A surface antigen from *Trypanosoma congolense*

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by

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The research in this Thesis
is my own original work except where
otherwise stated and has not been
submitted for any other degree.

CONTENTS

ABBREVIATIONS

APS	-	ammonium persulphate
ATPase	_	adenotriphosphatase
CO_2	-	carbon dioxide
CHAPS	-	3-[(3-chloroamidopropyl)dimethylammonio]-1-propanesulfonate
DMSO	-	dimethyl sulfoxide
DNA	-	decxyribonucleic acid
cDNA	-	complementary DNA
CON.A	-	concanavalin A
GLU	-	glutamine
GUGM	-	Glasgow University Genetics monoclonal
GPI	-	glycosylphosphatidylinositol
HCl	-	hydrochloric acid
HICE	-	hydrophobic interaction chromatography eluent
kD	-	kilodaltons
LDL	-	low density lipoprotein
LPG	-	lipophosphoglycan
NaOAc	-	sodium acetate
NaCl	-	sodium chloride
NaH_2PO_4	-	sodium hydrogen phosphate
Na_2HPO_4	-	di-sodium hydrogen phosphate
NaN_3	-	sodium azide

NP40 - Nonidet 40

NH_4OH - ammonium hydroxide

NaOH - sodium hydroxide

PAGE - polyacrylamide gel electrophoresis

PARP - procyclic acidic repetitive protein

PBS - phosphate buffered saline

PMSF - phenylmethylsulfonylfloride

PRO - proline

RLO - rickettsia like organism

RNA - ribonucleic acid

mRNA - messenger ribonucleic acid

SDS - sodium dodecylsulphate

STAINS-ALL - 1-ethyl-2-[3-(1-ethylnaphtol[1,2d]thiazolin-2-ylidene)-2-methyl-propenyl]-naphto[1-2d]thiazolium bromide

TLCK - 1-chloro-3-tosylamido-7-amino-2-heptanone

Tris - tris (hydroxymethyl) methylamine

TEMED - NNN'N'-Tetramethylethylenediamine

VAT - variable antigen type

VSG - variable surface glycoprotein

BEB - blot elution buffer

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SUMMARY

Using monoclonal antibodies, a surface membrane antigen was identified on *T.congolense* procyclic cells.

Various methods, including cation ion-exchange and H.P.L.C. were attempted to purify the antigen, anion exchange chromatography enriched it from whole cell extracts. An organic purification protocol, chromatography on Octyl-Sepharose yielded it pure and at the highest yields of these methods.

The antigen was found to be an acidic protein, whose molecular weight as determined by SDS PAGE was 29-40kD. When *T. congolense* procyclic cells were probed with an immunogold labelled monoclonal antibody the antigen was located on the surface of the cells. The amount of labelling seen with these experiments suggested the antigen was present in great abundance. Basic carbohydrate analysis on the antigen was carried out, including lectin binding studies and periodate treatment, positive results from these experiments suggested that the antigen was heavily glycosylated.

The protein exhibited many strange features; it did not stain with the conventional protein stains - Coomassie blue and Silver stain - and it is resistant to Trypsin, Chymotrypsin , Protease xvII-B and Proteinase K treatment digestion. When Western blots of SDS PAGE containing the protein were performed, the protein migrated through the first nitrocellulose membrane onto a second.

Certain properties of the protein including its stage

specificity, expression on the cell surface, and acidity of the protein suggest that it may be the *T. congolense* analogue of procyclin, the major surface protein of the procyclic stage of *T.brucei*.

CHAPTER 1

INTRODUCTION

1.1 LIFE-CYCLE OF GENUS *Trypanosoma* WITH PARTICULAR REFERENCE TO *Trypanosoma congolense*

1.1.1 General introduction

African trypanosomes have a complex life-cycle which involves several distinct stages in both a mammalian host and a tsetse fly vector. These stages can be distinguished by morphological, biochemical and antigenic features, which may be related to the very different environments the trypanosome meets within its two hosts.

The genus *Trypanosoma,* more specifically those which infect the tsetse fly *Glossina,* can be divided classically into species, including *Trypanosoma brucei* , *T. congolense,* T.vivax depending on, amongst other characters, which mammals they infect, where in the fly the different stages develop, the presence or absence of a free flagellum and the size of kinetoplast. The kinetoplast is a body of DNA unique to the order kinetoplastidae. Isolation of kinetoplasts in the early 1970s revealed that it is a massive network consisting of thousands of interlocked DNA circles and carrying genes similar to those on mitochondrial DNA in other eukaryotic cells, as well as a novel class of genes encoding guide RNAs (gRNAs), which specify RNA editing(1).

In humans *T.brucei rhodesiense* and *T.brucei gambiense* cause the debilitating disease Sleeping Sickness of which thousands of new cases are reported every year. If the disease is left untreated it can be fatal due to invasion and destruction of the central nervous system by the organism.

In other animals *T brucei, T congolense* and *T vivax* cause trypanosomiasis. Trypanosomiasis is a serious obstacle to human development due to the severe nutritional and economic problems it causes. *T congolense* can infect a wide range of livestock including cows, pigs, horses, goats, sheep and camels. Large numbers of cattle are affected and consequently the rearing of high meat and milk producing cattle is difficult.

1.1.2 Life cycle of *Trypanosoma congolense*

Trypanosoma congolense is a member of the sub-genus *Nannomonas* and measures from 8um to 24um in length (2,3). The sub genus is characterised by the presence of a large kinetoplast and the absence of a free flagellum in all known stages of its life-cycle (4,5).

In the tsetse fly three distinct morphological stages are observed. On ingestion by the fly the bloodstream trypanosomes enter the endoperitrophic space within the fly mid-gut. The peritrophic membrane which surrounds the endoperitrophic space separates the blood from the mid-gut epithelium.
At this stage the trypanosome becomes the elongated procyclic form, which is characterised by absence of the dense surface coat(variable surface glycoprotein; VSG) which covers all mammalian stages of the trypanosome. Branching is seen in the mitochondrion as the trypanosome changes its energy source from glucose -which it uses in the mammal- to proline. Proline is in abundant supply in the fly where it is the main energy source for flight muscle.

Procyclic trypanosomes divide repeatedly in the endoperitrophic space, establishing a population. Trypanosomes then appear in the mouthparts of the fly, where the epimastigote form appears. This divides and is attached to the walls of the labrum and food canal. Finally, the epimastigote form transforms to the metacyclic form, a non-dividing free living stage which enters the hypopharynx of the fly and is subsequently injected into the mammalian host at feeding. As it develops, from the epimastigote stage, the metacyclic cell acquires the VSG surface coat(6).

1.1.3 Major surface proteins of the different trypanosomes stages

1.1.3.1 VSG coat

One of the most prominent differences between the insect and the mammalian stages of trypanosomes is in the molecular structure of their surfaces(7).
The bloodstream forms are covered by a dense surface coat composed of a highly immunogenic glycoprotein known as the Variant Surface Glycoprotein (VSG), which endows on each trypanosome its Variable Antigen Type(VAT). The host mounts an antibody response against the particular VAT being expressed and clears the parasitaemia(8). However, within days the trypanosome population recrudesces expressing different VATs, thus eliciting a fresh immune response. The coat also protects against non-specific immune mechanisms. It has been shown in the absence of specific antibodies coatless forms of trypanosomes are engulfed and destroyed by host macrophages, whilst coated

forms are not. Complement activation by the alternative pathway appears also to be prevented by presence of the coat (9).

1.1.3.2 Procyclin or PARP

In the procyclic form of *T.brucei* the parasite is covered by a different molecular structure. Mowatt and Clayton (10) named it PARP (procyclic acidic repetitive protein) and Roditi *et al* termed it procyclin (11).

PARP appears to be a relatively abundant protein in *T.brucei* procyclic cells, It comprises about 1% of the total procyclic trypanosome soluble protein or 6 X 10^6 molecules per parasite (16). Richardson *et al* (14) were able to isolate procyclin from *T.brucei* procyclic cell lysates by affinity chromatography using cell surface specific monoclonal antibodies. The amino-terminal amino-acid sequence of the purified procyclin was found to be similar to the sequence predicted by Roditi *et al* (15) and Mowatt and Clayton(13). The yields of procyclin obtained were very low and a great deal less than had been predicted from mRNA levels. This contradiction was partially resolved by Clayton and Mowatt (16) who developed an improved purification method for procyclin, utilizing cation exchange and lectin affinity chromatography though their yield was still less than expected.

The pattern of expression of procyclin and its predicted conformation have been investigated (11,18). Interestingly there is a lag of several hours between the detection of procyclin mRNA and detection of protein, suggesting post-transcriptional control.

A structure for procyclin has been predicted (18) that has its long (Glu-Pro)repeat forming a rod- like structure 14-18nm long. During transition from blood stream form to procyclic cell form, when the quantity of VSG on the cell surface decreases as that of procyclin increases this structure is proposed to be inserted into the membrane via a glycosylphatidylinositol anchor (17) in between the VSG molecules corresponding to the observation that production of both overlaps (18).

In an attempt to quantitate the PARP mRNA Mowatt and Clayton (10) used the PARP cDNA to probe bloodstream form and procyclic form cDNA libraries. The frequency of homologous clones was 1 to 3% in the latter and less than 0.1% in the former libraries. The presence of the PARP mRNA on membrane bound polyribosomes and the nature of the sequence suggest that the mRNA is translated and probably encodes a glycosylated, membrane-associated protein. From amino-acid analysis of PARP performed by Mowatt and Clayton (10) and by Roditi *et al* (11) the protein was reported as containing a(Glu-Pro)29 repeat. Mowatt and Clayton found that injection of rabbits with a (Glu-Pro)6 repeat did not yield antibodies (10)

Roditi *et al* (11) have also reported another PARP cDNA which is closely related to the above cDNA, and which is termed PARP-1. The sequence isolated by Mowatt and Clayton is termed PARP-2. Nucleotide changes lead to amino-acid substitutions in the amino and carboxy terminal regions of the protein. Another difference between the two sequences is the number of Glu-Pro repeats. PARP-1 has 22 compared with 29 in PARP-2.

Mowatt and Clayton (13) were able to isolate two other PARP clones from their library, one which is similar to PARP-1. Both the cDNA clones give identical hybridisation patterns in DNA and RNA blots. The fourth clone lacked an N- glycosylation site and its repetitive domain consisted of six tandem copies of the pentapeptide Glu-Pro-Glu-Glu-Thr followed by three Glu-Pro repeats (13). All four proteins are stage specific,

Koenig al(7) have studied the organisation of procyclin genes in the Kenyan strain 227 and have shown that procyclin genes are tandemly repeated at three loci, at least two of which are transcribed.

At present a function for procyclin has not been determined, It could be envisaged that procyclin is present to protect the trypanosome from the very hostile environment it faces in the fly gut, although at present it has not been identified in any other trypanosome species.

1.2. MEMBRANE STRUCTURE

Trypanosomes, like all other eukaryotic cells, are surrounded by a plasma membrane, which is composed of a lipid bilayer: this layer is not water soluble, but can be effectively disrupted by detergents. Contrary to the situation in mammalian cells, the plasma membrane of trypanosomes is tightly associated with sub-pellicular micro tubules which do not disrupt with standard cell fractionation methods (19). This association of the plasma membrane with microtubules gives it a high density in sucrose gradients (20), allowing its identification in sub-cellular fractions of trypanosomes.

Typical plasma membrane marker enzymes such as 5'nucleotidase and oubain-sensitive ATPase have not been found in the plasma membrane from trypanosomes(20,21), which hampered initial analysis of the plasma membrane until markers could be identified ie. D-glucosidase and 3-nucleotidase being two of the first.

Trypanosomes possess another membrane that is exposed, in this case indirectly, to the exterior of the cell: the flagellar pocket membrane. This membrane is continuous with the plasma membrane. It is the only part of the trypanosome surface which is not supported by subpellicular microtubules and therefore is thought to be the only area on the cell's surface which has enough flexibility to allow invagination, and thus endocytosis (19)

Trypanosomes also transfer molecules from one side of the plasma membrane to the other using receptor mediated endocytosis. Opperdoes *et al* (19) has shown that *T.brucei*

has at least two receptors, one specifically for low-density lipoprotein and the other for transferrin. A gene encoding a transferrin binding protein has been cloned (22).

