, the extinction coefficient (1.1642 x 10⁵ M⁻¹.cm⁻¹ for the MBP-GPI-PLC) could be used, taking the molecular mass of the MBP-GPI-PLC as 83,000 Da, to estimate the final concentration of MBP-GPI-PLC obtained. The final concentration of MBP-GPI-PLC produced here was 0.31 mg.ml⁻¹. A gel and blot (using anti-GPI-PLC IgG) of the final product is shown in figure A.3.

Plate A.1. Induction and Release of Recombinant MBP-GPI-PLC from *E. coli*

Bacterial cultures were incubated to an A₆₀₀ of approximately 0.5, at 37 °C, before inducing expression of recombinant MBP-GPI-PLC by the addition of IPTG and lowering the incubation temperature to 18 °C, as described in earlier. Samples were taken before induction, and after overnight expression of recombinant fusion protein, for SDS-PAGE, shown in (a). The induced fusion protein is evident in lane 3 (arrow) as a thick band migrating above the 66 kDa marker.

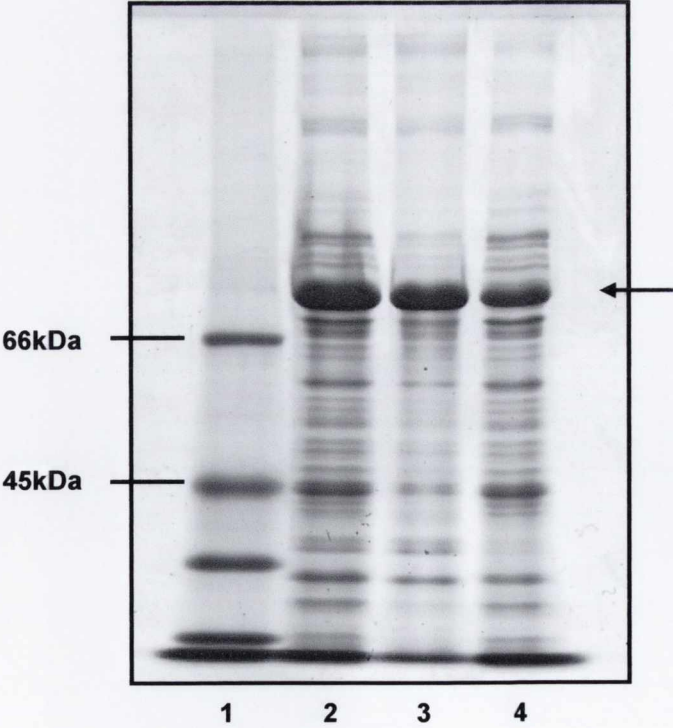
Induced bacteria were lysed by sonicating and centrifuged at 9,000 *g* for 30 mins at 4 °C. The pellet was vigorously resuspended in column buffer before adding boiling 5X sample buffer and preparing for SDS-PAGE. Samples of the *E. coli* pellet, and of the supernatant, were run on a 10% denaturing gel (b). Soluble protein released during cell breakage is evident in lane 4. Gel loading schemes are given below.

Gel (a):	Lane 1:	Molecular weight markers (Sigma-Aldrich)
	Lane 2:	Pre-induced sample
	Lane 3:	Post-induction sample
Gel (b):	Lane 1:	Molecular weight markers (Sigma-Aldrich)
	Lane 2:	Post-induction sample
	Lane 3:	Sample of the pellet following cell lysis
	Lane 4:	Sample of soluble protein released following cell lysis

(a)



(b)



A.3. Results and Discussion

Here, the recombinant MBP-GPI-PLC was expressed and purified without any of the contaminating bacterial chaperone GroEL, that co-purified in earlier strategies, present. The protocol presented here is based largely on a strategy developed by Hanrahan (personal communication), but with some changes, and allows for the purification of sufficient MBP-GPI-PLC for future anti-GPI-PLC antibody production. The key steps in removing GroEL from recombinant protein purification procedures were established by Rohman and Harrison-Lavoie (2000) for the removal of GroEL from glutathione-S-transferase (GST) fusion proteins expressed in *E. coli*, and these steps were adapted to earlier MBP-GPI-PLC purification strategies (see Hanrahan, 2006) by Hanrahan.

