

4 CHAPTER FOUR

MEMBRANE PROTEIN ISOLATION FROM *XENOPUS* OOCYTES AND PROTEIN GEL DETECTION

4.1 Introduction

4.1.1 Expression and isolation of *Plasmodium* proteins

Xenopus laevis oocytes have been used for expression of many proteins, including a few *P. falciparum* proteins (Woodrow *et al*, 1999; Krishna *et al*, 2001). However other expression systems have also been used for the expression and isolation of *Plasmodium* proteins, including *Escherichia coli* (Degen *et al*, 2000; Krause *et al*, 2000; Rohdich *et al*, 2001), yeast *Saccharomyces cerevisiae* (Wang *et al*, 2001) mammalian cells (Gamain *et al*, 2001), Chinese Hamster Ovaries (CHO; van Es *et al*, 1994; Buffet *et al*, 1999) and the slime mould, *Dictyostelium discoideum*. The latter system was used for the expression and purification of the nearly full-length circumsporozoite protein and has the closest codon preferences to *P. falciparum* than any of the other expression systems (van Bemmelen *et al*, 2000).

Plasma membrane proteins have been successfully isolated from *Xenopus* oocytes by repeated sedimentation in discontinuous sucrose gradients (Bretzel *et al*, 1986) and by varying high and low speed centrifugation steps (Camacho and Lechleiter, 1993). *P. falciparum* proteins such as the Ca²⁺-ATPase (PfATP4) have also been successfully isolated from *Xenopus* oocytes by the centrifugation method (R. Webb, St. George's Hospital Medical School, London, personal communication; Krishna *et al*, 2001) and used for Western blots. The method can therefore be very useful for the detection of dysfunctional recombinant mutant proteins that may have been inserted into the *Xenopus* oocyte membrane, but are undetectable using uptake assays. Reports on point mutated *P. falciparum* proteins expressed in *Xenopus* oocytes have been successful (Woodrow *et al*, 2000) but when dealing with chimaera proteins made by

the joining of a protein from two different species, the results can prove more difficult (K. Nel, University of Pretoria, South Africa, personal communication).

4.1.2 Chimaera proteins

Chimaera proteins are created from the joining of two or more regions from different proteins to create a new one (Lodish *et al*, 1995). This can either be done using completely different proteins, such as the attachment of the green fluorescent protein (GFP) to glucose transporters to monitor their trafficking within a cell (Oatey *et al*, 1997; Inukai *et al*, 1995; Powell *et al*, 1999), or with similar proteins such as those in the human glucose and fructose transporter family (Arbuckle *et al*, 1996).

The design and experimentation with chimaera proteins can give useful insight into the structure-function relationship of a transporter protein. Arbuckle *et al* (1996) and also Wu *et al* (1998) applied chimaeras in their studies to elucidate the fundamental difference between the mammalian glucose transporter GLUT3 and the mammalian hexose transporter GLUT2. Arbuckle *et al* (1996) created eight chimaera proteins from GLUT2 and GLUT3, all of which were functional, to identify the region or regions responsible for GLUT2's ability to transport fructose as well as glucose, and GLUT3's inability to. Each chimaera included a mixture of complete helices, thereby enabling the differences between GLUT2 and GLUT3 to be targeted to a specific helix. This method was relatively simple and enabled the researchers to pin point helix 7 as the region where differences that enable fructose transport in GLUT2 may lie. Buchs *et al* (1998) applied the same technique to chimaeras of GLUT3 and the mammalian fructose transporter GLUT5. Eight chimaeras were made, four of which were not functional. Research studies by Noel and Newgard (1997) also involved experimentation with GLUT1/GLUT2 chimaeras, except that it focused on the importance of the C-terminal tail for transport of fructose. All the above studies involved chimaera proteins made between proteins from the same group of transporters and from the same species. Coady *et al* (2000) applied a similar technique to different transporters namely, the Na⁺/glucose and Na⁺/myo-inositol co-transporters. These mammalian proteins transport different substrates, but fall within the same family. Twelve chimaeras were created, only two of which remained

functional, demonstrating the difficulty in maintaining functionality with increasing diversity between proteins (Coady *et al*, 2000).

An attempt was made to apply this technique to the *P. falciparum* hexose transporter PfHT1. No other *P. falciparum* sugar transporters have been reported yet. Chimaeras were therefore constructed between the *P. falciparum* and the mammalian glucose transporter GLUT1. Chimaeras of human and parasite glucose transporters were anticipated to establish differences between the two transporters that could be exploited for anti-malarial drug design. In the event that the chimaera transporters proved dysfunctional, isolation of the chimaera protein from the plasma membrane and Western blots would distinguish between problems with the expression or the trafficking of the protein from the ER/ Golgi to the plasma membrane or assembly of a functional protein. However, trafficking and assembly will be indistinguishable from one another.