1.3. TRANSMISSION BLOCKAGE

The trypanosome population evades the immune response of the mammal by constantly switching its variable antigenic coat. This property prevents development of an effective protective vaccine. However, the metacyclic VAT repertoire, that is the set of VATs expressed by the infective trypanosome stage developing in the mouthparts of the tsetse fly, comprises only 1->2% of the entire VAT repertoire (23). In one stock of *T congolense* only 12 VATs are expressed in the metacyclic population (23). With repeated fly transmissions, there is a gradual turnover in the metacyclic VAT repertoire of *T brucei*, ruling out any prospect for vaccination. Nevertheless, in the absence of repeated fly transmissions it is possible to vaccinate animals using large numbers of metacyclic cells. Both *T congolense* and *T vivax* produce few metacyclic cells at each fly bite, presenting an antigenic challenge which is not formidable (24). *T. congolense* metacyclic cells grown *in vitro* been used successfully for vaccination in the laboratory (26).

When entering the tsetse fly the trypanosome loses its variant surface coat. This stage of the life-cycle may prove an easier target for a vaccine, by being vulnerable to the effect of antibodies raised against exposed common surface antigens. Tsetse flies maintained on goats immunised with uncoated forms of *T congolense, T vivax* and *T brucei* showed significant suppression of mature and immature infection rates (26). Murray, Hirumi and Moloo (26) have shown that for

T.congolense suppression was not stock specific. However, there was no decrease in the infection rates by *T vivax* or *T brucei* in tsetse flies maintained on goats immunised against *T. congolense* . Thus the effect appears to be species specific but not stock specific (26).

Instead of looking for antibodies directed against unknown components on the surface of the trypanosome, an alternative approach to transmission blockage might be to search for biochemical features of the parasite with a view to interfering with them chemically. Evidence has been obtained for the presence of secondary messenger systems analogous to those used by other eukaryotic cells for transduction of extrinsic signals (eg.hormones, growth factors). Experiments on *T brucei* have revealed possible growth factor receptors with associated intracellular components of a secondary messenger system as well as a receptor for particular host factors (28).
It appears possible that a method could be found to interfere with infection rates based on the binding of extraneous molecules such as competitor antibodies to this receptor.

A third approach is to look at structural features which are unique to the trypanosome. Antibodies against these structures would not cross-react with components of the mammalian host.

The importance of the flagellar pocket as the site of macromolecular ingestion may imply a specificity in its molecular structure. It is possible that the flagellar pocket possesses some structurally invariant membrane

associated molecules which, by virtue of their receptor function, are exposed either directly or indirectly to circulating host macromolecules. Trypanosomes obtain sterol directly from the host. Sterol molecules circulate in the host attached to carrier proteins and require specific receptors for cellular uptake. In mammals cholesterol and low density lipoproteins (LDL) form a particulate complex which is ingested via surface specific LDL receptors. Uptake of LDL by *T brucei* by the kinetics of receptor-mediated ingestion. has been demonstrated (28). Olenick *et al* found that gold labelled LDL bound only to the membrane of the flagellar pocket suggesting this to be the site of the receptor (29); similar results have been obtained for the uptake of iron and its carrier protein transferrin. A gene encoding a transferrin binding protein has recently been cloned (22).

These receptor molecules could be targets for a vaccine, but it still has to be determined whether they are exposed on the exterior of the parasite or exclusively within the flagellar pocket, or indeed only transiently exposed.

Polyclonal antibodies have been raised against flagellar membrane fractions and by electronmicroscopy two major antigens have been localised to the pocket. When applied to intact living procyclic cells there were observed to bind specifically at the region of emergence of the flagellum from the pocket (29). In a preliminary experiment, mice immunised with these fractions were resistant to infection. This protection was independent of VSG as it afforded protection against two

VAT's but not a third.

It is known that the surface membranes of procyclic cells have exposed carbohydrate groups. These have been detected by the ability of particular lectins to agglutinate live procyclic cells and to bind to specific bands on Western blots of detergent solubilised procyclic cell lysates. Lectins are large molecules and it is possible that if these carbohydrate groups are accessible to lectins that they will be accessible to antibody (30,31).

A further candidate for a transmission blocking vaccine is procyclin, which is conserved between isolates of *T.brucei*. It is first expressed in transforming bloodstream forms of the trypanosome before the VSG coat is lost (11).

1.4 THE TSETSE-TRYPANOSOME INTERFACE

1.4.1. Lectins

Lectins are sugar binding proteins or glycoproteins of non-immune origin which agglutinate cells and/or precipitate glycoconjugates. Soluble lectins bear at least two sugar binding sites, the presence of which explains the cross-linking affect.

Ibrahim *et al* (32) showed that extracts from the gut of *Glossina austeni* were capable of agglutinating *T brucei* procyclic cells and that these agglutinins had specific activities when tested against erythrocytes from different mammalian species. They also found that the activity of tsetse gut extracts could be inhibited by specific monosaccharides, indicating that a lectin or lectin-like substance was present in the fly. Croft *et al* (33) have also shown that lectin-like substances may be present in the haemolymph of *G morsitans* with activity against *T brucei* procyclic cells.

Maudlin and Welburn (34,35) have demonstrated that some lines of *G morsitans morsitans* are more susceptible than others to trypanosome infection. They suggested that this refractoriness to infection is related to lectin presence. Their studies showed that feeding specific monosaccharides to flies with their infective feeds increased the likelihood of the trypanosome establishing a mid-gut infection. D+glucosamine is the most effective of these, in keeping with the demonstration by Ibrahim *et al* (32) that this monosaccharide specifically inhibits the *in vitro* agglutination activity from the mid-gut extracts.

Maudlin and Welburn (34) speculated that the mid-gut lectins are normally responsible for the killing of trypanosomes which have recently entered the flies' mid-guts.

Lectins may have another contrasting effect in the fly Maudlin and Welburn (35) have shown that *T.congolense* maturation rates were significantly reduced in flies fed with specific lectin inhibitor monosaccharides. They suggest that this phenomenon is related to the genotype of the trypanosome which determines its ability to bind lectin. If *T.brucei* and *T.congolense* maturation rates are compared,ie the ability to produce the infective metacyclic stage then in flies fed with lectin inhibitors, *T.brucei* maturation is less affected than is *T.congolense* is.

Lectins appear to have two distinct functions in flies: they are involved in establishment of mid-gut infection of trypanosomes, and they are involved in the maturation of mid-gut procyclic cells to infective metacyclics.

1.4.2 Rickettsia like organisms

As well as the genotype of the trypanosome being important for successful infection of tsetse flies it has been shown (36) that the genotype of the fly itself has also some part to play. Tsetse flies can be bred for refractoriness to trypanosome infection. This susceptibility is maternally inherited and Maudlin and Welburn (36) associate this mode of inheritance with the presence of a rickettsia-like-organism (RLO). The genotype of the fly determines whether it is infected with RLO.

In vitro culture of the RLO on a mosquito cell line reveals

that the bacteria produce chitinase (36). This enzyme can hydrolyse chitin to glucosamine (mainly N Acetyl glucosamine), which could compete for the mid-gut lectin thus enabling trypanosome infection to establish. This work was repeated by B.Beard (personal communication) who used an axenic culture of RLO derived fom Tsetse flies. This theory is still speculative, as it is not known whether this hydrolysis actually proceeds in the fly. Furthermore, the main product of chitin hydrolysis is N-acetyl D glucosamine rather than the non-acetylated form, which is the major known inhibitor of the mid-gut agglutinating actvity.

Welburn and Gibson (37,38) have cloned repetitive DNA sequences from RLO which can be used to identify the presence of RLO from homogenized tsetse mid-guts blotted onto nitrocellulose. RLO's are transmitted vertically in tsetse flies, susceptibility of tsetse flies to infection with trypanosomes has similarly been shown to be vertically transmitted (43) and a study of wild and laboratory flies has associated RLO load with such susceptibility (44). Use of these cloned DNA sequences will help in the examination of this association.

CHAPTER 2

MATERIALS AND METHODS

2.1. In vitro CULTURE OF Trypanosoma Congolense PROCYCLIC STAGE

2.1.1 Stocks

Trypanosoma congolense 1/148

Trypanosoma congolense TREU1627

2.1.2 Media

Eagle's Minimal essential medium MEM or 109-C was used as a growth medium for the *T congolense* procyclic stage.

MEM:-Stock solutions of the following were purchased-

MEM(Eagle's), 25mM HEPES with Earles salts without L-glutamine Gibco-Europe, Paisley,

L-glutamine 200mM (Northumbria Biologicals Limited) N.B.L.,

Foetal calf serum heat inactivated 56°c 30 minutes, Gibco-Europe, Paisley or (N.B.L).

To produce complete medium mix-

100ml of MEM (eagle) 25mM Hepes with Earles salts without glutamine,

2ml L-glutamine,

20ml of heat inactivated foetal calf serum.

Store at 4°C, heating to 28°C before use.

109-C: (44)

The following chemicals and stock solutions were purchased-

For one litre of medium mix the following-

0.3g L-proline (Sigma,Poole),

0.3g L-glutamine (Sigma,Poole),

1.0g glucose,

20mg adenosine (Sigma,Poole),

20ml amino-acid solution (50x),

20ml non-essential amino-acids (100x) (Gibco-Europe, Paisley),

60ml HEPES 1M (Gibco-Europe, Paisley),

950ml MEM (Eagles) 25mM HEPES with Earles salts without glutamine (Gibco-Europe, Paisley).

The pH of the solution is adjusted to 7.4 with 4M NaOH then filtered sterilised (0.22um Sartorius filter). Before use the medium is supplemented with 10% heat inactivated foetal calf serum and 0.1% of the haemin stock (see below).The medium is kept at 4°C, and heated to 28°C before use.

Haemin stock solution- 100mg haemin are dissolved in 20ml o.65% NaOH solution by heating to boiling. The solution is made up to 50ml with distilled water and autoclaved.

2.1.3 Culture of Procyclic cells

T congolense procyclics cells were grown to a density of 3-4x10^{6}cells/ml in 25cm^{2}and 75cm$_2$ plastic tissue culture flasks (Costar). At each passage the cells were gassed with 5%CO_2(B.O.C) The cultures were incubated at 28°C.

2.1.4 Cryopreservation

2x10^7 Procyclic cells were centrifuged at 600g for 10 minutes at room temperature and resuspended in 1ml of freezing solution (see below). The suspension of cells were transferred to 2ml cryotubes (Costar, Cambridge M.A.), wrapped in cotton wool and placed at -70°C overnight. The cryotubes were then directly transferred to liquid nitrogen.

Freezing solution: 660ul of MEM or 109-C,
330ul of 20% DMSO in PBS

2.1.5 Recovering Procyclic cells from liquid nitrogen

Cryotubes were transferred directly from liquid nitrogen to beaker of warm water, approximately 50°C. When thawed, procyclic cells were removed with a 1ml syringe attached to a wide bore needle and injected into 25cm^2 tissue culture flasks 5ml of MEM OR 109-C was added dropwise to the flask. The cells were then gassed for 30 seconds with 5% CO_2 and placed in 28°C

incubator. After 5-6 hours the contents of the flask were centrifuged 600g for 5 minutes at room temperature, then resuspended in 5ml of fresh medium, thus removing the DMSO from the culture.

2.2 POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-PAGE

SDS-page was performed according to Laemmli (45) using Hoeffer gel apparatus or Biorad minigel apparatus

2.2.1 Chemicals

Acrylamide-Bis-acrylamide 30:0.8 (National Diagnostics,New Jersey)

Tris base

S.D.S

Glycine

APS

2.2.2 Stock solutions

Stacking gel buffer solution

6.0g Tris were dissolved in 40ml distilled water, then titrated to pH 6.8 with 1M HCl. This solution was adjusted to a final volume of 100ml with distilled water, then filtered through Whatman No.1 filter paper and stored at room temperature.

