Characterisation of the ATPase activity and study of the chloroquine accumulation properties of purified *Plasmodium falciparum* plasma membranes

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ABSTRACT

The emergence and spread of chloroquine-resistant *Plasmodium falciparum* strains has severely compromised the effectiveness of this drug. The mechanism of chloroquine resistance has yet to be elucidated. This thesis describes a method for the purification of *Plasmodium falciparum* plasma membranes. Membranes were isolated in an high enough yield to allow an investigation of the chloroquine accumulation properties and a characterisation of the ATPase activity displayed by these purified membranes isolated from chloroquine-sensitive and –resistant parasites. Marker enzyme assays, transmission electron microscopy and gel electrophoresis demonstrated the purity of preparations from parasite cytosol and erythrocyte contaminants. Chloroquine accumulation capabilities of the plasma membranes isolated from *Plasmodium falciparum* exhibiting various degree of chloroquine sensitivity was evaluated. Parasite plasma membranes isolated from two chloroquine-sensitive strains accumulated significantly more chloroquine than those isolated from two of the three chloroquine-resistant strains tested. However, since erythrocytes infected with CQS parasites accumulate chloroquine at a higher level than their resistant counterparts, it is difficult to link these results to a possible role of the parasite plasma membrane in determining the global level of chloroquine accumulation in the parasite. Furthermore, chloroquine accumulation in *P. falciparum* plasma membrane was shown to be ATP-independent. Subcellular localisation of Pgh1 indicated that this protein is present in *Plasmodium falciparum* plasma membrane but no link between the overexpression of Pgh1 and chloroquine sensitivity of the *Plasmodium falciparum* strains tested could be found. In addition, polyclonal antibodies directed at Pgh1 were unable to inhibit chloroquine accumulation in purified plasma membranes, suggesting that Pgh1 is not involved as a chloroquine transporter in the plasma membrane of *Plasmodium falciparum*. Optimal *Plasmodium falciparum* plasma membrane ATPase activity was achieved with 2mM ATP and 2mM Mg^{2+}. The effect of V-type and P-type ATPase inhibitors on the ATPase activity of parasite plasma membrane indicated the presence of a vacuolar proton pump in the plasma membrane of *P. falciparum*. This thesis also showed that verapamil and other agents known to reverse chloroquine resistance by increasing chloroquine accumulation in parasitised erythrocytes did not affect either chloroquine accumulation or the ATPase activity of isolated membranes indicating that resistance reversal do not occur at the plasma membrane level.

CONFERENCE PRESENTATION

Part of the work completed in this thesis was presented in poster format at international conferences and published in the conference proceedings as follows:

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
AchE	acetylcholine esterase
ADP	adenosine diphosphate
AMP	adenosine monophosphate
APADH	reduced 3-acetylpyridine adenine dinucleotide
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BSA	bovine serum albumin
°C	degrees celcius
CHO	Chinese hamster ovaries
CQ	chloroquine
CQR	chloroquine-resistant
CQS	chloroquine-sensitive
CSP	circumsporozoite surface protein
CTP	cytosine triphosphate
DDT	dichloro-diphenyl-trichloroethane
DMA	dimethylamiloide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
Dnase1	deoxyribonuclease 1
E. coli	*escherichia coli*
E.D.T.A.	ethylenediaminetetra-aceticacid
EIPA	5-(N-ethyl-isopropyl) amiloride
f	femto
g	gram
FPIX	ferriprotoporphyrine IX
GDP	guanosine diphosphate
GST	glutathion-S-transferase

GTP	guanosine triphosphate
HBs	hepatitis b surface antigen
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethansulphonic acid
hr	hour
IC_{50}	inhibitory concentration 50
IPTG	isopropyl-β-D-thiogalactopyranoside
iRBC	infected red blood cells
ITP	inosine triphosphate
KDa	kilodalton
M	molar
m	milli
μ	Micro
MBS	maleimidobenzoyl-N-hydroxysuccinimide ester
MDR	multidrug resistant
min	minute/s
ml	milliliter
mV	millivolt
n	nano
NBT	nitro blue tetrazolium
N. D.	not determined
NEM	N-ethylmaleimide
NHE	Na^+/H^+-exchanger
P.	Plasmodium
PBS	phosphate buffer saline
PES	phenazine ethosulfate
P. falciparum	*Plasmodium falciparum*
Pfmdr1	*Plasmodium falciparum* multidrug resistance gene 1
PGEX	plasmid expression vectors
Pgh1	P-glycoprotein homologue 1
Pgp	P-glycoprotein

pH	negative logarithm of the hydrogen concentration ion
Pi	phosphate inorganic
pLDH	parasite lactate dehydrogenase
PMSF	phenylmethyl sulfonyl fluoride
PPM	parasite plasma membrane
PVM	parasitophorous vacuolar membrane
Rpm	rotation per minute
R. T.	room temperature
s. d.	Standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEM	transmission electron microscopy
UTP	uridine triphosphate

CHAPTER 1

INTRODUCTION

1.1 Introduction

Malaria remains one of the most serious infectious disease in tropical regions of the world, resulting in more than 400 million new cases every year mostly of young children and up to 1.5 to 2.7 million deaths (World Health Organisation, 1997). Human malaria is caused by four species of the *Plasmodium* parasite, namely *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malaria*. Of these species, the most abundant and fatal is *P. falciparum*. Despite major control campaigns, the situation is deteriorating in many areas. Drug resistance, especially multidrug resistance, and difficulties in eradicating the mosquito vector have been the basis of malaria resurgence over the past 20 years. In addition, massive problems with logistic, planning, allocation of resources, and a lack of operational research have contributed greatly to the failure of malaria eradication

An individual progressing episodically from shaking chills through intense fevers to drenching sweats is a classic description of the clinical manifestation of malaria. Other common symptoms includes headaches, jaundice, vomiting and diarrhea. Cerebral malaria caused by *Plasmodium falciparum* is responsible for the majority of deaths. Renal failure, hypoglycemia, severe anemia and pulmonary edema may also contribute to morbidity due to malaria. The severity of malaria illness depends largely on the immunological status of the infected individual. Partial immunity develops over time through repeated infections. Many infected people in areas where malaria is

endemic are asymptomatic and therefore contribute greatly to the spread of the disease.

1.2. Life cycle of the malaria parasite

The parasite requires two hosts, a female *Anopheles* mosquito and a human. The life cycle of *Plasmodium* is a complex process consisting of an exogenous sexual phase (sporogony) in the mosquito and an endogenous asexual phase (schizogony) in human. The latter phase is itself divided in two developmental cycles, namely exoerythrocytic and erythrocytic stage.