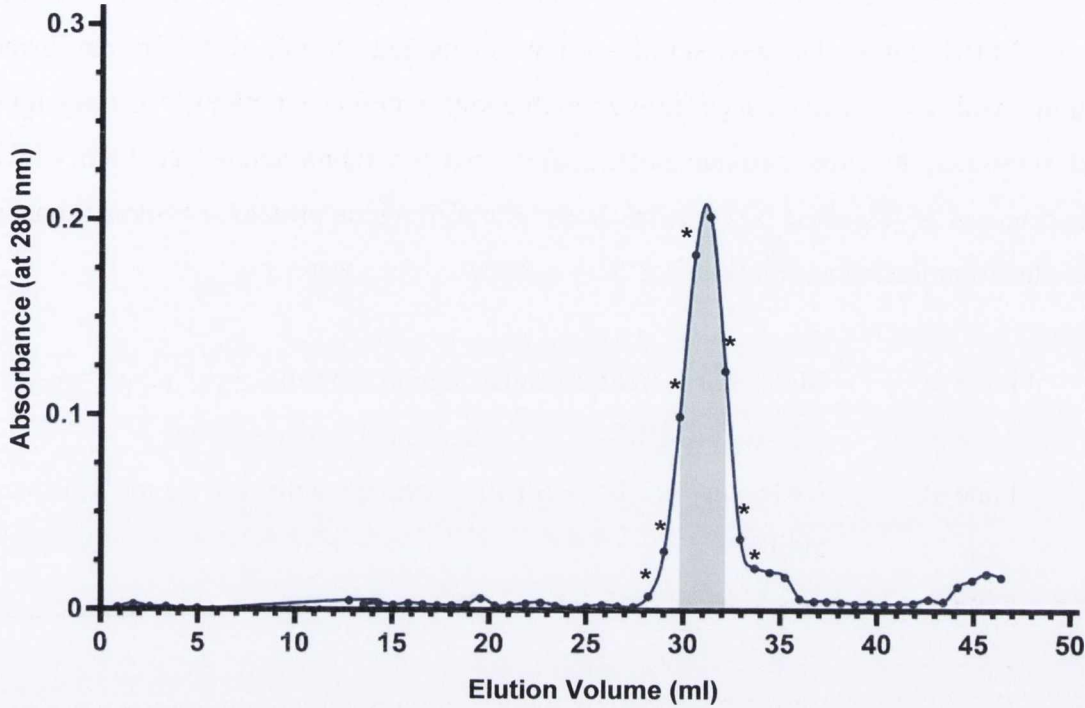
Although the purified MBP-GPI-PLC presented here may be sufficiently pure to begin crystallization trials, two considerations should first be made. Firstly, although recombinant MBP-GPI-PLC has been shown to be enzymatically active (Hanrahan, 2006), it is not known how this activity compares to the activity of natural GPI-PLC; biological activity of the MBP-GPI-PLC has not yet been quantified. Furthermore, the activity of MBP-GPI-PLC purified following the removal of the bacterial chaperone has not been determined. The second consideration is to continue attempts at separating the fused proteins, using a Tobacco Etch Virus (Tev) protease to specifically cleave the linker region between the MBP and GPI-PLC. Optimization of procedures for separating the GPI-PLC from the MBP fusion has not yet been achieved by this laboratory.

Figure A.2. Further Purification of Recombinant MBP-GPI-PLC by Size Exclusion Chromatography

Eluates from the amylose column were applied to an S-200 size exclusion column, which was then washed through with column buffer. Fractions, approximately 0.8 ml, were collected and the protein concentration monitored at 280 nm. The resulting elution profile is given in (a). Samples of fractions marked (*) were taken for gel electrophoresis, while the fractions shaded green were pooled, concentrated, aliquoted and stored at -20 °C. The 10% gel of eluates is shown in (b), with the recombinant MBP-GPI-PLC marked (arrow). The gel loading scheme is given below.