Resolving gel buffer solution

36.3g Tris was dissolved in 48ml 1M HCl, then titrated to pH 8.8 with 1M HCl.This solution was adjusted to a final volume of 100ml with distilled water, then filtered through Whatman No.1 filter

paper and stored at room temperature.

APS 10% or 1.5%

This solution is unstable and should be made up weekly and stored at 4°C.

10X Reservoir buffer

30.3g Tris, 144g glycine and 10g SDS were dissolved in and made up to 1000ml with distilled water. This solution should be approximately pH 8.3. The pH of this solution should not be adjusted.

2X Sample buffer

2.5ml stacking gel buffer,

2.0ml glycerol,

0.5ml distilled water,

1.0ml 2-mercaptoethanol,

4.0ml 10% S.D.S,

0.2ml 0.4% bromophenol blue.

2.2.3. Gel mixtures

10% resolving gel

10ml acrylamide-bisacrylamide,

3.75ml resolving gel buffer,

0.3ml 10% S.D.S,

1.5ml 1.5% ammonium persulphate,

14.45ml distilled water.

All the ingredients were added to a conical flask, and immediately before casting were mixed with 0.015ml TEMED (Sigma, Poole). The gel is overlaid with 0.1% SDS to

promote polymerisation.

4% stacking gel

2.5ml acrylamide-bisacrylamide,

5ml stacking gel buffer,

0.2ml 10% S.D.S,

1.0ml 1.5% ammonium persulphate

11.3ml distilled water.

All the ingredients were added to a conical flask, and immediately before casting were mixed with 0.015ml TEMED.

2.2.4 Electrophoresis conditions

Gels are run in 1X reservoir buffer. The larger gels are run at a constant current of 40mA until the bromophenol blue reaches the bottom of the gel, usually 3-4 hours. The mini-gels are run at a constant voltage of 150v until the bromophenol blue reaches the bottom of the gel, usually 1 hour 30 minutes.

2.2.5 Triton PAGE

Triton PAGE (18) is run according to the protocols for SDS PAGE except the 10% SDS stock solution is replaced with 10% Triton X 100

2.3 DETECTION OF PROTEIN AFTER COMPLETION OF ELECTROPHORESIS

2.3.1 Coomassie blue

Coomassie blue is a general protein stain which can detect proteins in acrylamide gels, it is sensitive to a minimum level of approximately 100ng.

Stock solutions

Stain

3g of Brilliant blue 350 (Sigma, Poole) were dissolved in 35% methanol, 10% acetic acid. The solution was filtered through Whatman No.13 filter paper

Destain

35% methanol, 10% acetic acid in distilled water.

Method

After electrophoresis placed gel in stain for 60 minutes, then place in destain overnight.

2.3.2 Silver staining

Silver stains is a general protein stain which can detect proteins in an acrylamide gels. It is sensitive to a minimum level of approximately 1ng.

Stock solutions

Solution A

0.8g silver nitrate was dissolved in 4ml distilled water.

Solution B

21ml of 0.36% NaoH was mixed with 1.4ml of 14.8M NH_4OH

Solution C

Solution A was added dropwise to solution B with constant stirring. Solution C should be used within 5 minutes.

Solution D

2.5ml of a 1% citric acid solution was mixed with 0.25ml of 38% formaldehyde, the volume is adjusted to 500ml with distilled water.

Use this solution immediately.

Method

All steps were carried out at 21°C - 25°C

After electrophoresis the gel was soaked in 50% methanol for 2 hours. The gel was placed in solution C for 15 minutes, then washed in distilled water for 5 minutes. The gel is then placed in developer solution D until bands appear on gel, stain development is stopped by placing the gel in a 10% acetic acid solution.

2.3.2 STAINS-ALL(40)

STAINS-ALL has a high affinity for acidic macromolecules

Stock solutions

Prepare a 0.1% stock solution of STAINS-ALL (Aldrich,)in formamide.

Working stain

The working stain was prepared before use by combining 10ml of the stock stain, 10ml formamide, 50ml isopropanol, 1ml 3.0M Tris-HCl pH 8.8 and distilled water up to a volume of 200ml.

Method

After electrophoresis the gel is placed in the staining solution in the dark overnight. The gel was then destained in several changes of distilled water. The acidic proteins are stained blue whilst other proteins are stained pink.

2.3.4. Schiff staining (46)

Schiff's stain is specific for glycoproteins

Stock solutions

0.5% periodic acid (Sigma, Poole)

Schiff's stain 1 in 4 dilution (Sigma, Poole)

Sodium arsenite

0.1% sodium metabisulphate in 10mM HCl

Method

After electrophoresis the gel is placed in 25% isopropanol, 10% acetic acid overnight. Then placed in 10% isopropanol, 10% acetic acid for 6-9 hours. The gel is then transferred to 10% acetic acid overnight. Treated gel with at least 10 volumes of 0.5% periodic acid for 2 hours in the dark at 4°C. Then transferred into 10 volumes 0.5% sodium arsenite, 5% acetic acid for 30-60 minutes. Transferred into 10 volumes 0.1% sodium arsenite, 5% acetic acid for 20 minutes. Repeat. Transferred into 10 volumes 5% acetic acid for 10 minutes. Transferred gel into 10 volumes schiff's reagent overnight in the dark at 4°C. Glycoproteins stain pink. To increase contrast and retard fading, wash out excess reagent with 0.1% sodium metabisulphate solution for several hours until the rinse solution is no longer pink upon the addition of formaldehyde.

2.4 AFFINITY CHROMATOGRAPHY

2.4.1 Protein-A-sepharose chromatography

Protein-A-sepharose was used to purify monoclonal antibodies

from ascites fluid or to purify specific proteins from a total cell lysate.

Stock solutions

Protein A coupled to Sepharose CL-4B (Sigma, Poole),

0.1M Citric acid,

0.1M $NaPO_4$ pH 8.0,

0.1M sodium citrate pH 3.5 (made by addition of NaoH to stock citric acid solution).

Method

0.25g of protein-A-sepharose is swollen in approximately 1ml of phosphate buffer. The swollen gel was added to a Pharmacia CL-10 column (Pharmacia) and the column connected to a peristaltic pump. The column is washed with 5ml $NaPO_4$ buffer, 2ml citric acid buffer and another 5ml $NaPO_4$ buffer. The column is now ready for use.

2.4.1.1 Purification of monoclonal antibody from ascites fluid.

1ml of ascites fluid was mixed with 1ml of $NaPO_4$ buffer and added to the column, which was then washed with 5ml $NaPO_4$ buffer. The bound material was then eluted by the addition of 5ml of 0.1M sodium citrate pH 3.5. The column was then regenerated with the addition of 2ml 0.1M citric acid, then 5ml $NaPO_4$ buffer. The column was run at 28ml/hour at the washing stages and 4ml/hour at the sample application and the elution stages.

2.4.1.2 Purification of specific proteins from a total cell lysate

1ml of purified monoclonal antibody was added to 1ml 0.1M

$NaPO_4$ buffer and added to the column, which was then washed with 0.1M $NaPO_4$ buffer. One ml of cell lysate was mixed with 0.1M $NaPO_4$ buffer and added to the column, which was again washed with 0.1M $NaPO_4$ buffer. The bound material was eluted by the addition of 0.1M sodium citrate pH 3.5. and the column then regenerated with the addition of 2ml 0.1M citric acid, followed by 5ml 0.1M $NaPO_4$ buffer.

2.4.2 Affi-gel blue

Stock solutions

DEAE Affi-gel blue (Bio-rad),

0.1M Acetic-acid pH 3.0 containing 1.4M NaOH and 40% isopropanol,

0.02M Tris-HCl pH 8.0 containing 0.05M NaCl.

Method

The DEAE affi-gel blue column is washed with the acetic-acid buffer then equilibriated in the tris-HCl buffer. One ml of ascites fluid was mixed with 1ml of tris-HCl buffer and added to the column. The column was washed with 5ml of 0.02M Tris-HCl, then the desired material eluted with 5ml Tris-HCl buffer containing 0.05M NaCl.

2.4.3 Cation ion-exchange chromatography

Cation ion exchange was used to enrich acidic proteins from total cell lysates.

Stock solutions

SP-Sephadex (Sigma, Poole),

Running buffer- 25mM sodium acetate pH 4.5 with 50mM sodium chloride and 2% CHAPS(Sigma, Poole).

Method

The SP-Sephadex was swollen in an excess of the running buffer by placing in a steamer for 2 hours. Ten ml was then added to a Pharmacia cl-10 column and the column attached to a peristaltic pump. The column was washed with 26ml of the running buffer. 0.2ml-1.0ml CHAPS cell lysate was mixed with an equal amount of running buffer and added to the column. Thirteen ml of running buffer was added to the column. The desired acidic protein should elute from the column in the void volume. one ml fractions were collected and a sample from each investigated by SDS PAGE to determine which fractions contained the protein of interest. Only the acidic proteins should pass through the column

2.4.4 Chromatography on Octyl-Sepharose

This method (adapted by M.Ferguson, personal communication) was used to purify the antigen from the other proteins in the organic extraction of *T.congolense* procyclic cells.

Stock Solutions

Octyl-Sepharose (Pharmacia),

Running buffer:-5% propan-1-ol in 0.1M ammonium acetate pH 5.0,

Elution buffer:-35% propan-1-ol in 0.1M ammonium acetate pH 5.0.

Method

Redissolve pellet, obtained from the organic extraction of 2.5 x 10^9procyclic cells, into the running buffer. Ammonium acetate is for salting onto column, propan-1-ol breaks miscelles. Mix with 1ml octyl-sepharose and leave for 30 minutes. Place mixture into column and wash with 5mls of running buffer then elute protein by washing column with 8mls elution buffer, Collect 1ml fractions. Determine which fraction contains protein by taking spectromotometer readings at 280nm.

2.4.5 H.P.L.C

H.P.L.C. was carried out in the Biochemistry Department of Glasgow University

2.4.6 F.P.L.C.

A Mono Q HR5/5 column was obtained from Pharmacia

Stock Solutions

Buffer A:-Tris-HCl 25mM, pH7.5 with 2% CHAPS,

Buffer B:- Buffer A with 1M NaCl added

Method

The column was run on Millipore Waters System

A sample of CHAPS lysate was loaded onto the column at 0.5ml/minute. Proteins were eluted with a linear gradient of buffer B. Fractions containing protein were monitered by simultaneously taken spectrophotometer readings at 280nm.

2.5. METHODS USED TO PREPARE LYSATES OF *TRYPANOSOMA CONGOLENSE* PROCYCLIC CELLS

2.5.1 NP40 Method

Buffers

Phosphate Buffered Saline (PBS): Mix 0.1M NaH_2PO_4 with 0.1M Na_2HPO_4 until pH 8 is obtained,

Wash buffer: PBS with 1% glucose added,

Lysis buffer PBS with 1% NP40 and 1mM PMSF (Sigma, Poole) added.

Method

Spun down $1x10^9$-$3x10^9$procyclic cells at 600g for 5 minutes, washed twice in wash buffer to remove serum proteins. Resuspended pellet by vortexing or flicking tube, added lysis buffer to a volume of $2x10^9$ cells/ml. Left on ice for 30 minutes. Microfuge for 5 minutes, keep supernatant, re-extracted pellet as above. Pooled both supernatants and stored at -20°C.

2.5.2 CHAPS method (16)

Buffers

Wash buffer: 10mM Hepes pH 7.4 (Gibco, Paisley), 0.9% NaCl, 1% glucose.

Lysis buffer 2% CHAPS (Sigma, Poole) was dissolved in distilled water containing the protein inhibitors leupeptin 5ug/ml and 0.1mM TLCK (Sigma, Poole)

Method

Spun down $1x10^9$-$4x10^9$ procyclic cells at 600g for 10 minutes, then washed twice in ice-cold wash buffer. The pellet was resuspended in distilled water containing protein inhibitors at $1x10^9$procyclic cells/ml. The procyclic cells were lysed by two incubations at 37°C, separated by rapid freezing on

dry ice/methanol. A membrane pellet was prepared by centrifugation at 800g for 5 minutes and washed twice with distilled water containing protein inhibitors, before extraction with lysis buffer for 15 minutes at room temperature. Insoluble material was removed by centrifugation and the supernatant stored at -20°C.