Plasmodium is transmitted to a human being by the bite of an infected female mosquito of the malaria-transmitting *Anopheles* species. The mosquito injects a small amount of saliva containing anticoagulants as well as parasite sporozoites. These sporozoites disappear rapidly from the circulation and enter the hepatic cells where they multiply. The merozoites released into the blood stream by rupture of the hepatocytes initiate the erythrocytic stage (Figure 1). During a 48-hr (or 72-hr for *Plasmodium malariae*) intraerythrocytic stage, the merozoite develops successively into a ring stage, a trophozoite, and finally a schizont, which divides into 16-24 merozoites. After several successive erythrocytic cycles, some trophozoites become differentiated into sexual forms, namely gametocytes. The male and female gametocytes are the forms which are infective to the vector in which they fuse to form a zygote and initiate sporogony. The zygote matures into an ookinete, which penetrates the stomach wall of mosquito. The resulting oocyst asexually multiplies to form sporozoites, which migrate to the mosquito salivary glands.

It is the intra-erythrocytic stages of the malaria parasite that produce the disease pathology. Most death occurs due to a complication of infections with *P. falciparum*, whereby erythrocytes infected with mature-stage parasites adhere to the vascular endothelium of post-capillary venules, particularly in the brain (For a review, see

Grau and De Kossodo, 1994). Whilst the blood forms of the parasites cause most of the pathology of the disease, they are also the stages that are most susceptible to attack by antimalarial drugs.

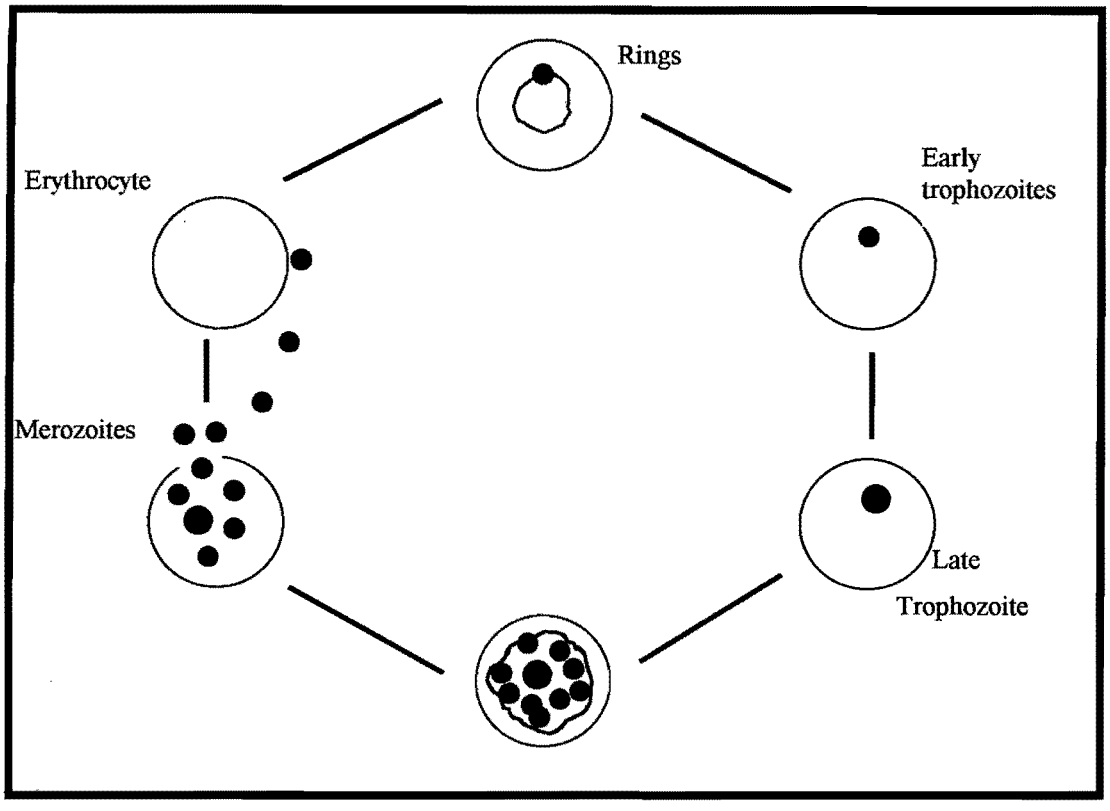

Figure 1: The intraerythrocytic cycle of the malaria parasite

1.3. Prevention and control

Attempts to eradicate mosquitoes have relied on environmental sanitation and use of insecticides. Larva stages of the mosquito are controlled by either eliminating potential breeding sites or by employing chemical and biological methods while the arsenal against the adult mosquitoes includes chlorinated hydrocarbons, organophosphate compounds, carbamates and pyrethroids. Although the extensive eradication programs of the 1950s, based on the insecticide control generated

impressive results in North America and Europe, by the mid-1960s, it became clear that eradication was not technically or economically feasible in many areas (World Health Organisation Expert Committee on Malaria, 1967). Due to selection pressure, mosquitoes have developed resistance to some pesticides, particularly to dichloro-diphenyl-trichloroethane (DDT), the most widely used pesticide in public health. In 1980, a total of 51 species of Anopheles showed resistance to pesticides, 34 of which were resistant to DDT and 30 of those were cross-resistant to other pesticides (Bruce-Chwatt, 1985). More recently, pyrethroid-resistant *Anopheles gambia* have been reported in Western Africa (Chandre *et al.*, 1999). Another approach consists of avoiding contact with the mosquitoes. Protective clothing, insect repellents, mosquito coils, and bednets have been shown to be effective in reducing the incidence and prevalence of malaria in some areas.

Vaccination would be a major advance in malaria control. The feasibility of a malaria vaccine is supported by two observations: first, volunteers immunised with irradiated sporozoites were protected against challenge with heterologous *Plasmodium falciparum* sporozoites (Clyde, 1975) and, second, 3-10 years of continuous exposure to naturally transmitted *Plasmodium falciparum* prevents severe disease and death as long as exposure is maintained (Mc Gregor *et al.*, 1956). Vaccination with SPf66, a synthetic peptide polymer containing sequences from several malarial proteins, had been promising showing 38.8% efficacy in human trials conducted in South America (Valero *et al.*, 1993) and evidence of borderline efficacy in Tanzania (Alonso *et al.*, 1996). Unfortunately, field trials in The Gambia (D'Alessandro *et al.*, 1995) and in Thailand (Nosten *et al.*, 1996) were not as successful and failed to show any protection against *Plasmodium falciparum* in infants. Another promising vaccine, the pre-erythrocytic RTS,S vaccine based on the circumsporozoite surface protein (CSP) of *Plasmodium falciparum* genetically linked to the hepatitis surface (HBs) antigen provided protection against homologous challenge in 6 out of 7 malaria-naïve volunteers (Stoute *et al.*, 1997). However, an ideal vaccine should induce immune responses against each stage of the *Plasmodium* spp life cycle. Antigens from sporozoites (CSP, SSP2/TRAP), the liver stage, merozoites (MSP-1, AMA-1),

trophozoites, gametocytes (Pfs48/45), toxic products released from infected erythrocytes and the infected erythrocytes are all under investigation for a vaccine development (Doolan *et al.*, 1997). Still other approaches to vaccine development such as the multiple gene or multiple antigen are expected.