Lane 1:	Molecular weight markers (Sigma-Aldrich)
Lane 2:	20 µl of fraction 37
Lane 3:	20 µl of fraction 38
Lane 4:	20 µl of fraction 39
Lane 5:	20 µl of fraction 40
Lane 6:	20 µl of fraction 41
Lane 7:	20 µl of fraction 42
Lane 8:	20 µl of fraction 43

(a)



(b)

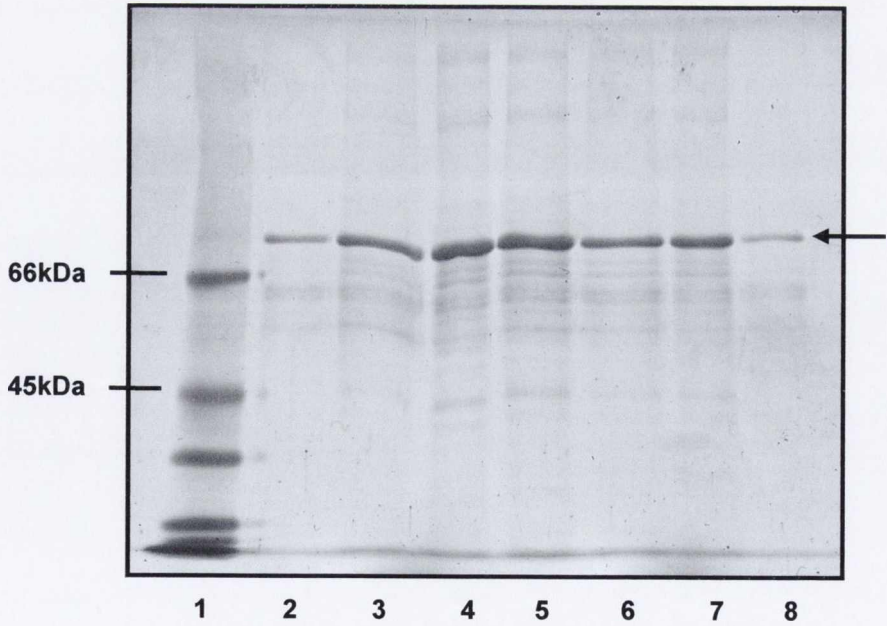


Figure A.3. Blot of purified MBP-GPI-PLC

Purified and concentrated MBP-GPI-PLC was boiled in the presence of 5X sample buffer and subjected to SDS-PAGE on a 10% gel. Protein was then transferred to a nitrocellulose membrane which was probed with rabbit anti-GPI-PLC primary IgG, and subsequently with peroxidise-conjugated goat-anti-rabbit secondary IgG, which was detected as described earlier in methods. Purified fusion protein is marked (arrow). Samples were loaded as below.

- | | |
|---------|---|
| Lane 1: | Molecular weight markers (Sigma-Aldrich) |
| Lane 2: | 6.25 μg of MBP-GPI-PLC stained with Coomassie |
| Lane 4: | 0.625 μg of MBP-GPI-PLC probed with 1.4 $\mu\text{g.ml}^{-1}$ primary antibody |