2.5.3 Organic extraction method

Stock Solutions

Wash buffer:-50mM $NaPO_4$ with 0.17M NaCl pH 7.3,

Chloroform/methanol/water in the ratio 4:8:3,

butanol saturated water.

Method

Wash 1.5 x 10^9 cells twice in the wash buffer, then resuspend pellet in 20 volumes of the $Chcl_3/Ch_3OH/H_2O$ solution and microfuge. Mix the pellet with 3mls of butanol saturated water and leave stirring overnight. Microfuge, discard pellet and dry down supernatent in a speedvac. The material is now ready to be fractionated on an Octyl-Sepharose column.

2.6 WESTERN BLOTTING FROM SDS PAGE

Western blotting was carried out according to the method of Towbin (47) with a Bio-rad blotting apparatus.

2.6.1 Chemicals and Buffers

Nitrocellulose

BEB:- 25mM tris with 192mM glycine and 20% methanol

2.6.2. Method

At the end of electrophoresis disassembled the gel from the apparatus and soaked in BEB for 5 minutes. Assemble the blotting sandwich in a container filled with BEB. The blotting sandwich comprised of the perforated perspex support, 2 pieces of 3MM filter paper, the gel and a sheet of nitrocellulose. All components were soaked in BEB and all air bubbles removed, particularly between the gel and the nitrocellulose. The sandwich was then placed in the blotting tank which already contained BEB,
A current of 0.25A was applied for one hour. At the end of the run diassembled the sandwich, and processed the nitrocellulose as required. The gel can be stained to assess efficiency of transfer.

2.7. DETECTION OF PROTEINS ON NITROCELLULOSE MEMBRANE

2.7.1 Staining for total protein

2.7.1.1 Amido-black

Materials

Stain

1% Amido-black (Naphthol blue-black) dissolved in 90% ethanol, 10% acetic-acid.

Destain

90% ethanol, 2% acetic-acid

Method

Placed membrane in stain for 10 minutes, then placed in destain until protein bands can be differentiated from the

background.

Amido-black is not very sensitive

2.7.1.2 Ponceau S

Stain

Ponceau S (Sigma, Poole)

Destain

TBS-Tween (See immuno-blotting protocol)

Method

Place membrane before blocking, into Ponceau S solution for 10 minutes. Destain until protein bands can be differentiated from the background. Ponceau S staining fades completeley if membrane is left for one hour in TBS-Tween.

2.7.2 Staining the membrane for specific proteins

2.7.2.1. Chloro-naphtol method

Immuno-blotting was carried out according to the method of Towbin (47)

Chemicals

Primary antibody - GUGM 2.2 characterised by A.Lainson,

Secondary antibody - Horseradish peroxidase labelled sheep anti-mouse antibody (SAPU),

Streptavidin peroxidase (Sigma, Poole),

chloro-naphthol (Sigma, Poole),

Hydrogen peroxide (Sigma, Poole),

Marvel.

E.C.L. detection system (Amersham).

Stock solutions

TBS - 10mM tris HCl pH 7.4, 0.14M NaCl

TBS-tween - TBS with 1% tween 20 (Sigma, Poole) added

HST - 10mM tris-HCl pH 7.4, 1M NaCl, 5% tween 20

Developer solution: 0.018g chloro-naphthol dissolved in 6ml methanol,
94ml TBS,
0.025ml hydrogen peroxidase.

Method

After blotting, placed nitrocelluse in TBS-tween containing 3% marvel for 1 hour to block non-specific binding of proteins on membrane. Diluted primary antibody 1/100 in HST buffer. Incubated nitrocellulose in a sealed plastic bag with primary antibody for 1 hour. Washed nitrocellulose 3X5 minutes in TBS-tween, 5 minutes in HST, then 3X5 minutes in TBS-tween. Diluted secondary antibody 1/100 in HST buffer. Incubated nitrocellulose in a sealed bag with secondary antibody for 1 hour. Washed nitrocellulose 3X5 minutes in TBS-tween, 10 minutes in HST, 3X5 minutes in TBS-tween and finally 5 minutes in TBS. The nitrocellulose was then placed in freshly prepared developer solution, bands should appear within 5 - 15 minutes. The reaction was st opped by washing the nitrocellulose in several changes of distilled water. The nitrocellulose should be kept protected from direct light to prevent fading of bands and discolouration of the membrane.

2.7.2.2 E.C.L. Western blotting detection system

Adapted from the protocol suggested by Amersham

Method

After blotting, place nitrocellulose into TBS-tween containing 5% marvel and 10% horse serum for 1 hour to block non-specific binding of proteins. Antibodies can be diluted to a greater extent than with the chloro-naphthol method, first antiboby is diluted 1:200, second HRP labelled antibody is diluted 1:2000

2.8 REMOVAL OF CARBOHYDRATE FROM GLYCOPROTEINS BOUND TO NITROCELLULOSE MEMBRANE

2.8.1 STOCK SOLUTIONS

Dissolve 0.01M sodium metaperiodate(Sigma, Poole) in

0.02M NaAc pH 5.4,

0.005M NaN_3,

0.05M NaCl.

0.15M NaCl, 20mMtris-HCl pH 7.2.

0.2M glycerol dissolved in above buffer.

METHOD

Placed nitrocellulose in sodium meta periodate solution for 1 hour. Then washed blot 3x in distilled water. The nitrocellulose is then placed in the glycerol solution for 20 minutes before rinsing 3x in 0.15M NaCl, 20mM-HCl pH 7.2. The nitrocellulose was then immunoblotted in the normal manner.

2.9. ENZYMATIC DIGESTION OF THE PROTEIN

Stock solutions

0.5% NH_4Bicarbonate pH8.5,

0.1%PBS pH8.0,

Trypsin, chymotrypsin and protease xviiB (Sigma, Poole).

Method

Trypsin experiments were carried out in the Ammonium bicarbonate buffer whilst the chymotrypsin and protease xviiB were carried out in the PBS buffer. Each reaction contained approximately 10ug of protein and 1 unit of enzyme. After addition of the enzyme the reaction is incubated at 37°C for 5 hours, more enzyme was added then the reaction incubated at 37°C overnight.

2.10 ENDOGLYCOSIDASE TREATMENT

Enzymes used

Endoglycosidase H 200u (Boehringer Mannheim)

Endoglycosidase F 120u(Boehringer Mannheim)

Method

Reactions were carried out in 10-50mM Sodium citrate. Approximately 10ug of protein were dissolved in 100ul of buffer,either 4ul of endoglycosidase H or 2u l of endoglycosidase F was added. The reactions were incubated at 37° C overnight.

2.11 AMINO-ACID ANALYSIS

Amino-acid analysis was carried out by the Molecular Paleontology group, Geology Department, Glasgow University.

CHAPTER 3

RESULTS

3.1. INITIAL IDENTIFICATION OF THE ANTIGEN

Immunogold labelled monoclonal antibody GUGM 2.2 recognises an epitope on the surface of *T.congolense* procyclic cells (Fig 1A). The negative control for this experiment, involving repeating the labelling without using first antibody, gave no binding. When the same mab was applied as a probe to a Western blot of a CHAPS lysate from *T.congolense* procyclic cells fractionated on SDS PAGE, an antigen was detected as a diffuse band in the size range 29-40kD (Fig 1B)

Purification and characterisation of this antigen are described in the ensuing sections.

Similar results were obtained with both the 1/148 and the TREU 1627 strains.

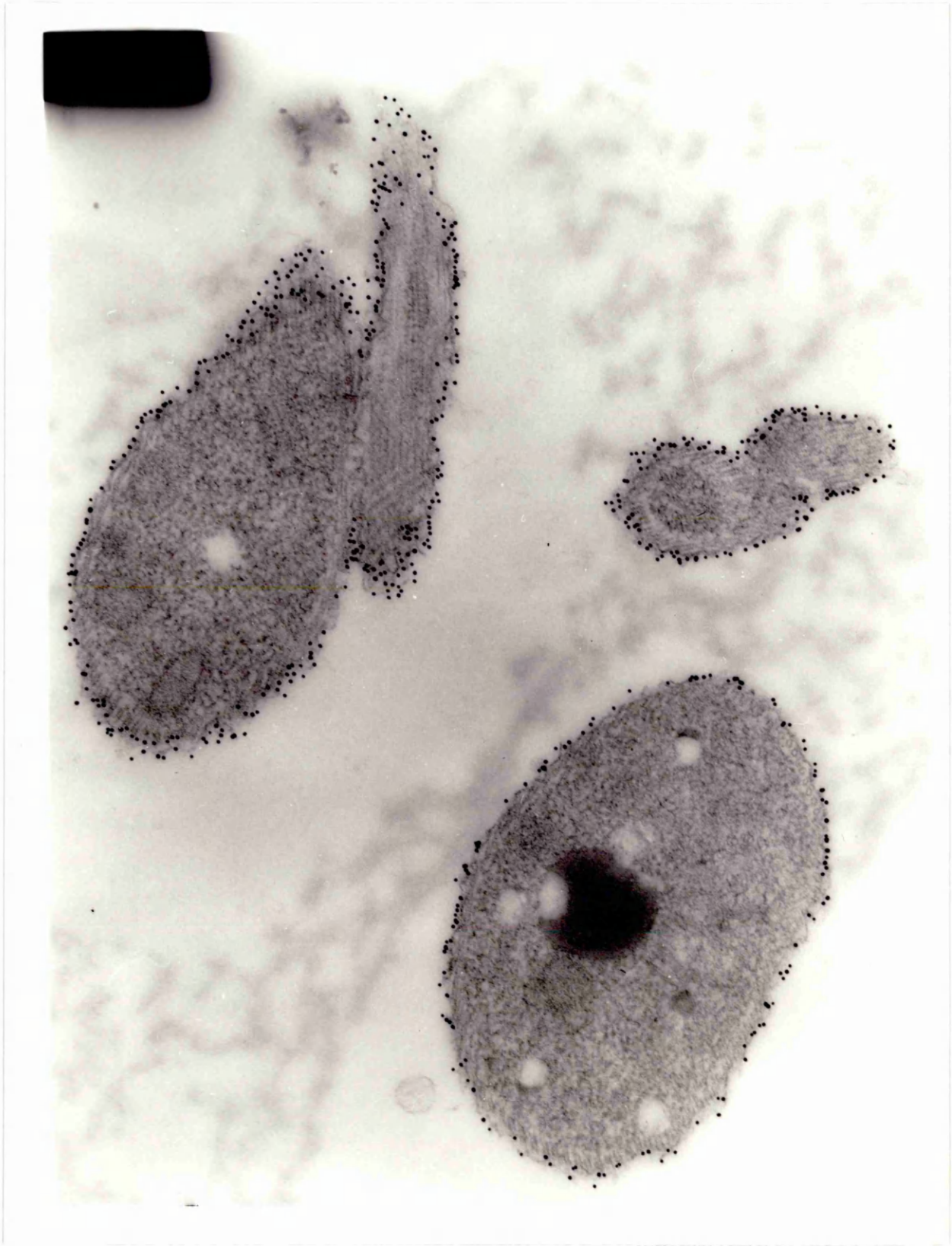


FIG.1.A: *T.congolense* procyclic cells probed with immunogold labelled GUGM 2.2. Magnification 18,000

(Electronmicroscopy by L.Tetley, Glasgow University).