As there is not yet a useful vaccine against this disease, the major forms of treatment and control still rely on the use of chemotherapeutic agents. Quinine extracted from the Cinchona tree was the first treatment for malaria to reach the Western world. Quinine has its origin in Peru, where Indians chewed the bark of the Cinchona tree to combat bad fever. In 1820, a method of purifying quinine from these trees was developed by Pelletier and Caventou in Paris (Russell, 1955). Salts of the isolated alkaloid, quinine soon became available commercially for clinical use and this remained the only source of this drug until it was synthesised in 1944. Quinine is now considered too toxic for prophylaxis or routine treatment of malaria, but it is still used as an intravenous injection to treat severe malaria. In 1946, as a result of a large-scale search for less toxic analogues of quinine, the 4-aminoquinoline, chloroquine was produced (Loeb *et al.*, 1946). Chloroquine has been used extensively ever since, because of its efficiency and safety, particularly in pregnant women, and its low cost.

Due to increases in the incidence of infection of U.S. army troops in Vietnam with chloroquine-resistant *Plasmodium falciparum*, a large drug screening programme was initiated by the Walter Reed Army Institute of research, that lead to the development of mefloquine and halofantrine (Ohnmacht *et al.*, 1971; Colwell *et al.*, 1972; Schmidt *et al.*, 1978a and 1978b). Mefloquine is most effective against multidrug resistant strains of *Plasmodium falciparum* although reports indicate that mefloquine resistance have emerged (Nosten *et al.*, 1991). Other antimalarials that have been developed include the quinine-like compounds such as amodiaquine and proguanil and the antifolate antagonists (pyrimethamine and sulfadoxine). More recently, a new class of compounds, the sesquiterpene endoperoxide has emerged with the isolation of artemisinin in 1972 from a traditional Chinese medicinal herb

Qinghao (Artemisia annua) (Klayman, 1985). Artemisinin and its derivatives have been shown to be particularly effective against most multi-resistant parasites. Furthermore, these drugs have been widely used in China where 1 million patients have been treated with no significant toxicity reported up to now (Hien and White, 1993). However, the generation of artemisinin-resistant mutants of *Plasmodium falciparum* after mutagenic treatment with N-methyl-N'-nitronitrosoguanosine has demonstrated the possibility of the development of resistance to this new antimalarial (Inselburg, 1985). Resistance of *Plasmodium falciparum* to all the major drugs used for the treatment of malaria has emphasised the need to develop novel antimalarial or to find a mechanism that circumvents the phenomena of drug-resistance.

1.4. Chloroquine

Chloroquine is the most common, well-tolerated and cost-effective drug for prophylaxis and therapy of malaria. It was widely used before the first resistant parasites were detected in 1959 in South America and a year later in South Asia. Since this time, resistance has spread rapidly and it is now present in all regions where malaria is endemic. Over the last 30 years, chloroquine resistance has become an increasingly serious problem. Chloroquine has been mixed with food, in the 1950s in order to control malaria at the population level. This has probably contributed to the development of chloroquine resistance by exposing parasites to sublethal concentrations. It is still not fully understood how *P. falciparum* becomes resistant to chloroquine.

1.4.1. Mechanism of chloroquine action

Early studies suggested that interaction of chloroquine with plasmodial DNA might be responsible for the antimalarial activity of the drug by inhibiting DNA replication and RNA synthesis. However, the concentrations of chloroquine required to inhibit

the growth of parasite were three orders of magnitude lower that those that had been reported to inhibit DNA synthesis (Parker and Irvin, 1952). Furthermore, chloroquine interacts more strongly with CG-rich DNA (Allison *et al.*, 1965) while the plasmodial DNA is strikingly AT-rich. Thus, this theory could not explain the preferential inhibition of malaria parasites as compared to the host cells.

Early clinical investigations of the antimalarial activities of chloroquine noted that drug action was limited to those stages of the parasite life cycle, which were actively degrading hemoglobin. It has been shown that rings, trophozoites and young gametocytes of *P. falciparum* were morphologically degenerated by chloroquine treatment while exoerythrocytic stages were unaffected (Fairley, 1947). After treatment with chloroquine, the first changes that are seen in ultrastructural examination are the swelling of the parasite food vacuoles and accumulation of undigested hemoglobin in endocytic vesicles (Macomber *et al.*, 1967; Aikawa, 1972) supporting the idea that the food vacuole is the site of chloroquine action. It has been assumed, therefore, that chloroquine interferes with the metabolic processes involved in the uptake or digestion of hemoglobin.

Chloroquine accumulates in the acid food vacuoles of *Plasmodium falciparum* partially as a result of its properties as a weak base. Chloroquine is a diprotic weak base (pKa_1=8.1, pKa_2=10.2). In its uncharged form, it can diffuse freely through membrane into the cell. In acidic compartments, it will be doubly protonated and membrane impermeable. Therefore, the extent of chloroquine accumulation depends on the difference in pH between the parasite food vacuole and the extracellular environment (Homewood *et al.*, 1972). Krogstad *et al.* (1985) measured the pH of the food vacuole (pH 5.2-5.4) by spectrofluorimetry. They showed that the vacuoles could be stimulated to acidify their interior by the addition of MgATP and have their internal pH increased by the addition of weak bases. They also demonstrated that chloroquine as well as NH_4Cl inhibited parasite growth at concentration virtually identical to those that increased parasite vacuole pH. The alkalinisation of the food vacuole as a result of chloroquine accumulation would inhibit the hydrolytic enzymes

responsible for hemoglobin degradation which function optimally at an acidic pH (Goldberg at al., 1990). Chloroquine has also been shown to directly inhibit the activity of partially purified hemoglobin-degrading proteases (Vander et Jagt, 1986) and the protein degradation in trophozoite-infected erythrocytes (Zarchin *et al.*, 1986). This lead to the hypothesis that inhibition of lysosomal function might lead to an inhibition of hemoglobin digestion and starvation of the parasite (Homewood *et al.*, 1972). However, none of these effects can explain the selective activity of chloroquine against the parasite. While the inhibition of hemoglobin digestion by protease inhibitors is reversible upon removal of the inhibitor (Rosenthal *et al.*, 1988), the inhibition of parasite growth by chloroquine is irreversible (Ginsburg and Krugliak, 1992). Moreover, mathematical modelling by Yahon *et al.* (1985) suggested that at therapeutic levels, chloroquine accumulation caused minor changes in the food vacuole pH. However, the idea that chloroquine accumulation leads to alkalinisation was supported by Krogstad and Schlesinger (1987b) as it was demonstrated that biologically active concentrations of chloroquine increase the pH of the parasite's food vacuole.