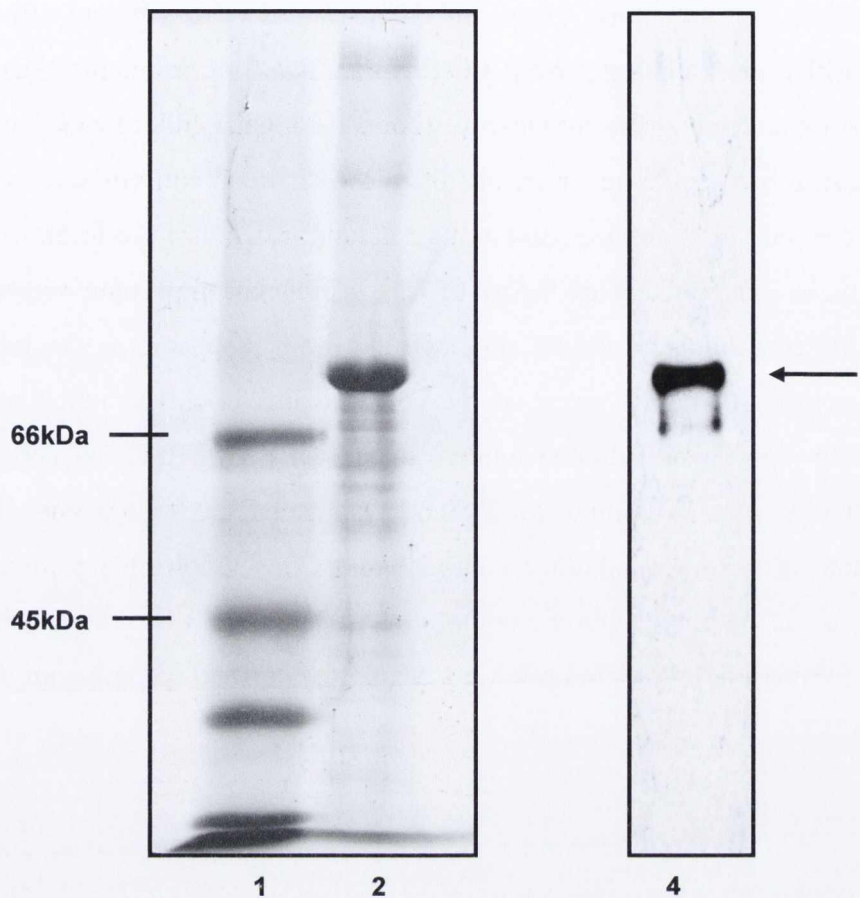


Figure A.3. Blot of Purified MBP-GPI-PLC

Purified and concentrated MBP-GPI-PLC was boiled in the presence of 5X sample buffer and subjected to SDS-PAGE on a 10% gel. Protein was then transferred to a nitrocellulose membrane which was probed with rabbit anti-GPI-PLC primary IgG, and subsequently with peroxidise-conjugated goat-anti-rabbit secondary IgG, which was detected as described earlier in methods. Purified fusion protein is marked (arrow). Samples were loaded as below.

- Lane 1: Molecular weight markers (Sigma-Aldrich)
- Lane 2: 6.25 μg of MBP-GPI-PLC stained with Coomassie
- Lane 4: 0.625 μg of MBP-GPI-PLC probed with 1.4 $\mu\text{g}.\text{ml}^{-1}$ primary antibody

Finally, one simple change to the protocol presented here may be worth considering. The use of size exclusion chromatography to separate affinity purified MBP-GPI-PLC from contaminating proteins may now not be required, as its use was largely introduced in earlier attempts to remove GroEL. Indeed many of the (feint) contaminating bands present in eluates from the amylose column are also present in eluates from the S-200 column, and may represent proteolysis products. Significantly, many of these contaminants are below 45 kDa in apparent molecular weight. Although some of the contaminants present approach the limit in separation capabilities of size exclusion chromatography, some of the contaminants could be much more quickly removed (or significantly diluted relative to the MBP-GPI-PLC) by concentration of MBP-GPI-PLC in a Vivaspin with a 50,000 Da molecular weight cut-off membrane (available from Sartorius and other manufactures). This simple change would eliminate the need for an overnight chromatography step, and should therefore also reduce the amount of proteolysis occurring prior to freezing the purified recombinant protein.

Appendix B

Correcting Absorbance Readings at 260 nm and 280 nm Using Standard Concentration Curves of Adenosine and Bovine Serum Albumin

B.1. Introduction

The de-energization of bloodstream form trypanosomes was previously found to causes a release of cellular material, over time, which absorbed light at 280 nm (Brabazon, 1999, Hanrahan *et al.*, 2009). This material was thought to represent predominantly surface proteins since SDS-PAGE of released material appeared to show one major protein band that corresponded to sVSG. However, Brabazon also recorded released material that absorbed light at 280 nm, but failed to stain with Coomassie blue. This later substance was speculated to be hypoxanthine, which trypanosomes were expected to produce under the de-energizing conditions used (Brabazon, 1999, Hanrahan, 2006). Since hypoxanthine absorbs maximally at 260 nm, the absorbance of material released over the time course of de-energization experiments was monitored at 260 nm and at 280 nm for this current work.

In an effort to more specifically follow surface protein release during de-energization, a procedure was developed to correct absorbance values measured at 280 nm for contaminating hypoxanthine, and possibly other purines. Adenosine was chosen as a standard molecule to test the absorbance of purines at 280 nm; establishing a standard curve of adenosine absorbance at 260 nm and at 280 nm would provide a way for correcting the absorbance values of de-energization supernatants (measured at 280 nm) for contaminating purines. Similar standard curves of BSA absorbance at 260 nm and at 280 nm would further allow for the absorbance values of de-energization supernatants (measured at 260 nm) to be corrected for contaminating protein.