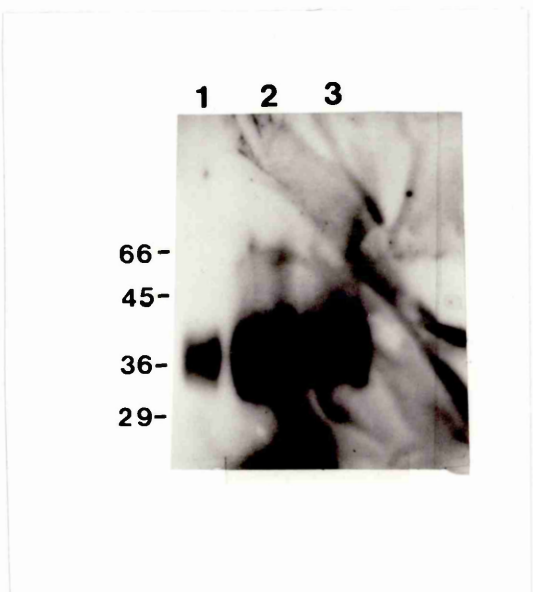


FIG.1.B: GUGM 2.2 probed onto a Western blot from an SDS PAGE
Molecular weight markers (kD) are shown.
lane 1- CHAPS lysate of *T.congolense* procyclic cells
lane 2- FPLC material
lane 3- HICE material

3.2 PURIFICATION APPROACHES

The first approach to isolate the antigen was to make detergent lysates from procyclic *T.congolense* cell cultures. Two detergents were used, NP40,a non-ionic detergent, and CHAPS,a zwitterionic detergent. Fig.2A shows the product of the two detergent lysate methods fractionated on SDS PAGE and stained with Coomassie Blue, Fig.2B and 2C are the same gel stained with STAINS-ALL. As described in section 5, the antigen does not stain with Coomassie Blue but is detected with STAINS-All The two staining methods show that although more proteins are detectable in the NP-40 lysate, the antigen is enriched by the CHAPS method. These gels also show the CHAPS lysate method can be used as a first step in the purification of the antigen from the other membrane proteins.

Two purification methods were attempted. One utilized the CHAPS lysate material whilst the other involved organic solvent extraction from whole procyclic cells.

CHAPS lysates were passed over an anionic FPLC column. Fig.2C track 1 shows the eluate from the column fractionated on an SDS PAGE gel then stained with STAINS-ALL. CHAPS lysates were also fractionated on TRITON PAGE and the relevant portion electro-eluted (Fig.2D).

The second purification method involves the use of organic solvents to extract the antigen from whole procyclic cells, then further purification by hydrophobic interaction chromatography (HIC) by passing the material over an octyl sepharose column.

The eluate from HIC was then fractionated on SDS PAGE and stained with STAINS-All (gel not shown). This approach to purification gave the same yield of antigen as the FPLC anionic column, but in terms of purity was more successful.

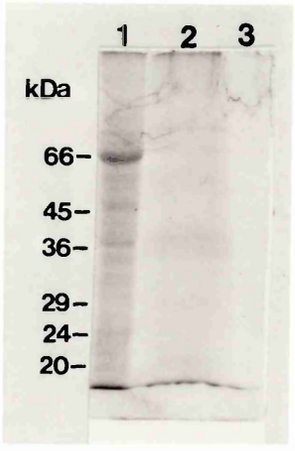


FIG 2.A. SDS PAGE stained with Coomassie blue.

lane 1- NP40 lysate

lane 2- CHAPS lysate

lane 3- FPLC

Molecular weight markers (kD) are shown

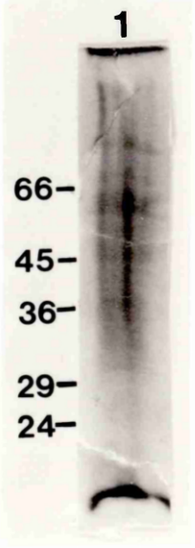


FIG 2.B. SDS PAGE stained with STAINS-ALL.

lane 1- NP40 lysate

Molecular weight markers (kD) are shown

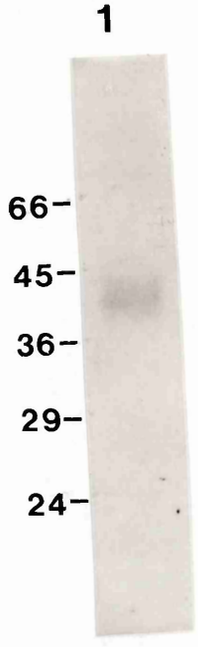


FIG 2.C. SDS PAGE stained with STAINS-ALL

lane 1- FPLC material

Molecular weight markers (kD) are shown

FIG 2.D. Triton PAGE stained with STAINS-ALL

lane 1- CHAPS lysate

3.3 AMINO-ACID ANALYSIS

Amino-acid composition analyses were performed of material obtained by electroelution of the appropriate region of an SDS PAGE gel separation of a CHAPS lysate from *T.congolense* procyclic cells, and of HICE purified material (Table 1). The antigen prepared by the different approaches give similar results: the antigen has 13% alanine residues and is 22-29% acidic. This correlates with its acidic pattern of staining with STAINS-ALL. The amino-acid composition is also similar to that of a protein purified in a similar way by T.Pearson,(personal communication). The major difference between the two proteins is the percentage of alanine residues. The protein purified by Pearson *et al* is alanine rich whereas the protein analyzed in this study is consistently only 13% alanine. One reason for this difference could be the grade of reagents used. All reagents used were Analar grade, and contaminants in them may have affected the amino-acid composition analysis. When the purification is repeated HPLC grade reagents will be used to determine whether this is the case. A second reason could be that the protein is contaminated with another protein.

Fig. 3

		PROTEIN pmol	%		PROTEIN pmol	%		DNA pmol	%		UPPER PROTEIN Pearson pmol	%		LOWER PROTEIN Pearson pmol	%	
Lys	K	307	6		361	7		16	6		55	4		414	11	
Arg	R	259	5		255	5		16	6		72	6		116	3	
His	H	74	2		68	1		4	2		13	1		12	0	
				13			13			15			11				14
Asp	D	560	11		742	14		20	8		119	9		500	13	
Glu	E	538	11		830	15		23	9		133	10		506	14	
				22			29			17			19				27
Gly	G	512	10		436	8		14	6		271	21		319	9	
Ser	S	298	6		428	8		18	7		93	7		228	6	
Thr	T	287	6		332	6		18	7		61	5		207	6	
Tyr	Y	124	3		107	2		0	0		12	1		17	0	
Cys	C	9	0		129	2		6	2		0	0			0	
				25			26			23			34				21
Ala	A	667	14		602	11		55	22		235	18		649	17	
Leu	L	369	8		309	6		23	9		75	6		211	6	
Val	V	260	5		248	5		20	8		84	6		211	6	
Pro	P	222	5		188	3		3	1		26	2		167	4	
Phe	F	170	3		125	2		3	1		21	2		34	1	
Ileu	I	168	3		144	3		4	2		19	1		141	4	
Met	M	75	2		99	2		5	2		9	1		8	0	
				39			32			46			36				38
pmol		4900			5404			248			1298			3740		

ALL NUMBERS HAVE BEEN ROUNDED UP.

3.4 EPITOPES OF ANTIGEN

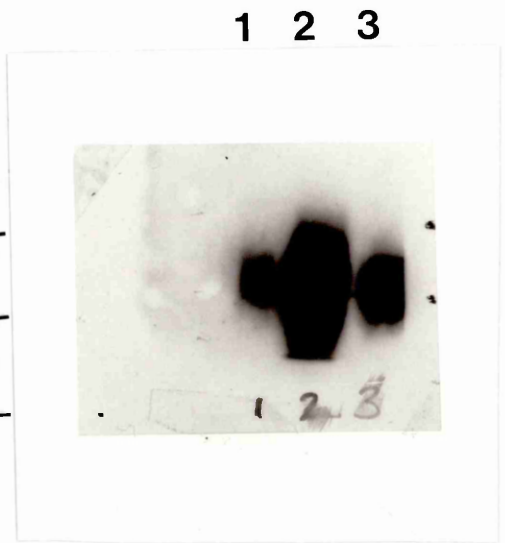
In Western blots of SDS PAGE fractionations of CHAPS lysate material, anionic FPLC eluate and HIC (Fig 4A) eluate, the monoclonal antibody GUGM 2.2 detects a diffuse band of around 29-40kD. The two tracks containing the column eluates contain more antigen than does the lysate track.

The western blot was also probed with antiserum ("anti-p4pGEX") raised against a protein expressed in *Escherichia coli* from a vector containing a translational fusion between a *T.congolense* procyclic specific cDNA and a gene encoding glutathione transferase. Anti-p4pGEX detects a band at 29kD and a diffuse band at 36-45kD (Fig4B). Fig 4B is the same immunoblot used in the periodate experiments described in section 3.6. but the steps carried out in the periodate control experiments do not affect the results seen in this section.

Controls of the immunoblots were carried out, using as probes second antibody alone, and a monoclonal antibody directed against an antigen in *Theileria* were negative.

Both GUGM 2.2 and anti-p4pGEX recognise epitopes of similar size but it can not be concluded at this stage whether thay are identifying the same protein. GUGM2.2 could be directed against several co-migrating proteins with similar carbohydrate residues, instead of one molecule with microheterogeneity of glycosylation whilst p4pGEX could be directed against one of the co-migrating proteins

FIG 4.A. Western blot of SDS PAGE probed with GUGM 2.2



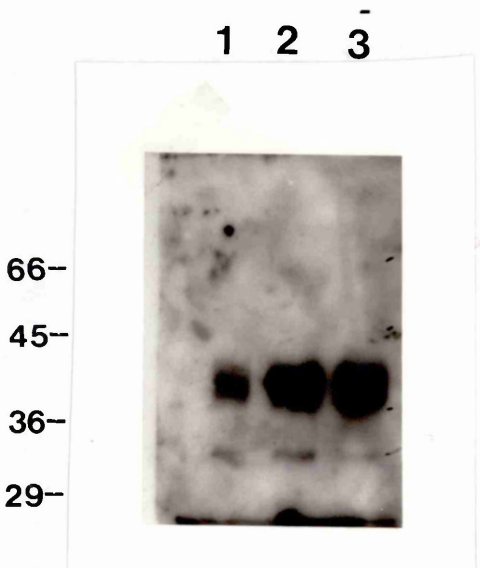
lane 1- Chaps lysate

lane 2- FPLC material

lane 3- HICE material

Molecular weight markers (kD) are shown.

FIG 4.B. Western blot of SDS PAGE probed with anti-p4pGEX



lane 1- CHAPS lysate

lane 2- FPLC material

lane 3- HICE material

Molecular weight markers (kD) are shown.

3.5. ABNORMAL STAINING OF ANTIGEN

Several conventional stains were applied to gels in which the antigen had been fractionated by PAGE, whether in whole cells CHAPS lysates (Fig.5,track 1) or anionic FPLC eluate (Fig.5, track 2). The protein detected with GUGM2.2 did not stain with Coomassie blue (Fig.5A) or with Silver stain (Fig.5B). With STAINS-ALL (Fig.5C) a region of the gel in the size range 29-40kD stained blue whilst the other proteins stained pink. A blue staining reaction indicates acidity, whilst pink indicates neutrality or basicity. When the carbohydrate specific Schiff's stain was applied to the gel a positive reaction was detected, in the corresponding region (Fig 5D).

The indication from these gels is that the 29-40kD antigen does not stain with Coomassie blue or Silver stain. The staining pattern with the STAINS-ALL suggests it is acidic and the positive result with the Schiff's stain means it contains carbohydrate.

It is not possible to distinguish at this stage whether the diffuse band seen on the gels and on the Western blots is one protein with varying degrees of glycosylation, or whether other molecules are being co-purified with the antigen.