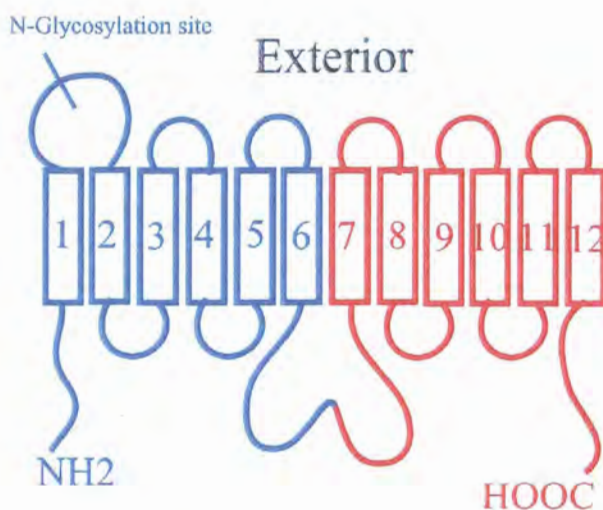
4.2 Materials and method

4.2.1 Construction of Chimaeras

4.2.1.1 *Chimaera design*

Two chimaera proteins were designed by K. Nel under the supervision of Professor A. I. Louw (University of Pretoria, South Africa), and were designated the names Chimaera 1 and Chimaera 2. The amino acid sequence from the *P. falciparum* hexose transporter PfHT1 was aligned with the amino acid sequence from the rat glucose transporter (*Rattus novogicus*, GenBank Accession number M13979), which is identical to the human glucose transporter GLUT1 (*Homo Sapiens*, GenBank Accession number K03195). Chimaera 1 contained the first 6 helices from PfHT1 and the last 6 helices from the rat glucose transporter. Chimaera 2 contained all 12 helices from PfHT1 and the C-terminal tail region from the rat glucose transporter (Figure 4.1, helical alignments in Appendix IIIa).

Chimaera 1



Chimaera 2

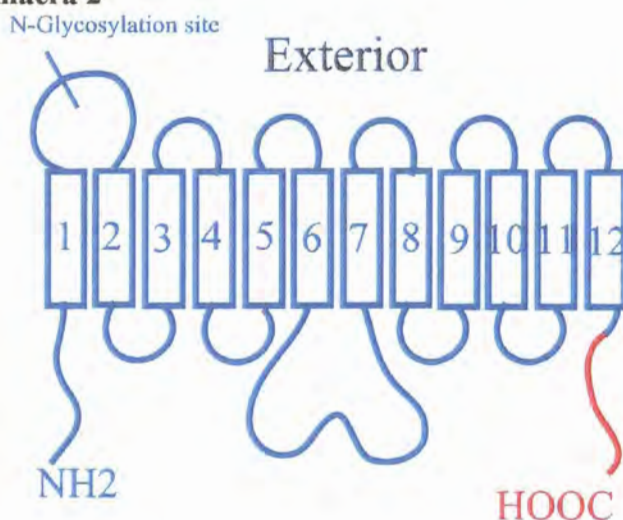


Figure 4.1: Schematic representation of Chimaera 1 and 2. Helices and joining strands in blue indicate regions from the *P. falciparum* hexose transporter PfHT1. Those in red indicate regions from the mammalian glucose transporter GLUT1. There is 29.8% identity between the mammalian and *P. falciparum* sugar transporters (Woodrow *et al*, 1999). The mammalian C-terminal tail is longer (42 amino acids) than that for PfHT1 (29 amino acids), whereas the N-terminal is shorter (GLUT1: 12 amino acids, PfHT1: 28 amino acids).

The rat glucose transporter gene was a gift from G. Gould (University of Glasgow, UK) and was contained in the pSP64T expression vector. The chimaeras were constructed by overlapping PCR as described in Paragraph 2.2.2. PCR 1 for Chimaera 1 used primers Mal/Rat F and R, and Chimaera 2 used primers Mal/Rat Tail F and R. PCR2 for both chimaeras used Mal 5' F and Rat 3' R for the amplification of the full-length gene (Table 4.1). PCR reactions were performed with 1.25 U ExTaq (Takara

Shuzo, Japan) and PCR conditions as described in Table 2.4. The PCR products were electrophoresed and isolated from agarose gel. The chimaera PCR products were then ligated into the pGEM[®]-T easy vector (Appendix Ib) and transformed into competent SURE *E. Coli* cells. The sequence of the chimaeras were verified with sequencing as described in Paragraph 2.2.8 with the T7 reverse primer in addition to the primers mentioned in Paragraph 2.2.8.

Table 4.1: Primer sequences for the primers used to construct Chimaera 1 and Chimaera 2. Mal = *P. falciparum* glucose transporter (in uppercase), Rat = rat glucose transporter (in lowercase), F = forward, R = reverse. Start and stop codons are in bold, *Bgl*III sites are underlined, and the Kozak sequence is given in italics.

Primer Name	Primer Sequence 5'-3'
Mal/Rat F	CAGATAATGTCGATGAACCAcaggagatgaaagaagag
Mal/Rat R	ctcttcttttcacatctcctgTGGTTCATCGACATTATCTG
Mal/Rat Tail F	TTATCAAAGAAACcaaaggccggaccttc
Mal/Rat Tail R	gaagggtccggccttttgGTTTCTTTGATAA
Mal 5' F	AAAGATCTCACC ATG ACGAAAAGTTCGAAGGATATATGTAGTG
Rat 3' R	aaagatct tca cacttgcgagtcagctcccagag

4.2.1.2 Ligation into pGEM[®]-T Easy Vector

The chimaera DNA from PCR reactions was inserted into the pGEM[®]-T Easy Vector (Promega, USA) according to the manufacturers instructions. Briefly a 3:1 insert to vector ratio was used for ligation, which resulted in 25 pmol of vector and 75 pmol of insert DNA. The vector and insert were added together with a 1× T4 DNA Ligase Buffer (30 mM Tris-HCl, pH 7.8; 10 mM MgCl₂; 10 mM DTT; 1 mM ATP) and 2 Weiss units of T4 DNA Ligase (Promega, USA). The reaction was made up to a final volume of 10 µl with distilled, deionised water. The reaction was then incubated overnight at 4°C, and stopped by inactivation of the T4 DNA Ligase enzyme at 70°C for 3 minutes.