One of the most likely hypotheses to explain the action of chloroquine is the decoupling of ferriprotoporphyrine IX sequestration into hemozoin. The parasite degrades the hemoglobin in the food vacuole, and uses the amino acids as a major nutrient source. When ferrous heme is released from globin it oxidizes into the ferriprotoporphyrine IX which is toxic to the parasite. This ferric form can damage membranes and inhibit various enzymes including proteases. Rather than degrading or excreting the heme, the parasite has evolved a novel pathway for its detoxification by incorporating it into an insoluble crystalline form called haemozoin or malaria pigment. The haemozoin is a non-toxic polymer of heme units linked by the central ferric ion of one unit to the carboxylic side group of the next heme group (Slater *et al.*, 1991).

Following early observations that free FPIX was able to form complexes with chloroquine (Cohen *et al.*, 1964; Macomber *et al.*, 1967) it was hypothesized that

chloroquine exerted its effect by forming toxic complexes with free FPIX released in situ (Chou and Fitch, 1980a). More recent work has shown that chloroquine is able to inhibit the polymerisation of hematin, suggesting a mechanism by which free FPIX or FPIX-chloroquine complexes may concentrate in the food vacuole causing the parasite death (Slater and Cerami, 1992). It was initially believed that this reaction was catalysed by a haem polymerase and that chloroquine inhibited this polymerase activity. The fact that this polymerase activity could survive extensive boiling and protease treatments argued against a proteinaceous catalyst (Dorn *et al.*, 1995). Instead, the heme polymerising activity of the parasite extract has been shown to be due to the presence of preformed heme polymers. These polymers of heme are thought to act as nucleation centres, allowing the efficient addition of further heme monomers. In addition, it was shown that spontaneous formation of β-haematin polymers can be induced in vitro and that this process is directly inhibited by chloroquine (Egan *et al.*, 1994). These data suggest that chloroquine inhibits hemozoin elongation, probably by formation of a chloroquine-heme complex. There is also evidence indicating that chloroquine depolymerise preformed complexes (Pandey and Tekwani, 1997). Further evidence that FPIX is the receptor for chloroquine came from studies using the plasmepsin I inhibitor, Ro 40-4388. This inhibitor was shown to be antagonistic to the antimalarial action of chloroquine on cultured parasites (Moon *et al.*, 1997; Mungthin *et al.*, 1998; Bray *et al.*, 1999). Plasmepsin I is an aspartic proteinase involved in hemoglobin degradation (Gluzmann *et al.*, 1994); its inhibition prevents the FPIX release from hemoglobin, thereby removing the chloroquine target. Inhibition of parasite enzymes (Ginsburg and Geary, 1987; Vander Jagt *et al.*, 1987) and disruption of membranes (Orjih, *et al.*, 1981) by free heme or chloroquine-heme complexes could account for the toxic effect mediated by chloroquine.

Recently, the inhibition of glutathione-dependant degradation of heme by chloroquine as been proposed as an explanation for its antimalarial action (Atamna and Ginsburg, 1995). Surprisingly, only 30% of the heme appears to be sequestrated into haemozoin in *Plasmodium falciparum* (Ginsburg *et al.*, 1998; Loria *et al.*, 1999). Atamna and

Ginsburg (1995) argued that the excess heme translocates across the food vacuole membrane to reach the parasite cytosol where it is degraded by glutathione. Recently, chloroquine was demonstrated to inhibit the gluthatione-dependent degradation of heme. Moreover, it has been shown that treatment of *Plasmodium falciparum*-infected erythrocytes with chloroquine results in a dose- and time-dependant accumulation of heme in the membrane fraction of these cells in correlation with the cytotoxic effect of the drug (Ginsburg *et al.*, 1998; Famin *et al.*, 1999). Thus the inhibition of glutathione-dependant degradation of heme would result in increased levels of free heme.

Another explanation for chloroquine action could be the inhibition of the peroxidase degradation of haem. The parasite has to face two major toxic waste problems: the release of heme and the production of H_2O_2. Upon degradation of hemoglobin, the heme is oxidised from the Fe(II) state to the Fe(III) state which results in the production of H_2O_2 (Atamna and Ginsburg, 1993). In the food vacuole, host-derived catalase and peroxidase might contribute to H_2O_2 breakdown although parasite proteases will rapidly destroy the host enzymes. It has been shown that heme can react with H_2O_2 displaying both catalase and peroxidase activities (Green *et al.*, 1996) and that chloroquine is an efficient inhibitor of the catalase activity of heme (Ribeiro *et al.*, 1997; Loria *et al.*, 1999). Moreover, under conditions designed to resemble those found in the food vacuole, i.e., at pH 5.2 in the presence of protein, it was found that the catalytic activity of haem could contribute significantly to the degradation of haem and to the breakdown of reactive oxygen species (Loria *et al.*, 1999). Therefore, a possible route to degradation of haem is by reacting with H_2O_2. Consequently, it has been proposed that chloroquine exerts its antimalarial activity by causing a build-up of H_2O_2 which may cause peroxidative damages to proteins and lipids.

1.4.2. Mechanism of chloroquine resistance

The biochemical basis of resistance is not completely understood. However, chloroquine is concentrated in the acidic vacuole of the parasite and it is clear that resistant parasites accumulate less chloroquine than sensitive isolates. The mechanism by which chloroquine resistant parasites accumulate less chloroquine is highly controversial but four basic possibilities exist. (1) A rapid efflux of chloroquine from chloroquine resistant parasites, (2) an insufficient chloroquine uptake mechanism in chloroquine resistant parasites due to a defective food vacuole acidification, an alteration in membrane permeability or in the specificity of an permease or other transporters or by sequestration of chloroquine in another compartment of the cell, (3) an alteration in the drug target site, (4) by metabolism of chloroquine into an inactive form.

1.4.2.1. Chloroquine metabolism by the parasite

Cytochrome P-450-dependant activities (Salganik *et al.*, 1987) have been demonstrated and an association between chloroquine susceptibility and cytochrome P-450 drug-metabolising enzyme activity has been found in both *Plasmodium berghei* and *Plasmodium falciparum* (Ndifor *et al.*, 1990). Cimetidime and other agents known to inhibit cytochrome P-450 enzymes were able to enhance parasite susceptibility in *Plasmodium falciparum* in vitro and *Plasmodium berghei* in vivo (Ndifor *et al.*, 1993). However, thin-layer chromatography studies showed that ^{3}H-chloroquine accumulated in resistant *Plasmodium falciparum* parasites was indistinguishable from the parent compound (Gluzmann *et al.*, 1987).