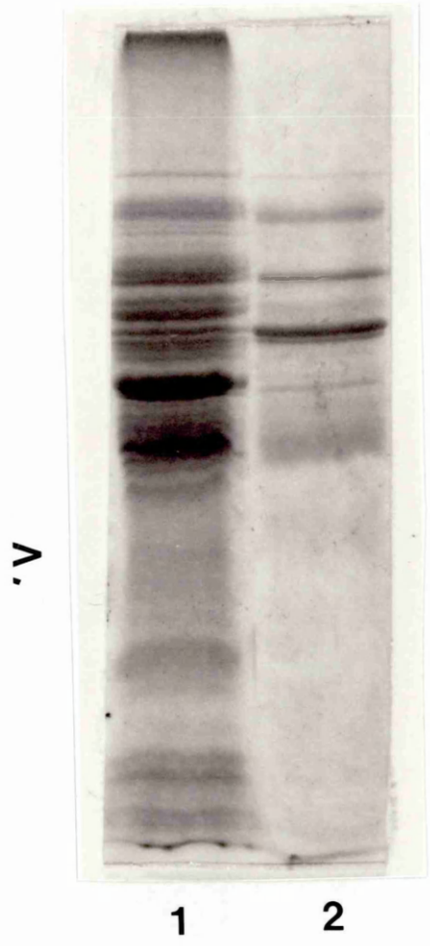


FIG 5.A. SDS PAGE stained with Comassie blue

lane 1- CHAPS lysate

lane 2- FPLC material

The arrow indicates the region of the gel where the antigen is expected to run to.

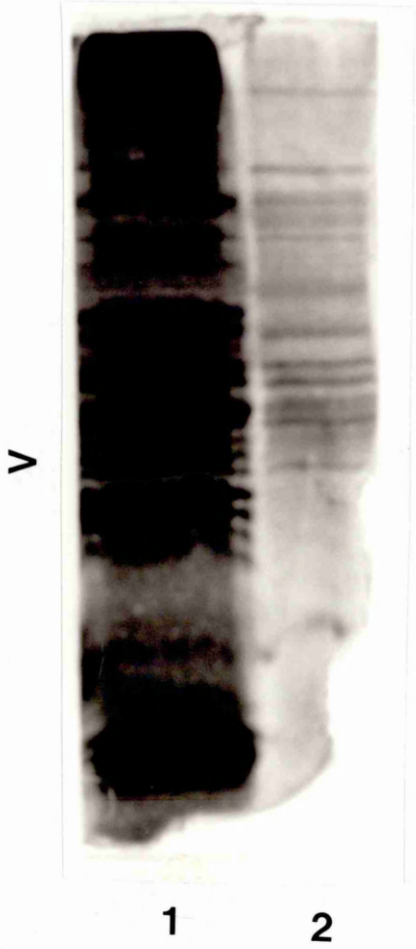


FIG 5.B. SDS PAGE stained with Silver stain

lane 1- CHAPS lysate

lane 2- FPLC material

The arrow indicates the region on the gel where the antigen is expected to run to.

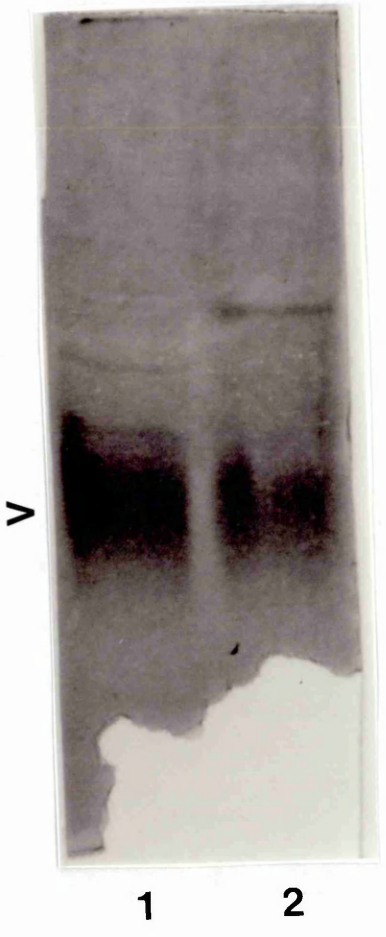


FIG 5.C. SDS PAGE stained with STAINS-ALL

lane 1- CHAPS lysate

lane 2- FPLC material

The arrow indicates the position of the antigen.

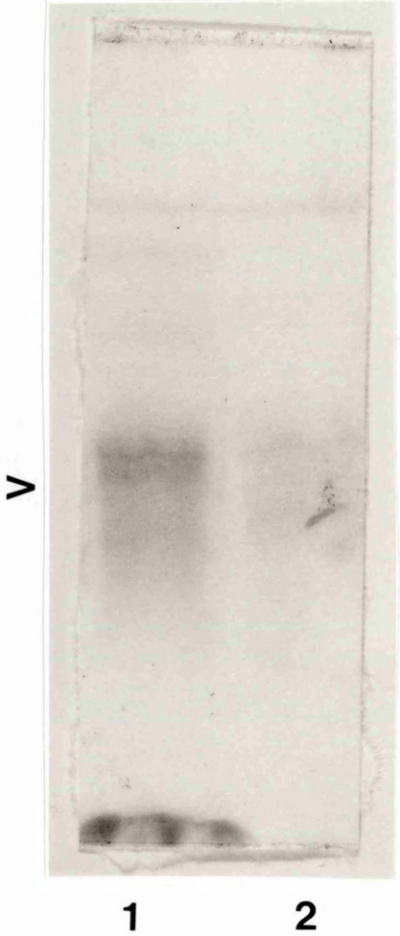


FIG 5.D. SDS PAGE stained with Schiff's stain

lane 1- CHAPS lysate

lane 2- FPLC material

The arrow indicates the position of the antigen.

3.6 CARBOHYDRATE ANALYSIS

3.6.1. Lectin binding

To obtain more information as to which carbohydrate groups may be present on the 29-40kDa protein, CHAPS lysate, anionic FPLC eluate and HIC eluate were fractionated on SDS PAGE, Western blotted and then probed with concanavalin A (ConA). Con A binds to components in the CHAPS lysate track but not in the region 29-40kDa. As would be expected no binding is seen in the tracks containing anionic FPLC eluate or HIC eluate

Since the probing of Con A blots produced negative results, it can be concluded that the protein does not contain the monosaccharides α-D-mannose or α-D-glucose, for which Con A has an affinity accessible on its surface. Control experiments were attempted using methyl-mannoside as a competitor sugar. Unfortunately the positive controls in this experiment were negative so it has not been included.

3.6.2. Deglycosylation by periodate treatment

CHAPS lysates (track 1), anionic FPLC eluate (track 2), and HIC eluate (track 3) were fractionated in duplicate by SDS PAGE and Western blotted. One blot was treated with periodate which reduces carbohydrate residues to dialdehydes, while the other was used as an untreated control. The two blots were initially probed with GUGM 2.2. In the periodate treated blot

(Fig.6A) GUGM 2.2 binding is totally absent in all three tracks. In the corresponding control gel(Fig.6B) the expected binding to the 29-40kDa region occurred.

To check that periodate treatment had not been too harsh and eliminated protein reactivity the two blots were stripped of the monoclonal probe and re-probed with anti-p4pGEX. A positive signal in both blots (Fig.6C and 6D) shows that the protein did not lose reactivity as a result of periodate treatment.

This experiment suggests that GUGM 2.2 is directed against a carbohydrate determinant on the protein and further, may explain why a diffuse band is always seen when the antigen is run on SDS PAGE: presence of carbohydrate chains can inhibit migration and microheterogeneity in the chains can lead to different degrees of inhibition.

3.6.3. Endoglycosidase treatment

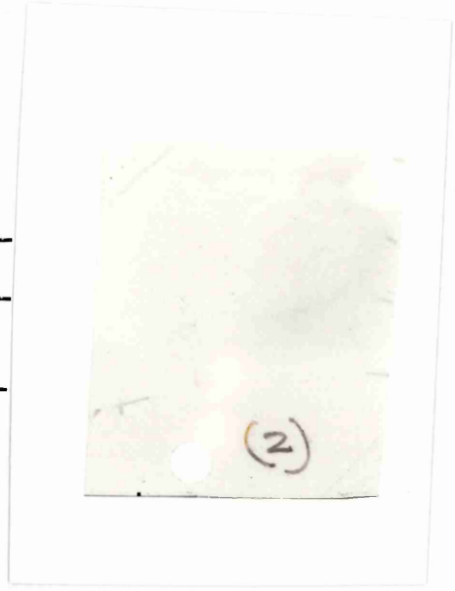
Endoglycosidases act on glycoproteins by releasing oligosaccharides from the conjugated glycans. Some endoglycosidases which specifically remove N-linked glycan chains are divided into two types: i)those which cleave peptide-glycan linkages removing the glycan totally and ii)those that cleave glycosidic bonds, liberating only part of the glycan.

The effects of treatment on the 29-40kD protein with both type

was examined. Endo H is specific for oligomannosidic and hybrid type glycans. Endo F is active on glycans of both the N-acetyllactosaminic and oligomannosidic types, but not glycans of the hybrid type.

Endo H and F were incubated separately with CHAPS lysate and HIC eluate and the products were then fractionated on SDS PAGE and susequently stained with STAINS-ALL. If the endoglycosidases had digested effectively, a change in mobility of the protein and/or a compaction of the diffuse band would have been seen. There was no alternation of the protein's mobility on these gels. Either glycosylation is O-linked or the glycans are inaccessible to the enzymes.

FIG 6.A. Western blot of SDS PAGE treated with periodate, then probed with GUGM 2.2.



lane 1- CHAPS lysate

lane 2- FPLC material

lane 3- HICE material

Molecular weight markers (kD) are shown

FIG 6.B. Western blot of SDS PAGE, not treated with periodate, then probed with GUGM 2.2.

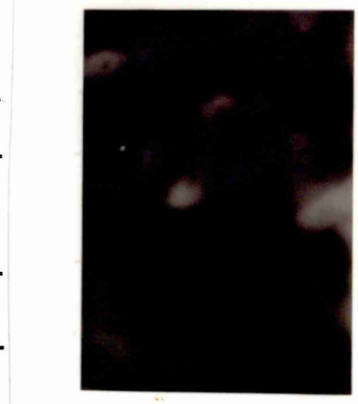
lane 1- CHAPS lysate

lane 2- FPLC material

lane 3- HICE material

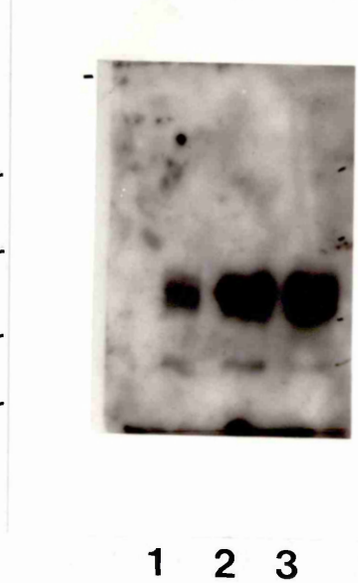
Molecular weight markers (kD) are shown

FIG 6.C. Nitrocellulose filters used in 6.A. treated to remove any antibody binding and reprobed with antip4pGEX



lane 1- CHAPS lysate
lane 2- FPLC material
lane 3- HICE material
Molecular weight markers (kD) are shown

FIG 6.D. Nitrocellulose filters treated to remove any antibody binding and reprobed with anti-p4pGEX



lane 1- CHAPS lysate
lane 2- FPLC material
lane 3 HICE material
Molecular weight markers (kD) are shown

3.7. Proteinase treatment

Anionic FPLC eluate was incubated with a range of proteinases: trypsin, chymotrypsin, V8 and proteinase K. The products of the reaction were fractionated by SDS PAGE then treated with STAINS-ALL (Fig 7A).The mobility and quantity of the 29-40kD antigen were unaffected, suggesting it is resistant to the action of these proteases. In a control experiment pepsin was substituted for the anionic FPLC eluate as a substrate in the reactions (Fig 7B) and complete digestion occurred. The bands detected on the gel, in lane 1 at 36kD is V8, and in lanes 2 and 3 at approximately 14kD are chymotrypsin and trypsin respectively

Anionic FPLC material was also treated with proteinase K under varying conditions, and no effect was seen (Fig 7C). Other proteins which are known to stain with STAINS-ALL, and whose molecular weights are known were included in this experiment. The track containing proteinase K on its own has more protein than the rest of the tracks explaining why it can be detected in these tracks but not in the experimental tracks.