4.2.1.3 Transformation into competent SURE *E. coli* cells

A few methods were tried for the preparation of competent cells and the calcium/magnesium-based method yielded the best results (Hanahan *et al.*, 1991).

SURE *E. coli* cells (Stratagene, La Jolla, CA, USA) were streaked on M9 minimal medium agar plates (0.05 M Na₂HPO₄-2H₂O, 0.02 M KH₂PO₄, 8 mM NaCl, 0.02 M NH₄Cl, 2 mM MgSO₄, 0.01 M D-glucose, 0.1 mM CaCl₂, 1 mM thiamine hydrochloride, 1.5% w/v agar, pH 7.4) and left to grow overnight at 30°C. Cells were picked from the M9 plates and streaked onto LB medium agar (1.5% w/v agar) containing 12.5 µg/ml tetracycline and grown overnight at 30°C. Colonies were picked from the plate and vortexed in 1 ml SOB medium (2% w/v Trypton, 0.5% w/v yeast extract, 10 mM NaCl, 2.5 mM KCl, pH 6.8-7.2). The 1 ml SOB with bacteria was inoculated into 49 ml SOB medium in a 500 ml flask and grown at 30°C while shaking at 250 rpm until an OD₆₀₀ of 0.3 was reached indicating an early exponential phase of growth. The cells were transferred to a 50 ml centrifuge tube and incubated for 10 minutes on ice. The cells were then centrifuged at 1000g for 15 minutes at 4°C. The supernatant was removed and the cells resuspended in one-third the volume CCMB80 medium (80 mM CaCl₂-2H₂O, 20 mM MnCl₂-4H₂O, 10 mM MgCl₂-6H₂O, 10 mM K-acetate, 10% v/v glycerol, pH 6.8) and incubated on ice for 20 minutes. The cells were centrifuged at 1000g for 10 minutes and the supernatant removed. The cells were resuspended in a twelfth the original volume of CCMB80 medium and aliquoted into microfuge tubes and frozen at -70°C.

The pGEM-T Easy Vector containing insert was transformed in competent SURE *E. coli* cells by the heat shock method (Sambrook *et al.*, 1989). Competent SURE *E. coli* cells were thawed on ice from storage at -70°C. In 5 ml plastic tubes, 2 µl of the deactivated ligation reaction mix was added to 100 µl of competent cells on ice. The same concentration of pBlueScript vector was used as a positive control, and 100 µl of competent cells without plasmid was used as a negative control. The cells were incubated on ice for 30 minutes. A heat-shock step followed, which involved immersing the cells into a 42°C water bath for 90 seconds and then transferring them immediately to ice for a 2-minute incubation. Preheated SOC medium (900 µl, 2%

w/v Trypton, 0.5% w/v yeast extract, 10 mM NaCl, 2,5 mM KCl, 20 mM D-glucose, pH 6.8-7.2) was added to the cells and incubated at 30°C for 1 hour while shaking at 250 rpm. The transformation reaction (100 µl) was plated on LB agar plates (1.5% w/v agar) containing 100 µg/ml ampicillin. The plates were first prepared with 32 µl of 20 mg/ml X-gal and 4 µl of 0.4 mM IPTG for blue/white selection. The plates were incubated at 30°C overnight.

4.2.2 Expression in *Xenopus* oocytes

For injection into *Xenopus* oocytes cRNA was prepared of Chimaera 1 and 2 and GLUT1 as described in Paragraph 2.2.9. *Xenopus* oocytes were assayed as described in Chapter 3. Uptake measurements were performed on batches of 6 oocytes from each cRNA injected and water injected controls at approximately 24, 48, 72 and 96 hours after injection. Oocytes were incubated in Barth's solution containing 2.5 µM D-[U-¹⁴C]-glucose (Amersham Life Sciences, UK, 323mCi/mmol) and 30 nM D-glucose (Sigma-Aldrich, UK) for 20 minutes and then washed three times in ice cold Barth's solution. Each oocyte was lysed in scintillation fluid in a 1.5 ml microfuge tube and then counted on a Wallac 1450 Microbeta Plus scintillation counter as decays per minute (DPM). Results were analysed with Microsoft Excel software.

4.2.3 Isolation of membrane proteins

Surface membrane proteins were isolated from Chimaera 1 and 2, GLUT1 cRNA injected and water injected oocytes 72 hours after injection and one hundred oocytes were used for each. A protein isolation procedure was followed published by Krishna *et al* (2001). Each batch of oocytes was separated into 1.9 ml microfuge tubes with 10 oocytes per tube. 500 µl of homogenisation buffer (83 mM NaCl, 1 mM MgCl₂, 10 mM HEPES, pH 7.9) was added to each microfuge tube. One Complete Protease Inhibitor Cocktail tablet (Roche, Germany) was added to every 10 ml homogenisation buffer before adding to oocytes. The oocytes were homogenised with 20 hand driven strokes with a homogeniser on ice. The homogenised solutions were centrifuged at 1000g for 5 minutes at 4°C to remove the yolk granules and melanosomes. The supernatant was removed and collected and 500 µl of homogenisation buffer was

added to the pellet and homogenised again and centrifuged at 1000g for 5 minutes at 4°C. The supernatant was collected and combined with the supernatant from the previous centrifugation. The pooled supernatants were centrifuged at 1000g for 5 minutes at 4°C to remove residual yolk protein. The resulting supernatant was centrifuged at 100 000g for 90 minutes at 4°C. The pellet was resuspended in homogenisation fluid; aliquoted into microfuge tubes and frozen at -70°C. Protein concentrations were determined with the Modified Lowry Kit for Protein Determination (Sigma-Aldrich, UK) and read on a Shimadzu UV 160 A spectrophotometer at 750 nm.