B.2. Methods

B.2.1. Standard Curves for the Absorbencies of Adenosine and BSA at 260 nm and at 280 nm

A series of dilutions of a stock 1 mM adenosine solution (in TSB) was prepared to yield adenosine concentrations ranging from 0.5 μM to 100 μM . Similarly, a range of BSA concentrations (11 $\mu\text{g}\cdot\text{ml}^{-1}$ to 2.25 $\text{mg}\cdot\text{ml}^{-1}$) was prepared from a stock 22.5 $\text{mg}\cdot\text{ml}^{-1}$ solution of BSA (also in TSB). The absorbance of each dilution at 260 nm and at 280 nm was measured and the resulting standard curves were plotted in figure B.1.

B.2.2. Derivation of the Correction Formulae

In the context of the VSG release experiments, the true absorbance at 260 nm is given by:

$$A_{260}^T = A_{260}^O - A_{280}^C \quad (1)$$

Where A_{260}^T is the true absorbance at 260 nm, A_{260}^O is the observed, or measured, absorbance at 260nm and A_{280}^C is the contribution to the observed A_{260} made by material that absorbs maximally at 280 nm. Similarly, the true A_{280} is given by:

$$A_{280}^T = A_{280}^O - A_{260}^C \quad (2)$$

Both A_{260}^C , and A_{280}^C , need to be defined, and this can be achieved using the standard curves for adenosine and BSA. From the adenosine standard curve (panel a, figure B.1.):

$$A_{260}^C = \frac{\text{slope of } A_{280}}{\text{slope of } A_{260}} (A_{260}^T)$$

$$\Rightarrow A_{260}^C = 0.1655(A_{260}^T) \quad (3)$$

From the BSA standard curve (panel b, figure B.2), A_{280}^C can be defined:

$$A_{280}^C = \frac{\text{slope of } A_{260}}{\text{slope of } A_{280}} (A_{280}^T)$$

$$\Rightarrow A_{280}^C = 0.5809(A_{280}^T) \quad (4)$$

Substituting (4) into (1) yields:

$$A_{260}^T = A_{260}^O - 0.5809(A_{280}^T) \quad (5)$$

Substituting (2) into (5), and the A_{260}^T can start to be re-written in terms of measurable quantities:

$$A_{260}^T = A_{260}^O - 0.5809(A_{280}^O - A_{260}^C)$$

A_{260}^C can now be replaced with (3):

$$A_{260}^T = A_{260}^O - 0.5809[A_{280}^O - 0.1655(A_{260}^T)]$$

Finally, re-arranging and simplifying yields the formula for correcting the observed absorbance at 260 nm:

$$A_{260}^T = \frac{A_{260}^O}{0.9039} - 0.6427(A_{280}^O) \quad (6)$$

By a similar method:

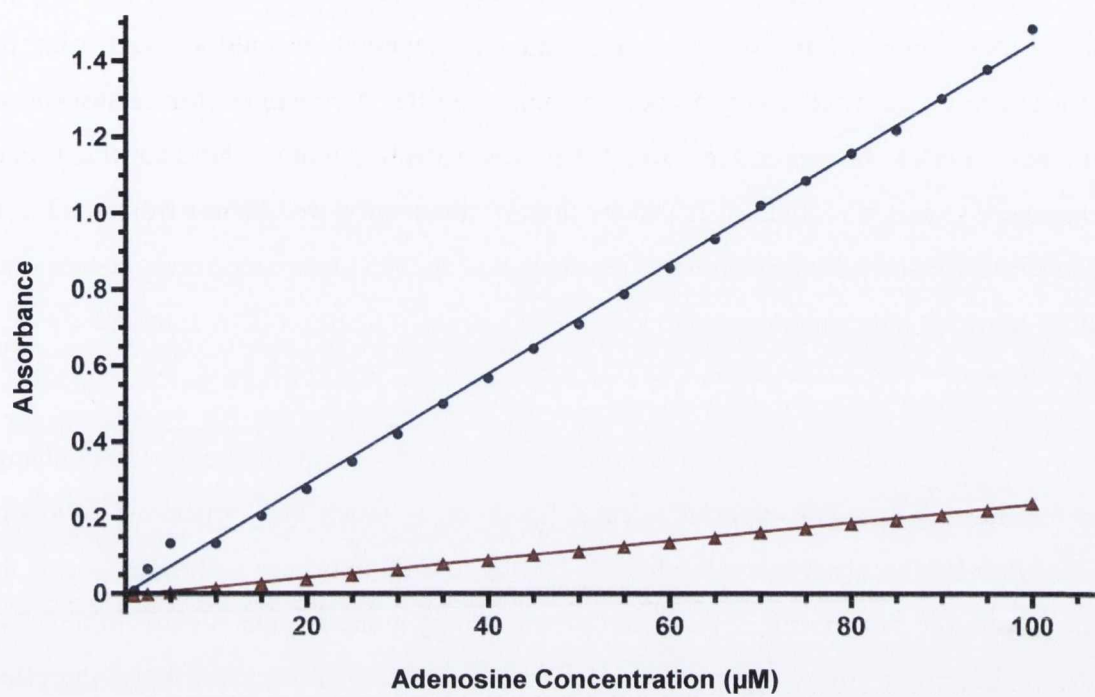
$$A_{280}^T = \frac{A_{280}^O}{0.9039} - 0.1831(A_{260}^O) \quad (7)$$