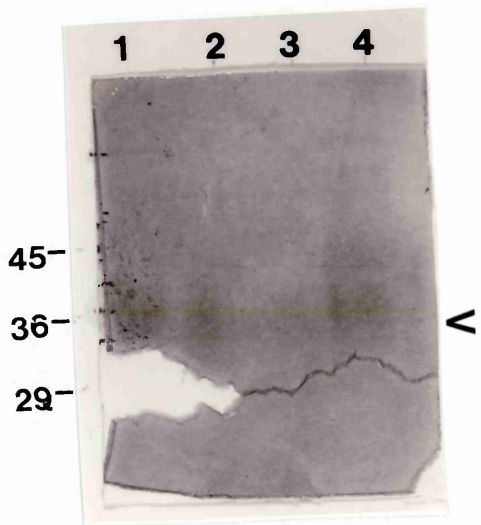


FIG 7.A. SDS PAGE stained with STAINS-ALL

Lane 1- FPLC material treated with V8

Lane 2- FPLC material treated with chymotrypsin

Lane 3- FPLC material treated with trypsin

Lane 4- FPLC material untreated

Molecular weight markers (kD) are shown

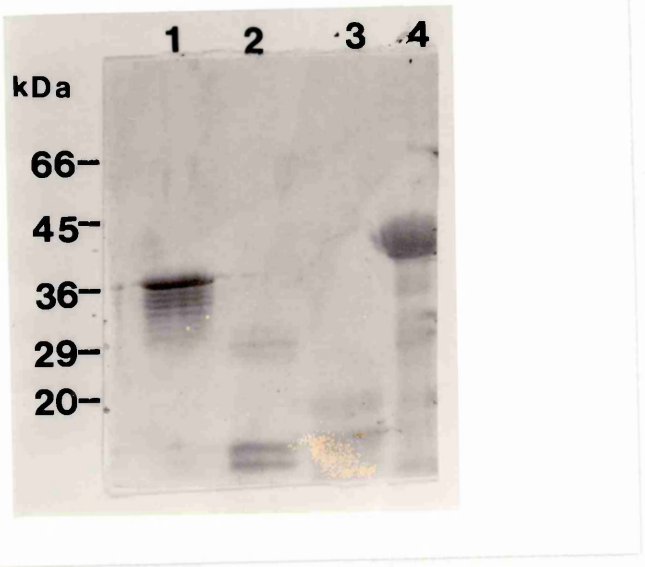


FIG 7.B. SDS PAGE stained with Comassie blue

Lane 1- pepsin treated with V8

Lane 2- pepsin treated with chymotrypsin

Lane 3- pepsin treated with trypsin

Lane 4- pepsin untreated

Molecular weight markers (kD) are shown

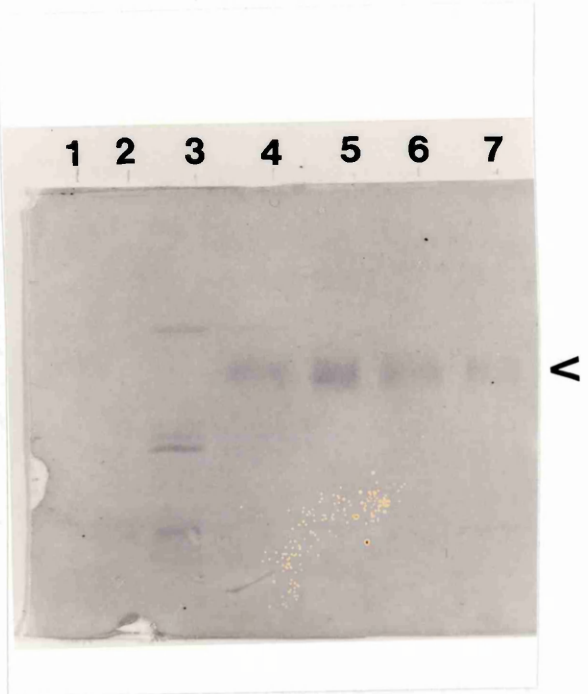


FIG 7.C. SDS PAGE stained with STAINS-ALL

Lane 1- 36kD control treated with proteinase K

lane 2- 29kD control treated with proteinase K

Lane 3- proteinase k

Lane 4- FPLC material treated with proteinase K

Lane 5- FPLC material treated with SDS

Lane 6- FPLC material treated with proteinase k and SDS

Lane 7- FPLC material untreated

The arrow indicates the position of the antigen

CHAPTER 4

DISCUSSION

4.1. Introduction

4.1.1 General introduction

For a large part of its life-cycle *T.congolense* is covered by a dense surface coat composed of the VSG which it uses as the basis of a mechanism to evade the immune responses of its mammalian host (39,41). As the trypanosomes develop to the procyclic stage after entering the tsetse fly, this coat is shed and a set of'common' surface antigens become exposed (15).

A wide aim of the programme of which this study is a part, is to understand the molecular nature of the common surface antigens and their role in the interaction between fly and parasite An aim is to exploit the universality of these antigens to develop a vaccine which would block transmission of the parasite through the tsetse fly.

To achieve these aims it is necessary to identify and characterise the surface molecules.

The surface of *T.brucei* procyclic cells has been extensively studied and one major protein, procyclin, identified, Procyclin is a membrane associated protein which is probably glycosylated (16). Procyclin is specific to the procyclic stage of *T.brucei*. The aim of this work was to identify an abundant surface molecule from *T.congolense* procyclic cells and to compare it with procyclin in order to determine whether they are functionally analogous proteins.

4.1.2. IDENTIFICATION OF A PROTEIN ON THE SURFACE OF THE PROCYCLIC FORM OF *T.congolense*

Monoclonal antibodies were raised against whole *T.congolense* procyclic cells in mice. Procyclic cells can be grown readily in cell culture and by the addition of non-ionic detergent, lysates are made which are enriched in surface proteins. Injection of these proteins into mice elicits antibodies that can be purified and used to identify and characterise the corresponding antigens.

Initial work on the project (F.A. Lainson) had resulted in the production of several monoclonal antibodies (mab). One particular mab, GUGM 2.2. had been shown in immunofluorescence studies to bind to the surface of acetone-fixed smears of *T.congolense* procyclic cells. When immuno-blots were performed using NP40 lysates of *T.congolense* as the source of antigen and GUGM 2.2 as the probing antibody, a diffuse band was detected ranging in size from 29kD-40kD. Combining these data, it was decided that this molecule would be a suitable candidate to attempt to purify and subsequently characterize.

4.1.3. PURIFICATION OF THE SURFACE PROTEIN IDENTIFIED BY GUGM 2.2

The first purification method attempted was immunoprecipitation. *T.congolense* procyclic cells were either directly iodinated or biotinylated. Lysates were prepared, and used in an immunoprecipitation with GUGM 2.2, which should have precipitated the antigen for which it is specific.
This method was not successful as only non-specific binding

was seen.

The next method attempted was affinity chromatography, using as matrix GUGM 2.2 bound to protein A sepharose. *T.congolense* procyclic cell lysates were passed over the column, and any antigen binding specifically to GUGM 2.2 would be eluted by competition with a high concentration of salt. This was slightly more successful in that eluted fractions, when examined by SDS PAGE subsequently stained with Coomassie blue, contained a faint band in the 29kD region, or when Western blotted and probed with GUGM 2.2 revealed a strong band in the same region.

When the initial attempts at purification were carried out the only detection method attempted was SDS PAGE, subsequently stained with either Coomassie blue or the silver stain. As the project developed another detection method STAINS-ALL was employed. STAINS-ALL had been used by Clayton and Mowatt (16) to stain procyclin. Another method to prepare procyclic cell lysates was attempted, again this had been developed to increase the yields of procyclin extracted from *T.brucei* procyclic cells. The method will be discussed in greater detail below. The immunoprecipitation and the antibody bound affinity chromatography experiments were repeated using the improved lysate protocol and detection systems. The yields obtained, assessed by the amount of staining with STAINS-ALL, were not increased, so these approaches were therefore unsuccessful in producing enough material for subsequent experiments.

As seen with later purifications, these methods were

inefficient, with most of the antigen being lost. One possibility could be that the antigen might be complexed in some way or insoluble during the preparation of the lysate, making it inaccessible to the GUGM 2.2 antibody.

The strategy of Clayton and Mowatt (16) involved incorporating a freeze-thaw step into the lysate method procedure and also used the detergent CHAPS instead of NP40. This method enriches the resultant lysate for membrane proteins. The CHAPS lysate is passed over an ion-exchange chromatography column and the flow-through fractions collected, substantially enriching for procyclin. When this adapted protocol was applied to *T.congolense* procyclics and analyzed by SDS PAGE and Coomassie blue staining the prevalent species was a protein in the size range 29kD-40kD. Judging by the degree of staining on Coomassie stained SDS PAGE, the antigen does not appear to be present in a large amount. As previously mentioned procyclin, for which the purification method was developed, does not stain with Coomassie blue but can be detected with STAINS-ALL, which was therefore employed for detection. When these SDS PAGE gels were analyzed, the amount of antigen present now correlates more closely with the results obtained with the immunofluorescence studies.

STAINS-ALL (41) stains DNA, RNA and protein in gels, but more importantly it differentially stains acidic proteins blue and non-acidic proteins pink. Thus, STAINS-ALL helps monitor protein contamination in any subsequent purification steps.

CHAPS lysates run on SDS PAGE subsequently stained with

STAINS-ALL detected a large amount of blue staining protein in the region 29-40kd. Thus, it can be concluded from these gels that the protein purified over the cationic exchange chromatography column is probably the same protein detected by GUGM 2.2. Also in agreement with its exclusion from the column it is acidic. The only method available to quantitate the amounts of protein present is to compare it to a known standard. Differing known amounts of procyclin fractionated on SDS PAGE subsequently visualised with STAINS-ALL were used. However it must be borne in mind that procyclin may be more or less sensitive than the *T.congolense* protein to STAINS-ALL

One problem with using STAINS-ALL to detect the antigen in SDS PAGE is that SDS inhibits its ability to penetrate the gels (41). Although it is able to detect the *T.congolense* antigen in SDS gels, its sensitivity may be impaired. Since the abundance of the antigen was indeterminate, it was important to use the most sensitive staining method available.

The problem of the incompatibility of SDS and STAINS-ALL was solved by using a PAGE system developed by Ziegelbauer *et al* (18), in which Triton X100 rather than SDS is incorporated into the gel. As Triton X100 is non-ionic, proteins migrate according to intrinsic charge, with acidic proteins migrating faster towards the cathode. The drawbacks are that a mixture of acidic proteins will co-migrate and no estimate of size can be made. However, because the 29-40kD range was the only region of the SDS gels to stain detectably with STAINS-ALL the reasonable assumption was made that this protein was the major component

detected by STAINS-ALL and a combination of the Triton and SDS PAGE analysis would be useful to help monitor purification. Indeed when CHAPS lysates were fractionated by Triton PAGE, an intense blue, broad band was detected migrating ahead of a similar pink band.

4.2 ATTEMPTS TO PURIFY THE *T.congolense* PROTEIN FROM THE MIXTURE PRESENT IN THE TOTAL LYSATE .

4.2.1.Electroelution of protein by PAGE.

One advantage of using Triton X100 PAGE is that the majority of proteins are unable to migrate far into the gel matrix, so this property was exploited in attempted purification of the protein.

CHAPS lysates of *T.congolense* procyclic cells were applied to preparative Triton X100 gels. Electro-elutions from the Triton X100 gels gave an 80% yield of the initial amount of protein applied to the gel, assessed by running a small amount of the recovered protein on another Triton X100 PAGE, but as anticipated the band excised from the gel, when analysed by SDS PAGE and Coomassie blue staining was found to contain more than one protein species, thus complicating subsequent analyses.

Electro-elution of protein from SDS PAGE was also attempted. This was successful though the yield, determined by running a sample of the electro-eluted material on a Triton X100 PAGE, was less than that obtained with the Triton system.

There was time to perform this type of electro-elution only twice, so a proper evaluation was not possible.