4.2.4 Protein separation using SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

A Mini-Protean II Electrophoresis Cell (Bio-Rad, UK) was used for the separation of the protein samples collected from the membrane protein isolation step. Ten ml of separation gel (8% w/v acrylamide; 0.375 M Tris buffer, pH 8.8; 0.06% v/v TEMED; 0.1% w/v sodium dodecyl sulphate, 0.1% w/v ammonium persulphate) was made and poured between a glass sandwich set on a casting stand. The gel was covered with butanol and left to set. Once set the butanol was removed from the gel and a 10 well comb was placed on top of the gel. Five ml stacking gel (5% w/v acrylamide; 0.126 M Tris buffer, pH 6.8; 0.1% v/v TEMED; 0.1% w/v sodium dodecyl sulphate, 0.1% w/v ammonium persulphate) was made and poured around the comb and the gel was left to set. The glass sandwich containing the gel was placed in the electrophoresis tank and 1× PAGE running buffer (0.4 M Tris base, pH 8.3, 14.41% w/v glycine, 0.1% sodium dodecyl sulphate) was poured into the tank. Approximately 150 µg of each protein sample was loaded into each well alongside 10 µl BenchMark™ Prestained Protein Ladder (Life Technologies, UK). The gel was run at 200 Volts for 45 minutes. The gels were either stained in Coomassie Brilliant Blue R250 Stain (Sigma-Aldrich, UK) or used for Western blots. Gels not used for Western blots were stained in 0.1% w/v Coomassie Brilliant Blue for 2 hours and then transferred to a 1:10 dilution of Coomassie Brilliant Blue and stained overnight. The gels were then destained in 10% v/v acetic acid for 1-2 hours. The stained gels were dried at 60°C on Whatman paper

on a Hoefer GD 2000 Vacuum Gel Dryer System (Amersham Pharmacia Biotech, USA).

4.2.5 Western blots

Gels were sandwiched between nitrocellulose paper and blotting paper and placed in a Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad, UK) and run at 100 Volts for 1 hour. Gels were removed and placed in Coomassie Brilliant Blue as in Paragraph 4.2.4 to check the transfer from the gels. The nitrocellulose blots were blocked in Tris buffered saline (TBS)/ Tween (0.145 M NaCl; 0.05 M Tris/ HCL, pH 7.5; 1% v/v Tween) with 5% w/v powdered milk for 30 minutes. The nitrocellulose blots were then transferred to TBS/ Tween buffer containing 1:5000 dilution of rabbit anti-GLUT-1 polyclonal antiserum (Chemicon International, CA, USA) and incubated for 1-2 hours on a rotator. The blots were washed 3 times in TBS/ Tween for 5 minutes each wash. The blots were then incubated in TBS/ Tween containing a 1:500 dilution of anti-rabbit alkaline phosphate conjugated secondary antibody for 1 hour. The blots were washed 3 times in TBS/ Tween for 5 minutes each wash. Two tablets of Sigma Fast™ 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma-Aldrich, UK) were dissolved in distilled water and the blots were soaked in the solution for 5-10 minutes. The blots were then rinsed in distilled water and left in the dark to dry.

4.3 Results

4.3.1 Expression in *Xenopus* oocytes

Uptake measurements of D-[U-¹⁴C]-glucose were conducted 24, 48, 72 and 96 hours after injection. As seen in Figure 4.2, 96 hours after injection the counts for Chimaera 1 and Chimaera 2 are similar to those obtained for the water-injected controls, which remained low (\approx 100 counts per minute). The uptake measurements obtained for the GLUT1 injected oocytes were high (2000-4500 counts per minute).

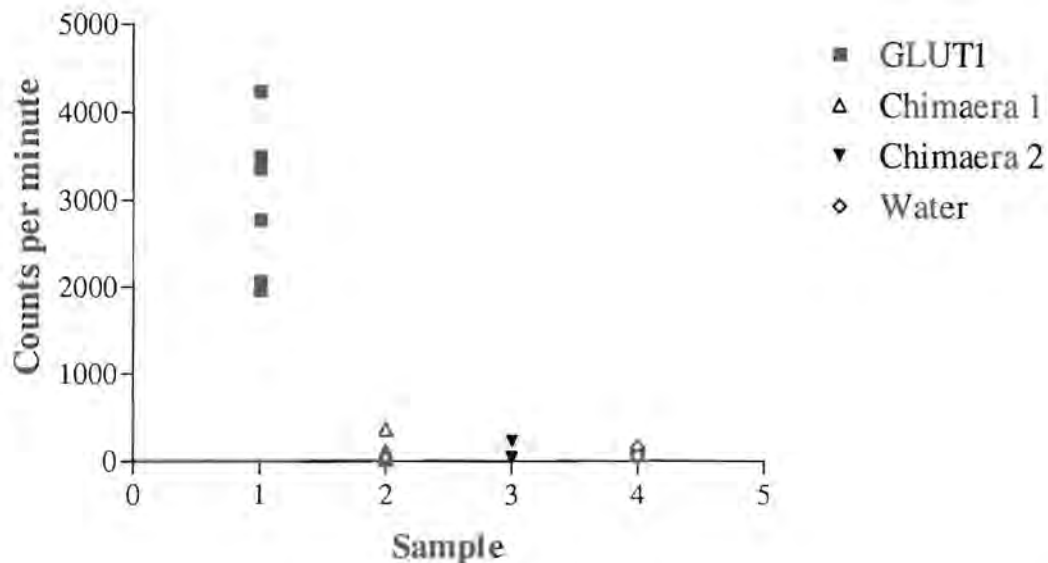


Figure 4.2: Uptake measurements of D-[U-¹⁴C]-glucose by chimaeras compared to GLUT1 96 hours after injection. The *Xenopus* oocytes injected with GLUT1 cRNA showed uptake measurements 200-400 fold higher than those obtained for Chimaera 1 and 2 cRNA injected oocytes, which were as low as water-injected oocytes.