1.4.2.2. Increased chloroquine efflux

It was originally proposed that chloroquine-resistance was associated with an efflux mechanism. This hypothesis relied on the existence of a verapamil-sensitive drug exporter which is thought to be responsible for an apparent increase in the rate of efflux of chloroquine from chloroquine resistant parasites compared with chloroquine sensitive parasites, resulting in a greater reduction of drug accumulation within the infected erythrocyte in chloroquine-resistant parasites than in chloroquine-sensitive parasites (Krogstad *et al.*, 1987a). It was found that the initial rate of chloroquine uptake was identical in sensitive and resistant strains. Furthermore the initial rate of efflux was demonstrated to be more rapid from the resistant parasites than the sensitive even when equivalent amounts of chloroquine were preaccumulated by the sensitive and resistant parasites. This accelerated efflux of chloroquine is energy-dependent as it is inhibited by the removal of glucose or the addition of the ATPase inhibitor vanadate (Krogstad *et al.*, 1992). However, the biochemical data indicating an increased level of chloroquine efflux in chloroquine-resistant parasites have since been questioned (Ginsburg and Kruigliak, 1992) and subsequent studies have indicated that efflux rates of chloroquine-resistant and –sensitive strains are similar (Bray *et al.*, 1992a).

It has been suggested that the mechanism of chloroquine resistance has similarities with the multidrug resistance phenotype of mammalian tumor cells, as verapamil, an agent which reverses multidrug resistance of tumor cells is also able to modulate chloroquine resistance (Martin *et al.*, 1987). The mechanism of resistance in tumor cells can be defined as increased drug efflux that is mediated by increased expression of the P-glycoprotein (Cornwell *et al.*, 1986). This P-glycoprotein is an ATP-dependent pump encoded by the *mdr* gene which is amplified in tumor lines resistant to multiple drugs.

Plasmodium falciparum has been shown to possess at least two *mdr*-like genes (*pfmdr1* and *pfmdr2*), one of which has been suggested to confer the chloroquine

resistant phenotype. *Pfmdr1* has been sequenced (Foote *et al.*, 1989) and has been shown to encode a 160 kDa P-glycoprotein homologue (Pgh1). Mammalian *mdr* genes typically share 50% homology with *pfmdr1* (Foote *et al.*, 1989). The *Plasmodium falciparum* P-glycoprotein is of interest because its putative transport function is unknown and because it has been linked to the chloroquine resistance phenotype. Cowman *et al.* (1991) showed that Pgh1 is localised primarily to the membrane of the digestive vacuole of *Plasmodium falciparum* trophozoites and that a small fraction appears to be localised to the parasite membrane. However, photoaffinity studies using a photoreactive analogue of chloroquine failed to demonstrate an interaction of chloroquine with Pgh1 in *P. falciparum* (Foley *et al.*, 1994).

Pgh1 belongs to the superfamily of ATP- binding cassette transporters (ABC). Members of this group include the cystic fibrosis transmembrane conductance regulator (CFTR), the mating pheromone transporter in *Saccharomyces cerevisiae* (ste6), the mammalian multidrug resistance-associated protein (MRP), hlyb and others transporters found in bacteria (Higgins, 1992). The typical ABC transporter, including Pgh1, is formed by two highly homologous halves (62% homology) connected by a hydrophilic linker region. Each half contains six predicted transmembrane domains, glycosylation consensus sequences, and one consensus site for ATP-binding. The latter site contains two short motifs, the Walker motif associated with ATP-binding properties (Walker *et al.*, 1982).

It has been demonstrated that transport is dependent on ATP-binding and hydrolysis (Cornwell *et al.*, 1987b). It has also been shown that chemosensitisers reverse MDR by increasing accumulation of drug within cells, bind specifically to membrane vesicles from MDR cells and inhibit vinblastine analogue photoaffinity labelling of the P-170 (Cornwell, 1987a). Photoaffinity data reveals that some drugs bind to the MDR protein (Cornwell, 1986), although this is indirect evidence for active transport since many transporters bind compounds specifically but do not transport them (amiloride and Na^+/H^+ exchanger, stilbene and Cl^-/HCO_3^- exchanger, etc.).

Measurement of intracellular pH (pHi) for MDR cells reveals that it is closely correlated with the relative steady-state of efflux, suggesting that the relevant function of MDR-protein expression in these cells may be to raise intracellular pH directly or indirectly such that less drug is sequestered in a charge-dependent manner (Roepe, 1992). This author also found that verapamil affects pHi significantly in the resistant cells and suggested that the MDR protein modulates intracellular pH and indirectly promotes the efflux of chemotherapeutic without performing active drug transport. It has been reported that Pgp can function as an ATP-dependent, cell volume-regulated chloride channel (Gill *et al.*, 1992; Valverde *et al.*, 1992). However, it is possible that Pgp rather act as a chloride channel regulator (hardy *et al.*, 1995; Bond *et al.*, 1998).

Pgh1 is expected to share at least some of these features with other P-glycoproteins, although presently only ATP-binding has been demonstrated for Pgh1 (Karcz and Cowman, 1991). Initially, Pgh1 present on the food vacuole membrane was thought to expel drugs from this organelle and therefore overexpression of Pgh1 was expected to increase the rate of removal of chloroquine from *Plasmodium falciparum* thereby rendering the parasite chloroquine resistant. It was also proposed that amino-acid substitution favouring expulsion might occur in the protein. However, the protein is orientated with its nucleotide-binding domain exposed to the parasite cytoplasm (Karz *et al.*, 1993b), which suggests pumping of substrate into the food vacuole rather than out. Nevertheless, mutation in *pfmdr1* may have changed the substrate specificity of the wild-type (CQS) protein in such a way that chloroquine became a poor substrate. The same mutation associated with chloroquine resistance may also have affected translocation of essential endogenous substrates of Pgh1 into or out of the digestive vacuole. It has been difficult to correlate any overexpression or mutation in this protein with chloroquine resistance. Foote *et al.* (1989) found that the *mdr* gene was amplified in some resistant isolates but not in any sensitive ones, while Wilson *et al.* (1989) correlated amplification of the same gene with increased mefloquine, but not chloroquine resistance. To determine if *pfmdr1* was linked to chloroquine resistance, Wellems *et al.* (1990) performed a genetic cross between

chloroquine-resistant and chloroquine-sensitive clones of *Plasmodium falciparum*. Their result showed that the rapid efflux is controlled by a single gene or a closely related group of genes but there was no linkage between the rapid efflux, chloroquine-resistant phenotype and *pfmdr1*. Analysing the complete sequence of two chloroquine-resistant (CQR) and 5 chloroquine-sensitive (CQS), Foote *et al.* (1990) were able to predict the CQS/CQR status of a further 34 out of 36 isolates. They predicted that both a *pfmdr1* allele competent for chloroquine-resistance and a mutation in a second unknown gene is required for chloroquine-resistance. However, further studies showed that the level of chloroquine resistance in a number of field isolates of a *Plasmodium falciparum* did not correlate with the level of Pgh1 expression (Cowman *et al.*, 1994) and there was no correlation between particular alleles of *pfmdr1* and chloroquine resistance (Wilson *et al.*, 1993). It is therefore unlikely that an amplification of the *pfmdr1* is responsible for chloroquine resistance.