4.2.2 Ion-exchange chromatography

Clayton and Mowatt (16) had used a cation exchange method in conjunction with lectin affinity chromatography to purify procyclin from CHAPS lysates. The *T.brucei* procyclic

lysates were passed over a column containing SP-sephadex, through which acidic proteins should flow, whilst all other proteins should bind. The flow through fractions, which contain procyclin, were further purified by passing over a column containing Con A. In the present study this method was applied to *T.congolense* procyclic lysates, and the flow-through fractions were seen to contain a protein of 29-40kD when analysed on Triton X100 and SDS gels. The fractions containing the desired protein were contaminated with other proteins. If the flow-through fractions were passed over a column containing ConA, then recovery of the 29-40kD protein was minimal. One other minor problem with this technique is that the running buffer is not suitable for amino-acid or protein sequence analysis and further loss of protein occurs in subsequent dialysis.

4.2.3. HPLC and FPLC

Since all the previous methods described did not purify the 29-40kD protein sufficiently or, in the case of electro-elution, in a non-denatured state the more refined HPLC and FPLC were also examined.

CHAPS lysate was passed over a $C18H_2O$ reverse phase hydrophobic column and spectrophotometer readings taken at 220nm of all the eluted fractions. This method was unsuccessful, in that no proteins staining blue with STAINS-ALL could be detected following Triton PAGE analysis of all fractions.

Several factors could have contributed to the failure.

such as interference by the detergent, or irreversible binding of the protein to the column following removal of the detergent. The subsequent demonstration of a fatty acid containing GPI anchor on this protein (T.Pearson, personal communication.), makes this a likely explanation.

FPLC anion exchange chromatography was attempted using a CHAPS lysate of *T.congolense* procyclic cells. The column was washed with a standard Tris-HCl pH7 buffer to which 1M NaCl was added. Chloride ions acted as the counter ion in the elution buffer. The approach was very successful, resulting in the 29-40kD protein being eluted in a pure form in three consecutive fractions. Unfortunately after three runs with this system the column became unusable due to internal blockages. In an attempt to remove the obstruction the column was washed with buffer containing CHAPS detergent. This was successful, and the protocol was modified to include CHAPS detergent in all buffers. If the protein was insoluble without CHAPS being present, resulting in its precipitation within the column, then the modified protocol would prevent this occurrence, producing higher yields. However, this was not the case as the yields obtained with column runs, with or without detergent, were similar.

Of all the purification methods attempted the FPLC technique seemed the most successful. Unfortunately this method was not without its problems: in particular, although the protein is always eluted from the column, it is not always collected in the same fractions, therefore not at identical

salt concentrations each time, thus the protein is behaving in an abnormal manner in this system. In addition, with the Mono-Q-Sepharose system the column becomes blocked very easily, suggesting that CHAPS lysates of procyclic cells may not be ideal starting material for this system. A reverse of this problem was also seen; in some runs of the column all the lysate proteins washing straight through the column, suggesting an equilibration problem. Treating the column in identical ways between runs did not determine success or failure of the subsequent runs. This irrepeatability caused problems as the project proceeded, as a lot of time and material were expended in attempts to standardise the technique. Finally in the latter successful runs of the Mono-Q-Sepharose column, a contaminating protein co-eluted with the desired protein, creating a problem when analysis of the protein was undertaken.

4.2.4. Extraction of the 29-40kD protein using organic solvents

The final method employed to extract the 29-40kD protein *T.congolense* procyclic cells used organic solvents. The method was suggested by M Ferguson (personal communication) who was looking in *T.brucei* for Lipophosphoglycan like molecules as on *Leishmania*, and purified procyclin instead.

T.congolense procyclic cells were treated with organic solvents, as detailed in the Materials and Methods section, with the resultant protein mixture being passed over an octyl-sepharose column. The eluate from this column contained the 29-40kD protein.

This purification protocol proved very successful, The amounts of protein obtained were comparable to the FPLC method

but there were less contaminating proteins present. With this approach the 29-40kD protein was eluted from the column at a specific organic solvent concentration, which resulted in a greater reproducibility than either of the previous methods.

4.3. CHARACTERISATION OF the 29-40kD PROTEIN

In spite of the problems encountered with the purification of the 29-40kD protein in sufficient quantities and of desired purity some analysis was able to be carried out.

4.3.1 Amino-acid analysis

A sample of the protein obtained from the initial FPLC runs was subjected to amino-acid composition analysis and protein sequencing. The amino-acid composition confirmed a high acidic : basic residue composition, and showed the protein is also rich in alanines. This is in agreement with the earlier observation of acidity and the way the protein performed in Triton X100 PAGE and in the purification methods used in its preparation. However it does not agree with the derived cDNA and gene sequences nor with the T.W. Pearson amino-acid sequence.

Protein sequencing analysis was unsuccessful, as the amino-terminal of the protein appeared to be blocked or protected.

4.3.2 Proteinase treatment

Since the protein could not be sequenced in its entirety, it was hoped that by digestion with proteases partial sequence could be obtained. Three enzymes were used, Trypsin, Chymotrypsin and V8. However not one of these digested the protein.For some reason the protein seems either inaccessible or resistant to protease treatment. The fact

that the protein appears to be glycosylated might explain these results which would also explain the failure in the sequencing attempts.

4.3.3 Carbohydrate analysis

Positive staining of the protein in SDS PAGE with Schiff's reagent suggested that carbohydrate is conjugated to the 29-40kD protein. Some crude analysis was attempted in order to try and address some of the inexplicable results obtained earlier. Would the protein behave normally ie. run as a single band on SDS PAGE, or be accessible to enzymatic digestion, when stripped of its carbohydrate residues?

Would the antibody GUGM 2.2 still recognise bands on an immuno-blot of the protein when stripped of carbohydrate ? Is the Glycan N- or O- linked to the protein?
If these questions could be answered, then a hypothesis could be proposed suggesting a function for the protein in the living *T.congolense* procyclic cells.

Immuno-blots of CHAPS lysates of *T.congolense* cells gave positive results when probed with the lectins Concanavalin A and Wheat Germ Agglutinin. Similar initial experiments have been carried out with the 29-40kD protein from the FPLC eluate. Here there was binding to the filter but it was not in the region to where the 29-40kD protein was expected to have migrated. These experiments would have to be repeated before conclusions could be drawn.

The FPLC eluate was treated with endoglycosidases F and H to determine what type of glycan linkage was present on the

protein. Endoglycosidases F and H specifically remove N linked glycans. When the products of these reactions were run on SDS PAGE their mobility was unaltered, suggesting that deglycosylation had not occurred. In comparison with amounts of protein in control tracks, it could be concluded that all the protein treated was detected on the gel.

Another deglycosylation method was attempted, this involving treating Western blots of FPLC eluate with periodate in an attempt to remove some of the carbohydrate residues from the protein. The blots were then probed with GUGM 2.2 to ascertain whether the antibody was still able to recognise the antigen. No binding was obtained and so it can be concluded that the 29-40kD protein is glycosylated and that GUGM 2.2 is directed against a carbohydrate moiety of the protein.

The 29-40kD protein was probed with another antibody, anti-p4pGEX, anti-p4pGEX is an antiserum raised against a translational fusion between a *T. congolense* procyclic cell specific cDNA and a gene encoding glutathione-S-transferase expressed in *E.coli*, Two bands were detected. It is believed that this anti-serum is binding the unglycosylated form of the 29kD-40kD protein, or it is identifying only one of the many peptides that GUGM 2.2 recognises. This will have to be investigated further.

4.3.4 Abnormal behaviour of the 29-40kD Protein during Western blotting

When CHAPS lysate or FPLC eluate were fractionated

on SDS PAGE, and Western blotted in the normal manner a strange phenomenon was observed. Some of the 29-40kD protein passed through one sheet of nitrocellulose membrane and onto a second. If blotting was continued then some of the protein would also pass through the second membrane, although the marker track proteins and any other contaminating bands remained bound to the first membrane. It could not be determined whether this abnormal behaviour is an intrinsic property of the protein or whether it is due to the the protein's solvent. This characteristic of the protein may be exploited in further attempts at its purification. Total CHAPS lysate could be fractionated on SDS PAGE and Western blotted onto two membranes, and the 29-40kD protein would pass straight through to the second membrane whilst the contaminating proteins would be left behind. The protein would then be eluted from the nitrocellulose membrane. This has not been attempted yet.

Other people working in the same field have experienced the same problems in their attempts to purify and subsequently analyse surface proteins from *T.congolense* procyclic cells. T.Pearson (personal communication), using the cationic exchange method of Clayton and Mowatt (16), purified from the surface of the procyclic cells two antigens reactive with the same mabs. These two species, when fractionated on SDS PAGE, produced distinct diffuse bands which stained blue with STAINS-ALL. The proteins' apparent molecular weights were 50-60kD and 22-28kD. If the amino-acid compositions of these two proteins are compared with the 29-40kD protein then all three are found

to be similar (this study, and T.Pearson,personal communication). The major difference between the two molecules purified by Pearson *et al* and the 29-40kDa protein is the percentage of alanine residues. The 29-40kD molecule has 13% whereas the other two have 17%. Pearson *et al* (personal communication) have recently had some success in their attempt to fragment the 50-60kD protein with cyanogen bromide and have obtained some peptide sequence.

Two major peptides were sequenced. One shares 26 of 30 amino-acids predicted from a cDNA clone, selected after differential screening of a *T.congolense* procyclic cell cDNA library in this laboratory (R.Bayne, personal communication). Antisera raised against a translational fusion between the cDNA and a gene encoding glutathione-S- transferase expressed in *Escherichia coli* identified two bands, one 29kD the other 36-40kD when probed onto a Western blot containing the FPLC eluate and the HICE eluate material.

Comparing the 29-40kD protein with the two surface molecules obtained by T.Pearson there are similarities; both are acidic and both contain a large amount of alanine residues, but there are also some differences. Firstly, and most obviously, is the size difference on SDS PAGE. This could be explained by microhetergeneity. Secondly, the 50-60kD molecule reacts with Coomassie blue and silver stains but the 22-28kD molecule just with STAINS-ALL, suggesting that the 29-40kD protein and the lower molecular weight molecule have more similarity, though all three molecules have similar amino-acid compositions.

Is the 29-40kD protein the *T.congolense* analogue of

T.brucei procyclin? Both proteins are abundant molecules, and purification strategies designed to purify procyclin also helped purify the *T.congolense* antigen. Both proteins are expressed on the cell surface, are stage specific and immunodominant, are highly acidic and do not stain with either Coomassie blue or silver. Although there are some similarities between the two proteins there are several differences. Procyclin is proline rich, and is also predominantly composed of a dipeptide repeat, at least some of which properties are not seen in the 29-40kD protein or in the two proteins purified by Pearson *et al*.

Nevertheless the 29-40kD protein and procyclin could have been developed by the two trypanosome species for the same purpose, that is to survive in the Tsetse fly gut. The habitat of the trypanosomes at this stage is very hostile. To prevent killing by the digestive enzymes of the fly the organisms may have to cover itself in a heavily glycosylated molecule.

CHAPTER 5

CONCLUSIONS

The initial aims of the project were to identify a surface membrane protein from *T.congolense* procyclic cells, purify it, and then characterise it. The protein chosen for analysis was identified in immunofluorescence studies when *T.congolense* procyclic cells were probed with GUGM 2.2, a mab raised in mice against this stage. In this study GUGM 2.2 detected an abundant surface antigen. Purification of the desired protein from the surface of the procyclic cells using several standard biochemical and immunochemical methods was attempted. These approaches led to the identification of a very intriguing protein which exhibited some unusual properties. It will be interesting to carry out more extensive carbohydrate analysis to help elucidate the structure of this molecule. When this is carried out it may be able to propose a biological reason for the protein being present. If the information obtained about the protein is to be used successfully in an attempt to control trypanosomiasis, then we need to clone the gene that codes for it. As previously mentioned cDNA clones are already made (R.Bayne) from *T.congolense* procyclic cells encoding a putative protein whose amino-acid sequence corresponds to the peptides obtained by Pearson in the cyanogen bromide cleavage experiments. Therefore it may turn out that this work has already been initiated.

The information obtained in the study of the 29-40kD protein has not only been advantagous to the study of trypanosomiasis, but the analytical methods used in its purification and characterisation will be useful when studying similar proteins from any organism.

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