4.3.2 SDS-PAGE gel and Western blots

The SDS-PAGE gels stained with Coomassie Blue revealed lanes cluttered with membrane proteins originating mainly from endogenously expressed *X. laevis* proteins (Figure 4.3). The expected size for the chimaera proteins was around 60 kDa and the expected size for the GLUT1 protein was 56.4 kDa (Woodrow *et al*, 1999). Each lane for the cRNA and water injected oocytes showed similar patterns of protein separation and no distinct band was visible around 60 kDa for the cRNA injected oocytes compared to the water-injected oocytes.

The Western blot revealed a distinct staining around 60 kDa for the GLUT1 injected oocyte with a smear below 60 kDa (Figure 4.4). The primary rabbit anti-GLUT1 antibody recognises the C-terminal section of GLUT1 in human and rat tissue. Since the chimaeras both have C-terminals from the rat glucose transporter the antibody will bind to either chimaera protein if present on the nitrocellulose as well as to the GLUT1 positive controls. No bands were detected for Chimaera 1 or 2 or for the water injected control.

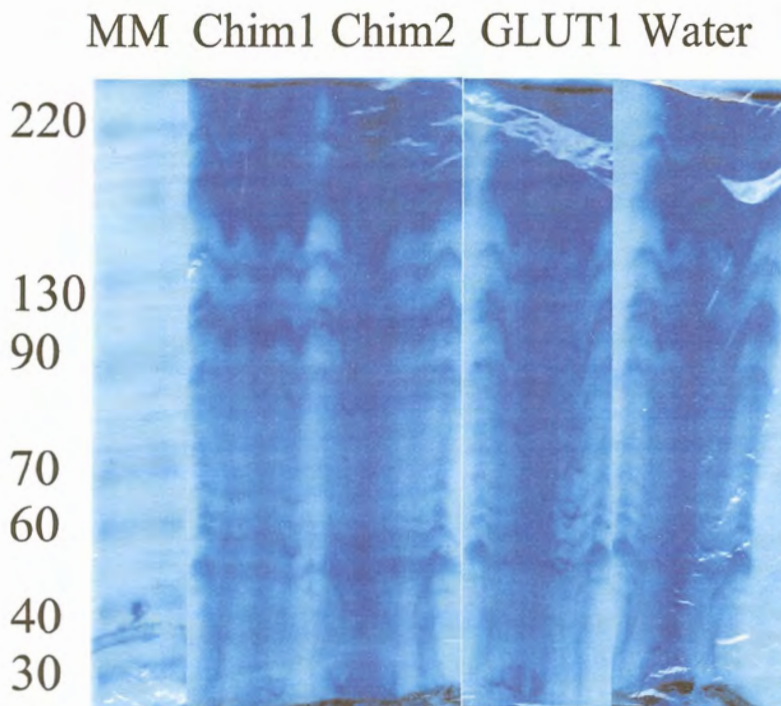


Figure 4.3: SDS-PAGE gel stained with Coomassie Blue. Molecular markers (MM) are in kDa and the 70 kDa band is stained pink for easy identification. Chim1 and Chim2 stand for Chimaera 1 and 2, respectively.

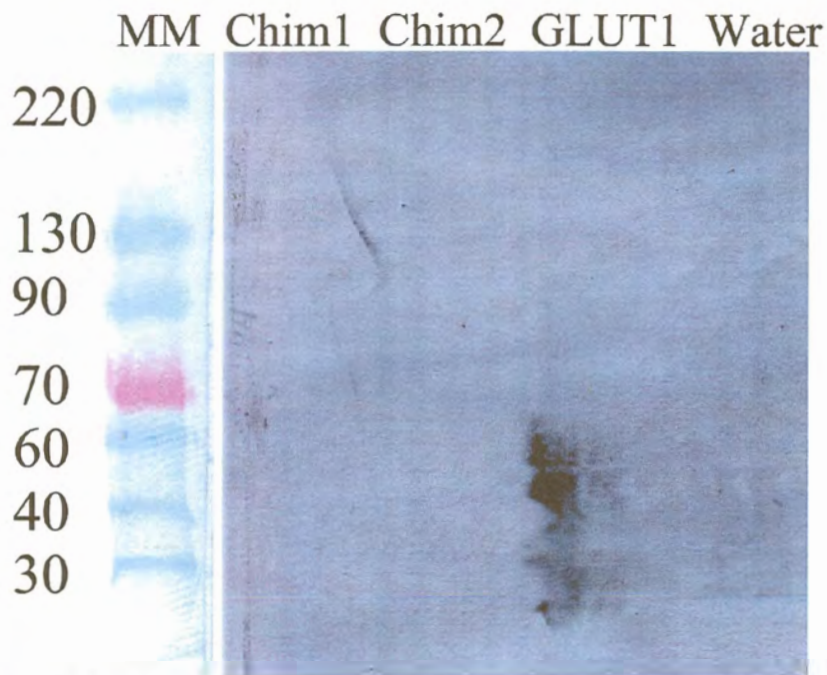


Figure 4.4: Western blots of chimaera proteins and GLUT1. Molecular markers (MM) are in kDa and the 70 kDa band is stained pink for easy identification. Chim1 and Chim2 stand for Chimaera 1 and 2 respectively. The lane containing membrane proteins isolated from GLUT1 cRNA injected oocytes has a smear from 60 kDa and below.