Figure B.1. Standard Curves for the Absorbencies of Adenosine and BSA at 260 nm and at 280 nm

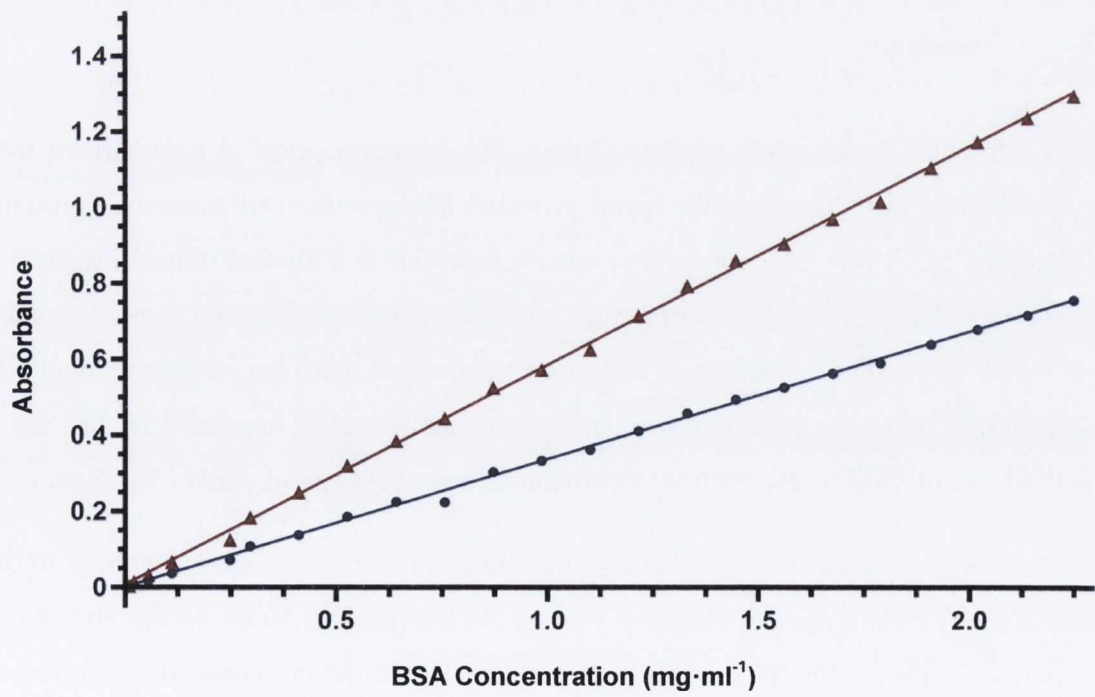
The absorbencies of adenosine and BSA were measured over a range of concentrations, at both 260 nm and 280 nm. The resulting values for adenosine are graphed in (a), with absorbencies for BSA shown in (b). In each graph $A_{260\text{nm}}$ values are shown with a blue trace (—●—), while $A_{280\text{nm}}$ values are shown with a red trace (—▲—). The slope of each trace is listed below:

Adenosine:	Slope of the A_{260} trace = 0.0145	(correlation coefficient = 0.9984)
	Slope of the A_{280} trace = 0.0024	(correlation coefficient = 0.9993)
BSA:	Slope of the A_{260} trace = 0.3351	(correlation coefficient = 0.9987)
	Slope of the A_{280} trace = 0.5768	(correlation coefficient = 0.9992)

(a)



(b)



B.3. Results

To correct the measurements of released protein for interfering purines, the absorbance values from a trial de-energization experiment were all adjusted using the formula derived earlier (figure B.2). Application of the formulae to correct absorbance readings at both 260 nm and at 280 nm has a noticeable effect on the quantification of material present; a reduction in the maximum absorbance at 280 nm from 0.8222 to 0.6217 was observed for example. This change of 0.2005 absorbance units corresponds to an approximate protein concentration of $125 \mu\text{g}\cdot\text{ml}^{-1}$ (using a BSA standard curve as a reference).

The change in the rate of material release in de-energizing cells, or the change in the time of onset of material release, however, is much less noticeable following correction of the absorbance readings. In the case shown here (GPI-PLC^{-/-} cell de-energization in the presence of calcium), there is only a one minute “delay” in the onset of protein release following correction of the absorbance values. Similarly, the effect on the maximum rate of protein release is a decrease in rate of approximately 0.0077 absorbance units per minute.

B.4. Discussion

In early experiments performed here, the de-energization of bloodstream form trypanosomes was unexpectedly found to result in the release of numerous proteins. The ability of GPI-PLC^{-/-} cells to also release material, in a manner almost identical to wild-type cells, was puzzling; originally, protein release was assumed to be dependent on GPI-PLC activity. Since it had been speculated that hypoxanthine could be contributing to, and interfering with, the absorbencies being measured at 280 nm, a method for filtering contaminating absorbencies was necessary to clarify the situation.

The procedure described here for correcting absorbance readings, similar to that described by Warburg and Christian (1942), was applied to some of the absorbance readings obtained from de-energization experiments. However, no significant effect on the trend of protein release following correction was found. Only a noticeable decrease in the quantity of released protein (and purines) was observed.

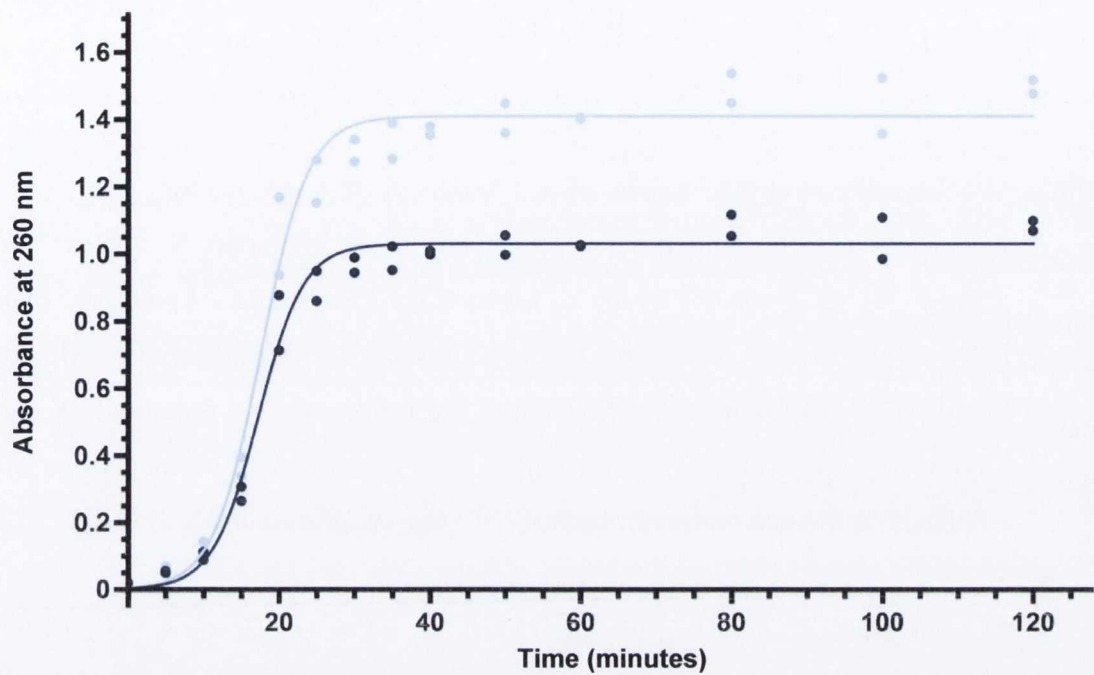
Later findings have shown that the de-energization of trypanosomes results in the release of much more cellular matter than previously reported. In particular, the presence of a single broad peak (at 260 nm) in the absorption spectrum of released material (section 4.2.2) has highlighted that attempting to obtain specific information about the release of proteins during the de-energization should not be pursued through simple absorbance measurements.