Using an heterologous expression system for the *pfmdr1* gene to express Pgh1 in CHO cells, Van Es *et al.* (1994a) found that wild-type Pgh1 can mediate chloroquine accumulation whereas CQR-associated mutated form of the protein cannot. This finding demonstrates that there is more than one CQR mechanism which probably involves different sets of genes, depending on the *Plasmodium falciparum* isolate analysed.

It was first demonstrated that CQR strains of *Plasmodium falciparum* could be sensitised by verapamil (Martin *et al.*, 1987). Later studies revealed that CQR in this parasite could also be reversed by tricyclic anti-depressants including desipramine (Basco *et al.*, 1990) and tricyclic anti-histamines such as cyproheptadin. The ability of verapamil to increase steady-state chloroquine accumulation was found to be totally insufficient to explain the increase in chloroquine activity by the drug. In contrast, when chloroquine accumulation was increased by raising the medium pH, the corresponding shift in sensitivity to chloroquine could be accurately predicted (Bray *et al.*, 1994). It is possible that verapamil could inhibit the efflux of chloroquine from a low capacity, high affinity site inside the parasite. These data have suggested

that at least in highly chloroquine-resistant parasites, two phenotypically distinct mechanisms for chloroquine-resistance exist, one that is verapamil reversible, and one that is not.

1.4.2.3. Reduced uptake

Many studies have demonstrated that chloroquine resistance is associated with a reduced initial rate of drug uptake (Ginsburg *et al.*, 1991; Bray *et al.*, 1994; Martiney *et al.*, 1995). Early studies have demonstrated that chloroquine uptake into *Plasmodium falciparum* consisted of a component which is saturable at nanomolar concentrations and a component which is non-saturable in the submicromolar range (Fitch, 1970). There are number of hypotheses accounting for a reduced uptake.

1.4.2.3.1. Weakened proton pump

As chloroquine accumulates in the food vacuole due to its weak base properties, the reduced accumulation of chloroquine in resistant parasites could result from a modified pH (Geary *et al.*, 1990). Kinetic modeling of chloroquine uptake by sensitive and resistant *Plasmodium falciparum* was shown to be consistant with the decrease activity of a vacuolar proton pump (Ginsburg and Stein, 1991). Bray *et al.* (1992b) hypothesize that the intravacuolar pH of resistant strains is higher than for sensitive strains as a consequence of a weakened pump in the vacuole of resistant strains. This hypothesis was supported by the fact that Bafilomycin A1 significantly reduces uptake of [^{3}H]-chloroquine into both chloroquine resistant and chloroquine sensitive strains of *Plasmodium falciparum*. Furthermore, chloroquine resistant strains were found more sensitive to Bafilomycin A1 than chloroquine sensitive strains. Although, the genes encoding the A subunit (VAP-A) and the B subunit (VAP-B) of the plasmodial vacuolar proton pump have been cloned, no mutations

have been found that can account for chloroquine resistance (Karcz *et al.*, 1993a; Karcz *et al.*, 1994).

1.4.2.3.2. Regulation of a chloride-channel

Ferrari *et al.* (1991) claimed that chloroquine resistance is not the consequence of a specialised drug-exporter in erythrocytes infected with chloroquine resistant parasites, which is absent or deficient in chloroquine sensitive strains, but rather the consequence of the presence of a drug importer in erythrocytes infected with chloroquine sensitive parasites, which is deficient in chloroquine resistant parasites.

Van Es *et al.* (1994b) investigated the role of Pgh1 in chloroquine transport using a heterologous expression system. Transfection of Chinese hamster ovary cells with *pfmdr1* led to expression of Pgh1 in the lysosomes, which was associated with a decrease in lysosomal pH. Moreover, Chinese hamster ovary cells expressing the Pgh1 protein demonstrated an increased verapamil-sensitive susceptibility to chloroquine while those expressing a mutated Pgh1 did not (Van Es *et al.*,1994a). This result suggests that Pgh1 acts as a chloride channel, enhancing chloroquine accumulation by modulating the *P. falciparum* vacuolar pH, as has been demonstrated for some other members of the P-glycoprotein family (Valverde *et al.*, 1992).

When Martiney *et al.* (1995) forced CQR trophozoites to become phenotypically sensitive by weak base manipulation, they found that verapamil increased the susceptibility of the CQR but has no effect on the toxicity of chloroquine on the CQS. They concluded that verapamil specifically increases chloroquine accumulation in genetically resistant *Plasmodium falciparum* trophozoites independently of weak base processes and phenotypic susceptibility due to external pH manipulation. They raised the hypothesis that the mutation conferring CQR in *Plasmodium falciparum* up-regulates a chloride channel regulator protein, which alters ion conductances that

indirectly control drug transit within the parasite's cytoplasm by setting the cytoplasmic pH and this chloride channel regulator is inhibited by verapamil during reversal of resistance. Thus, while genetic studies indicate that *pfmdr1* mutations are not sufficient to confer chloroquine resistance, it is possible that they may be involved in modulating the level of drug resistance.

1.4.2.3.3. Reduced affinity of a chloroquine importer

Warhust (1988) has suggested that a drug carrier or permease exists within the membrane of the intraerythrocytic parasite that transports chloroquine from the host erythrocyte cytosol to the parasite cytosol, thus facilitating drug uptake into the parasite. Drug resistance could then arise from either a decrease in the number or efficiency of the permease units.

More recent work has implicated a plasmodial Na^+/H^+ exchanger (NHE) in the facilitated import of chloroquine (Sanchez *et al.*, 1997). This group demonstrated that both CQR and CQS *P. falciparum* possess a chloroquine importer with a single binding site for chloroquine and linked altered saturation kinetics of initial chloroquine uptake to the chloroquine-resistant phenotype. All the progeny of a genetic cross between a CQS and strain and a CQR strain of *P. falciparum* (Wellems *et al.*, 1991) exhibit comparable kinetics of one or the other of the parental strains, indicating the influence of a single gene on this phenotype (Sanchez *et al.*, 1997). Amiloride derivatives known as specific inhibitors of Na^+/H^+ antiport, competitively inhibited the initial uptake of chloroquine into infected-erythrocytes leading to the suggestion that chloroquine is directly transported by the parasite plasma membrane NHE in place of sodium and in exchange of protons. Further studies indicated that the NHE of CQS parasites was stimulated by chloroquine suggesting that this drug is taken up by the NHE in the ensuing rapid burst of sodium proton exchange (Wunsch *et al.*, 1998). Subsequently, it was proposed that the NHE of CQR parasites was unable to transport chloroquine, since it was constitutively activated and insensitive

to further stimulation by chloroquine. The reversal of chloroquine resistance by verapamil was proposed to occur via a calcium/calmodulin-dependent pathway, although no evidence was presented (Sanchez *et al.*, 1997).