4.4 Discussion

The fate of chimaera proteins originating from a conjugation of sugar transporters from distinctly different species such as the malaria parasite and its host, are multifaceted. In the case where expression of the proteins in an exogenous expression system is unsuccessful, the problem may lie in various areas. A chimaera containing the first 6 helices of the PfHT1 hexose transporter and the last 6 helices of the rat glucose transporter; and a chimaera containing all 12 helices of the PfHT1 hexose transporter and the tail of the rat glucose transporter were constructed. Injection of the chimaeras into *Xenopus* oocytes and uptake measurements with D-glucose revealed no transport of substrate by the chimaera proteins. This could indicate a number of possibilities. Since the protein is made from two transporters that differ slightly in their substrate selectivity, the lack of transport indicated by the chimaera proteins could originate from dysfunctionality of the chimaera as a result of this difference in substrate selectivity between the two parent proteins. Another possibility is that the protein is expressed and folded in the ER but is not trafficked to the oocyte membrane due to a lack of some signal sequence removed by the construction of the chimaera. More likely in the case of a chimaera protein originating from two phylogenetically distinct species, the protein may be expressed and not folded properly and instead aggregates within the cell and is destroyed. Whether the protein is inserted or not into the oocyte membrane can easily be resolved with membrane protein isolation and Western blots.

Membrane protein isolation from the cRNA and water-injected oocytes and their subsequent separation on a SDS-PAGE gel revealed a collection of membrane proteins ranging in size from approximately 20 kDa and larger. The protein bands from the cRNA injected oocytes were identical to those obtained from the water injected oocytes which indicates that they originate from the endogenously expressed *X. laevis* membrane proteins. If any protein is present originating from the injected cRNA, it is lost within the high concentrations of endogenous protein.

The Western blots revealed binding of GLUT1-specific antibody to the nitrocellulose in the lane corresponding to the GLUT1 injected oocytes. This resulted in a smear

from 60 kDa and smaller that could indicate that the expected 60 kDa GLUT1 protein was slightly degraded possibly during the membrane protein isolation step. However no similar bands were obtained for either Chimaera 1 or Chimaera 2 cRNA injected oocytes. No bands were expected for the water-injected oocyte used as negative controls. This indicates that whereas the GLUT1 protein was functionally inserted into the oocyte membrane, the chimaera proteins were not inserted at all.

The trafficking mechanisms within the malaria parasite differ in some ways from higher eukaryotes as reviewed in Chapter 1. It is fairly agreed upon that the parasite has a functional ER at the nucleus periphery. The presence of a Golgi apparatus is less well defined and Golgi functions have been found scattered throughout the cell as well as in the TVM. This difference in trafficking may cause certain malaria proteins expressed in exogenous expression systems to not be trafficked to their proper destinations. However in the case of PfHT1, the wild type transporter is inserted functionally into the *Xenopus* oocyte membrane (Woodrow *et al*, 1999), as is the GLUT1 glucose transporter seen in Figure 4.2. Therefore the absence of the chimaera proteins in the oocyte membrane made from these two parent transporters must be as a result of misfolding of the chimaera within the ER.

Chimaera proteins can be useful in identifying regions between different proteins that are important for the proteins functions. However most chimaeras, except for GFP conjugated chimaera proteins, are usually made from two proteins originating from the same species. In the case of the chimaeras designed here they originated from two species that are distantly related. Parasitic protozoa such as *P. falciparum* and *T. brucei* may have sugar transporters that belong to the same superfamily as GLUT1, but are evolutionary distant (Walmsley *et al*, 1998). The idea behind the design of Chimaera 1 and Chimaera 2 was centred on a direct approach to elucidate differences between the malaria parasites hexose transporter and its hosts transporter GLUT1 by combining the two transporters.

Deletion of most of the C-terminus (37 out of 42 amino acids) of GLUT1 by Oka *et al* (1990) still resulted in the transporter being inserted in CHO cell membranes determined by Western blots. However the transporter was no longer able to transport

glucose into the cell and was hypothesised to be locked in an inward facing form due its ability to bind the endofacial ligand cytochalasin B but not the exofacial ligand ATB-BMPA. Chimaera 2 was constructed with the 12 helices from PfHT1 truncated 4 amino acids after the 12th transmembrane helix and joined to the last 36 amino acids of the rat glucose transporter tail, the same amino acids deleted in the experiments conducted by Oka *et al* (1990). This leaves 7 amino acids (residues 449-455) left out from the GLUT1 tail that lie directly after the GLUT1 12th transmembrane helix. Had these amino acids been included in Chimaera 2, this protein may have been inserted into the oocyte membrane. Alternatively, if seven amino acids directly after PfHT1 helix 12 had been included instead of only four, the transporter may have been inserted in the membrane or may have even been functional. These amino acids may be important for the correct folding or insertion into the ER membrane during expression into the ER lumen.

It appears that in order for studies of this nature to be successful, the chimaeras need to be constructed from parts originating from the same species or genus. Until an additional sugar transporter is identified from *P. falciparum* or any other *Plasmodium* species, chimaeras of this nature may not be successfully expressed. For a parasite to survive inside its host it must adapt to many challenges, one being the challenge to obtain nutrients in competition with its hosts own nutrient requirements. In the case of glucose transporters this could result in such evolutionary differences between host and parasite transporters that they will not function when combined in a chimaera form. Further glucose transporters for *Plasmodium* species, or in particular *P. falciparum*, will have to be identified before such chimaera experiments would be successful.