Although the correction procedure described here may be useful in certain circumstances, it seems unwise to apply a correction procedure based on the absorption properties of only two compounds (adenosine and BSA) to mixtures that have since been shown to contain many unknown compounds, with unknown absorption characteristics. Therefore, the correction formulae were not applied to the absorbance readings in chapter 4 of this thesis. Instead, protein release was followed more accurately using a specific protein quantification assay as described.

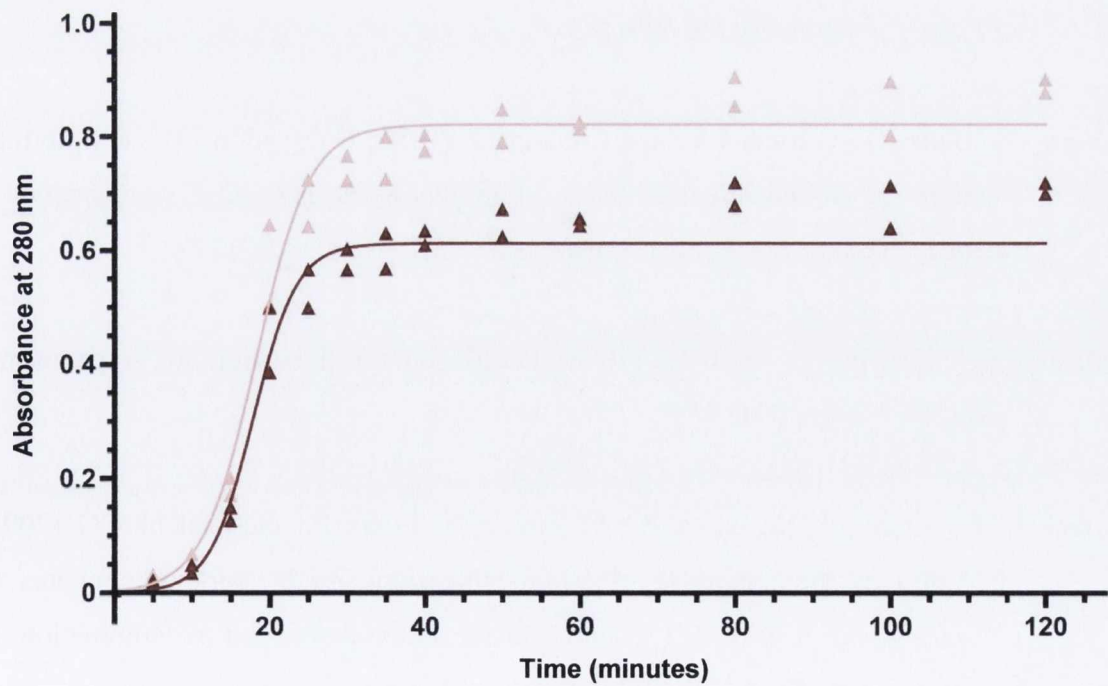
Figure B.2. Applying the Correction Formulae to the Absorbance Measurements of a De-energization Experiment

GPI-PLC^{-/-} trypanosomes were de-energized in the presence of calcium, as described in section 2.6.2, and the absorbance of released material was measured at 260 nm (a) and at 280 nm (b). The correction described in section B.2.2 was applied to both sets of data. In (a), uncorrected A_{260} values are shown by a light blue trace (—○—), while corrected A_{260} values are shown in a dark blue trace (—●—). In (b), uncorrected A_{280} values are shown by a light red trace (—△—), while corrected A_{280} values are shown by a dark red trace (—▲—). In each case, data were fit to a sigmoidal curve, using the mean of absorbance values from two separate de-energization experiments (the individual measurements are shown).

(a)



(b)



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