Bray *et al.* (1999) have questioned the validity of this model since replacement of sodium in the incubation buffer by a non-transportable cation such as choline did not result in enhanced uptake by isolated CQS parasites, suggesting that any ability of chloroquine to stimulate the NHE of CQS parasites (Wunsch *et al.*, 1998) is unrelated to the mechanism of chloroquine uptake. In addition, stimulation of chloroquine uptake into CQR parasites was retained in sodium-free buffer. Therefore, these results are incompatible with the notion that verapamil modulates the NHE activity via a calcium/calmodulin-dependent pathway (Sanchez *et al.*, 1997).

Analysis of a genetic cross between a chloroquine-sensitive and chloroquine-resistant strain of *Plasmodium falciparum* identified a single chloroquine-resistant locus within a 400kb segment of chromosome 7 (Wellems *et al.*, 1990, 1991). Recently, this locus was mapped to a 36-kb region and a candidate gene, *cg2*, has been identified within this region (Su *et al.*, 1997). The cg2 protein encodes a 330-kDa protein, which has been shown by immunogold electron microscopy to be located at both the parasitophorous membrane, the space separating the parasite from the erythrocyte host, and the food vacuole. One particular set of polymorphism correlates with chloroquine resistance while numerous differences were observed in sensitive parasites. However, one clone from Sudan was found to have the polymorphism associated with the chloroquine resistant phenotype, but exhibited chloroquine sensitivity. The possibility that the cg2 protein is an integrated Na^+/H^+ exchanger was raised, although, there is no evidence to support this hypothesis (Sanchez *et al.*, 1998). However, the function of cg2 has not been established and another promising candidate, pfcrt, is also being investigated for its role in the mechanism of chloroquine-resistance (Wellems, personal communication to our laboratory).

1.4.2.3.4. Reduced affinity of chloroquine-FPIX binding

Chloroquine has been shown to form complexes with free hematin in vitro (Chou *et al.*, 1980b) and it was originally suggested that saturable uptake into cells was due to the binding of chloroquine to hematin in the plasmodial food vacuoles. This hypothesis was refuted since the amount of FPIX present in the food vacuole was estimated to be insufficient to account for the total chloroquine uptake in the infected-erythrocytes (Ginsburg *et al.*, 1987). However, it has been since demonstrated that a high proportion of total uptake is non-saturable and only the saturable component of drug uptake is relevant to antimalarial activity (Bray *et al.*, 1998). Recent evidence found that FPIX produced by the parasite is enough to fully account for the saturable accumulation of chloroquine. The number of chloroquine binding sites in the parasite is significantly reduced by the presence of Ro 40 –4388, a specific inhibitor of hemoglobin proteolysis, indicating that hematin binding is required for chloroquine concentration into the food vacuole. Bray *et al.* (1998) suggested that the specific accumulation and antimalarial activity of chloroquine are all determined by the saturable equilibrium binding of chloroquine to FPIX. Recently, it was found that chloroquine-resistant parasites have a reduced apparent affinity of chloroquine-FPIX binding that is reversible by verapamil, compared with chloroquine-sensitive parasites (Bray *et al.*, 1999). Therefore, it has been postulated that the altered binding of chloroquine to FPIX could be achieved by direct binding to FPIX or by altering vesicular pH or buffering capacities.

1.5. Scope of the study

Chloroquine has to cross several membranes, the erythrocyte plasma membrane, the parasitophorous membrane, the parasite plasma membrane and the membrane of the food vacuole before reaching the food vacuole cytosol where it accumulates. The contribution of these membranes to the process of chloroquine accumulation has not yet been clearly defined; neither has their possible role in resistance. Chloroquine

accumulation has been extensively studied in purified food vacuoles and it has been shown that chloroquine accumulates approximately 20-fold less in isolated food vacuoles than in the same number of infected-erythrocytes (Saliba *et al.*, 1998), suggesting that (1) the food vacuole might not be the major site of accumulation or might require a cytosolic factor to allow chloroquine transport, (2) the isolated food vacuoles might be leaky, (3) the purified food vacuoles are not producing heme. However, another likely explanation could be that other membranes might have a role in concentrating chloroquine in the parasitised erythrocytes. Erythrocyte ghosts from infected erythrocytes as well as membrane vesicles from infected erythrocytes have been prepared (Herwaldt *et al.*, 1990). Unfortunately these membrane vesicles comprised several membranes present in the infected-erythrocytes, therefore impeding the elucidation of their participation in the mechanism of chloroquine accumulation. Purified preparation of parasite plasma membranes would represent a major experimental advance for approaching this problem. For this reason, a method was developed to prepare pure and intact parasite plasma membranes from *Plasmodium falciparum* infected-erythrocytes in order to study its possible involvement in the mechanism of chloroquine accumulation and resistance.

This study aims to investigate whether parasite plasma membrane vesicles isolated from sensitive and resistant *Plasmodium falciparum* are capable of differential chloroquine accumulation and whether reversal of chloroquine resistance occurs at the parasite plasma membrane level. Optimal condition for assaying the ATPase activity of these membranes will be determined and P-type and V-type ATPase inhibitors will be used to investigate any correlation between the ATPase activities and the level of chloroquine accumulation in parasite plasma membranes isolated from sensitive and resistant strains of *Plasmodium falciparum*. Since Pgh1 has been reported to be expressed on the parasite plasma membrane, antibodies against Pgh1 will be used to identify and locate this protein in the parasitised erythrocytes and plasma membranes isolated from various strains of *P. falciparum* in order to determine whether its expression on the plasma membrane correlates with the chloroquine sensitivity of the strain. Moreover, the effect of these antibodies on

chloroquine accumulation by purified parasite plasma membranes will be assessed in order to determine the role of Pgh1 in chloroquine accumulation at the parasite plasma membrane level.