5 CHAPTER FIVE:

CONCLUDING DISCUSSION

At present, about 500 million people are affected by malaria globally, and there are up to 3 million malaria deaths per year. Malaria is generally endemic in the tropics, with extensions into the subtropics (Bradley, 1996). Early diagnosis and correct treatment form the basic elements of any malaria control program. The need to discover and validate new targets to generate new drug candidates has elevated because of the emergence of resistance to widely used antimalarial drugs, such as chloroquine (Hastings and D'Alessandro, 2000). Several enzymes from a number of biochemical pathways in the human malaria parasite *P. falciparum* have been identified and proposed as potential drug targets, although few of them have been validated (Chapter 1, Table 1.1).

One of these potential drug targets, a putative glucose transporter named PfHT1, was isolated, cloned and expressed in *Xenopus* oocytes (Woodrow *et al*, 1999). PfHT1 transports D-glucose at a higher affinity than GLUT1 and also transports D-fructose with a relatively high affinity. The most important carbons on glucose with which PfHT1 interacts are C-3 and C-4. The most interesting observation to date is the discovery that mutation of Q169 resulted in no fructose being transported by PfHT1 (Woodrow *et al*, 2000).

GLUT1 is the most studied glucose transporter to date, and along with most transporters within the major facilitative superfamily, is predicted to form 12 transmembrane helices (Mueckler *et al*, 1985). Without the availability of a crystal structure, indirect approaches remain the best means for obtaining structure-function information on transporters within this family. From indirect approaches a number of interesting details of GLUT1 have emerged. Five of the 12 transmembrane helices are thought to be amphipathic and form an aqueous pore through which the hydrophilic substrate is proposed to cross the lipid membrane. Hence most studies have concentrated on these five key helices as discussed in Chapter 2, Paragraph 2.1.

Mutational studies provide information into structure-function relationships and extensive studies have already been carried out on mammalian GLUT1. Cysteine-scanning mutagenesis studies on GLUT1 helix 7 have provided insight into which of the helix 7 amino acids are exposed to the substrate for interaction. The highly conserved QLSQQLSGINAVFY region in helix 7 has been the main subject for investigation, and has revealed five amino acids within this region as essential for protein-substrate interaction namely, Q282, Q283, I287, V290 and F291 (Olsowski *et al*, 2000; Olsowski *et al*, 1998). GLUT1 helix 7 residues that have been suggested to be involved in substrate binding, such as Q282 and Q283, are located in the centre of the transmembrane helix, and it has therefore been suggested that helix 7 may move up in the membrane to accept exofacial ligands (Hashiramoto *et al*, 1992). Glutamine 161 in GLUT1 helix 5 that corresponds to glutamine 169 in PfHT1 (Q169) was predicted to form part of the exofacial ligand binding site of GLUT1. Glutamine 161 (in GLUT1 helix 5) when mutated to leucine reduced the transport activity by 50-fold as discussed in Chapter 2 (Mueckler *et al*, 1994). Studies such as these on PfHT1 would provide valuable information on key amino acids that are involved in ligand binding.

Site directed mutagenesis studies have already been applied to PfHT1 and resulted in the discovery that mutation of Q169 to asparagine ablated PfHT1's ability to transport fructose (Woodrow *et al*, 2000). In this study, a site directed mutagenesis approach was applied to identify key amino acid residues within helix 5 and 7 responsible for PfHT1-hexose interactions. The amino acids that were focused on were Q169 in helix 5, and S302 and L304 in helix 7. These amino acids were mutated to residues differing in atomic composition and size to the original. Results concerning the helix 5 amino acid (Q169) had already been published (Woodrow *et al*, 2000), but kinetic studies had not yet been completed. Studies on the helix 7 amino acids were novel. The helix 7 amino acids are contained within a motif (SGL), whose equivalent in GLUT1 (QLS, Figure 2.1A) has been implicated in differential transport of fructose and glucose. An attempt was made to mutate the PfHT1 SGL motif to the GLUT1 QLS motif. These attempts failed to produce a functional transporter (Krishna, St. George's Hospital Medical School, London, unpublished results) and therefore an alternative was needed. The *T. brucei* equivalent motif (AGT) was decided on since a

correlation can be drawn between these two parasites. *P. falciparum* and *T. brucei* share similar living conditions within their vertebrate hosts and may have evolved similarly due to pressure exerted by harsh living conditions and also by a common necessity to obtain glucose from their hosts. The mutations were achieved by PCR reactions discussed in Chapter 2 and the resulting mutants were cloned into a *Xenopus* expression vector (pSP64T). Kinetic parameters K_i and K_m were obtained from uptake experiments with the mutant transporters and glucose, fructose and their analogues and are presented in Chapter 3.

The involvement of the helix 7 tripeptide motif (SGL) in PfHT1 in the discrimination between glucose and fructose substrate is now questionable. It was thought that this motif was associated with fructose transport. Based on the results obtained in this study in comparison with those obtained by Seatter *et al* (1998), it appears that the conserved QLS motif in mammalian glucose transporters is only important for D-glucose transport. Transporters with this motif cannot transport D-fructose, whereas those without it can. This is most evident when replacement of GLUT2 HVA by QLS resulted in a notable decrease in D-glucose transport inhibition by D-fructose (Seatter *et al*, 1998). It would have been interesting to see what effect substitution of GLUT1 279QLS for PfHT1 302SGL would have had on the PfHT1 transporters activities and properties. However, such a mutation resulted in no transport of any substrate across the *Xenopus* oocyte membrane. This could be due to either misfolding of the mutant protein or to disruption of the mutant protein's trafficking to the oocyte membrane (Krishna, St. Georges Hospital Medical School, London, unpublished results).

With the PfHT1 helix 7 SGL motif mutated to the *T. brucei* THT1 AGT motif, a lower uptake was observed after 24, 48 and 72 hours after microinjection (Chapter 3, Figure 3.5). The S302A and L304T separately also produced lower uptakes at 24, 48 and 72 hours after microinjection. From Figure 3.5 (Chapter 3) it can be postulated that both the S203A and L304T mutations were causing the lower uptake seen for XF2.4. However, the L304T mutant may have a greater effect on lowering the uptake measurements seen for XF2.4 as it has a significantly lower ($P < 0.04$) uptake measurement compared with S302A. From Figure 3.6 (Chapter 3) it can be postulated that XT1.10 may follow a similar expression pattern to XF2.4 when one compares the

percentages of the maximum expression (48h), particularly at 24 and 48 hours. The same can be said for XA1.4 that appears to follow a similar expression pattern to the wild type at all time points.

The results obtained from kinetic analysis of the mutants are summarised in Chapter 3 Table 3.1. The results suggest that the helix 5 residue (Q169) may interact with glucose at C-2, C-3, C-5 and C-6. Mutation of this amino acid results in the ablation of fructose transport as previously reported, but it appears that this amino acid is not as important for glucose transport. This glutamine may either be interacting directly with glucose or it may be interacting with another amino acid which itself interacts directly with the glucose.

Mutation of the two amino acids in helix 7 (S302 and L304, XF2.4) produced a significant change in fructose transport. However, no significant change in fructose transport was observed with the single mutants. However, all three helix 7 mutants (XF2.4, XA1.4 and XT1.10) showed no difference in affinity for 2,5-AHM compared with the wild type. Therefore the involvement of the PfHT1 SGL motif in fructose transport does not appear to be important.

It is already proposed that PfHT1 hydrogen bonds to C-1 and C-3 (Woodrow *et al.*, 1999). Analogue studies with the helix 7 mutants produced changes in affinity between PfHT1 and 1-DOG, 3-OMG, 5-thio-D-glucose and 6-DOG. However the significances of the results for 1-DOG and 5-thio-D-glucose were greater than those for 3-OMG and 6-DOG. It therefore appears that the helix 7 SGL motif interacts more strongly with C-1 and C-5. If one looks at the two amino acids (S302 and L304) separately, it becomes apparent from the results in Table 3.1 that the significant changes seen for XF2.4 can be attributed to the amino acid S302. Therefore it can be said that S302 may interact with glucose at C-1 and C-5. The significance of the results obtained with the C-3 or C-6 analogues (3-OMG and 6-DOG, respectively) could be further investigated using alternative analogues such as 3-DOG, which has the hydroxyl removed at C-3, and D-xylose, which has C-6 removed. This may clarify whether the PfHT1 transporter forms any interactions with these two carbon atoms in glucose.

Another approach to structure-function studies was explored using chimaeras constructed from the rat GLUT1 and *P. falciparum* PfHT1. Two chimaeras were constructed, one consisting of the first 6 helices from PfHT1 and the last 6 helices from GLUT1, and the other consisting of all 12 helices from PfHT1 and the GLUT1 tail. Neither chimaera produced uptake measurements with D-glucose. Western blots revealed that the transporters were not inserted into the oocyte membrane. Therefore the chimaeras were either not expressed or folded properly, or they were not trafficked through the ER and Golgi apparatus of the *Xenopus* oocyte. The literature confirms the difficulties experienced when trying to express chimaeras of varying diversity between the counterparts. Here the chimaeras were made from two species that are placed very far apart on the evolutionary tree (Walmsley *et al*, 1998).

Chimaera 2 was constructed with the 12 helices from PfHT1 truncated 4 amino acids after the 12th transmembrane helix and joined to the last 37 amino acids of the rat glucose transporter tail. In an experiment conducted by Oka *et al* (1990), 37 amino acids of the GLUT1 tail were truncated, which resulted in the transporter being unable to transport substrate into CHO cells (Chapter 4). This same transporter constructed by Oka *et al* did however contain 7 amino acids that lie directly after helix 7. With these 7 amino acids the transporter was inserted correctly into the oocyte CHO membrane, yet without the rest of the tail the transporter would not transport glucose into the cell. Therefore, if 7 or more amino acids directly after PfHT1 helix 12 had been included instead of only four this protein may have been inserted into the oocyte membrane. These 7 amino acids directly after PfHT1 helix 12 may be important for the correct expression or functioning of the transporter.

The mutational and chimaera studies conducted here and the results obtained open the way for further investigations into PfHT1 helices that could be important for protein-substrate interactions. These results could be extended by studies with additional analogues such as those used by Woodrow *et al* (2000). The use of only one glucose analogue per C-atom position may not be extensive enough to give conclusive results. The importance of the amino acids investigated in helix 5 and 7 could also be further investigated by mutating them to a variety of alternative amino acids that differ in

polarity and size. Investigations into the affect of alternative amino acids in place of Q169 are already underway (Krishna, St. George's Hospital Medical School, London, personal communication). If one were to consider the extent to which GLUT1 has been studied and that this transporter is still being studied, there is still a lot of potential for investigation into structure/ function relationships of PfHT1.