CHAPTER 2

Isolation of trophozoites and parasite plasma membranes

2.1 Introduction

Plasmodium falciparum can be obtained by continuous culture of parasitised human erythrocytes (Trager and Jensen, 1976). Although extracellular, axenic development of the erythrocyte cycle of *Plasmodium falciparum* has been obtained (Trager *et al.*, 1992; Williams *et al.*, 1995; Trager *et al.*, 1996), the number of merozoites completing the cycle is not sufficient to permit continuous extracellular culture. Under the best conditions, only 1% of the merozoites further develops into trophozoites. Therefore, the isolation of trophozoites still relies on their liberation from the host cells.

Several methods for the isolation of trophozoites had been developed (for review, see Hamburger and Kreier, 1980) including: osmotic lysis (Dulaney and Stratman-Thomas, 1940), ultrasound disruption (Verain and Verain, 1956), the French press method (D'Antonio *et al.*, 1966), nitrogen cavitation method (Wallach and Conley, 1977), mechanical shearing (Heidrich *et al.*, 1979). However, to prepare intact trophozoites in high yield and of high purity remains technically difficult. These methods either affect the integrity of parasites or fail to remove host materials from parasites.

The most widely used method for freeing parasites from their host cells is the saponin lysis (Christopher and Fulton, 1939) but electron microscopy studies have shown that these parasites are still trapped within the erythrocyte plasma membrane. The most recent procedures include agglutination and lysis by passage through a

series of filters (Heidrich *et al.*, 1982), glycerol-enhanced haemolysis (Wunderlich *et al.*, 1987), and sorbitol lysis (Hoppe *et al.*, 1992). Isolation of viable free parasites from *P. knowlesi*-infected erythrocytes has been obtained by nitrogen cavitation (Nillni *et al.*, 1985).

Released parasites need to be further purified from host cell materials, in particular erythrocytes and their ghosts, as well as unlysed infected erythrocytes. Published methods to achieve this include differential centrifugation (Trager *et al.*, 1990), Percoll gradient (Wunderlish *et al.*, 1987), Ficoll gradient (Nillni *et al.*, 1985), free flow electrophoresis (Heidrich *et al.*, 1982) and chromatography on an immunoaffinity column (Hoppe *et al.*, 1992).

The use of enzyme markers of *Plasmodium falciparum* and human erythrocytes as indicators of parasite purity is well established. The purity of the parasite preparation is commonly assayed for the erythrocyte membrane protein, acetylcholine esterase (AchE) and the parasite cytosolic protein, lactate dehydrogenase (pLDH) in order to evaluate the degree of erythrocyte membrane or parasite cytosol contamination, respectively (Vander Jagt *et al.*, 1982).

Collecting released parasites is only part of the preparation process. The next step involves obtaining plasmodial constituents from isolated trophozoites. A certain number of techniques have been applied for the disruption of freed parasites, including lysis by distilled water (Cook *et al.*, 1971), by Triton X-100 (Sherman *et al.*, 1975), by freezing and thawing (Sherman and Hull, 1960), by sonication (Diggs, 1966), by homogeneization (Rock *et al.*, 1971), or by treatment with a French pressure cell (D'Antonio *et al.*, 1966).

While methods to isolate host cell ghosts from parasitized erythrocytes have been developed (Gruendberg *et al.*, 1983; Wunderlich *et al.*, 1987), there is no published method to isolate the parasite plasma membrane. The isolation of the parasite plasma membrane presents technical difficulties due to the presence of three closely related membranes, the erythrocyte plasma membrane, the parasitophorous vacuolar

membrane (PVM), the endoplasmic reticulum membrane, and the parasite plasma membrane (PPM), as well as the food vacuole membrane. An attempt has been made to prepare membrane vesicles from parasitized erythrocytes (Herwaldt *et al.*, 1990); unfortunately these preparations contain membrane elements of both parasites and host cells. In this method, disruption of the trophozoites was achieved by nitrogen cavitation and the membranes were separated from parasite lysates by discontinuous sucrose gradient centrifugation.

A number of criteria have to be met when isolating subcellular fractions for biochemical experiments. The subcellular constituents have to be pure, intact, isolated in high yield and retain their normal physiological capabilities. This chapter describes a procedure of parasite plasma membrane isolation from *Plasmodium falciparum*-infected erythrocytes without any cross contamination at high yield. In this method, the trophozoites released by saponin treatment were purified from erythrocyte membranes using anti-erythrocyte antibodies fixed to polystyrene beads. These trophozoites were then biotinylated and the parasite plasma membrane was disrupted by nitrogen cavitation. This process allows the membranes to reform into vesicles. The magnetic streptavidin beads bind specifically to the biotinylated parasite plasma membrane vesicles facilitating their recovery with a magnet. These vesicles can then be easily released from the magnetic beads by treatment with dithiotreithol, the key steps in the purification are set out in figure 2.

2.2 Results

2.2.1 Purification of *Plasmodium falciparum* trophozoites

Two methods of lysis for the isolation of *Plasmodium falciparum* trophozoites were examined. For a detailed description of the method see section 7.2. Briefly, parasites synchronised at the trophozoite stage by treatment with 5% sorbitol were released from infected erythrocytes either by saponin or sorbitol treatment. Removal of erythrocytes was achieved by immuno-affinity chromatography. The immuno-

affinity column was prepared with anti-erythrocyte antibodies fixed to polystyrene beads. After lysis of the erythrocytes, the protease inhibitors aprotinin, leupeptin and phenylmethylsulfonyl fluoride (PMSF) were added. Unlysed erythrocytes and erythrocyte membranes were adsorbed onto the immuno-affinity column. The trophozoites were recovered by pelleting.

2.2.2 Trophozoite purity

2.2.2.1 Acetylcholine esterase activity of *Plasmodium falciparum* trophozoites

Detailed methodology is described in chapter 7, section 5. Acetylcholine esterase (AchE) activity of isolated trophozoites was low. AchE activities of trophozoites isolated by saponin lysis, sorbitol treatment followed by immunoaffinity purification and saponin treatment followed by immunoaffinity purification was 0.14 ± 0.02 (n=3), 0.08 ± 0.04 (n=3), 0.05 ± 0.01 μmol/min/mg protein (n=3), respectively. By comparison, the specific activity of AchE in erythrocyte ghosts was 2.30 ± 0.15 μmol/min/mg protein (n=3) (Table 1). Therefore, trophozoites isolated by saponin lysis, sorbitol treatment followed by immunoaffinity purification and saponin treatment followed by immunoaffinity purification exhibit 6%, 3.5% and 2% AchE activity, respectively, when compared to isolated erythrocyte ghosts.

The AchE activity in the isolated trophozoites differed significantly from erythrocyte ghosts AchE activity ($p<0.0001$). The AchE activity in the trophozoites isolated by saponin lysis differed significantly from those further purified by immunoaffinity chromatography ($p= 0.0091$). There is no statistically significant difference between the AchE activities in the immunopurified trophozoites isolated by sorbitol or saponin lysis ($p=0.3659$).

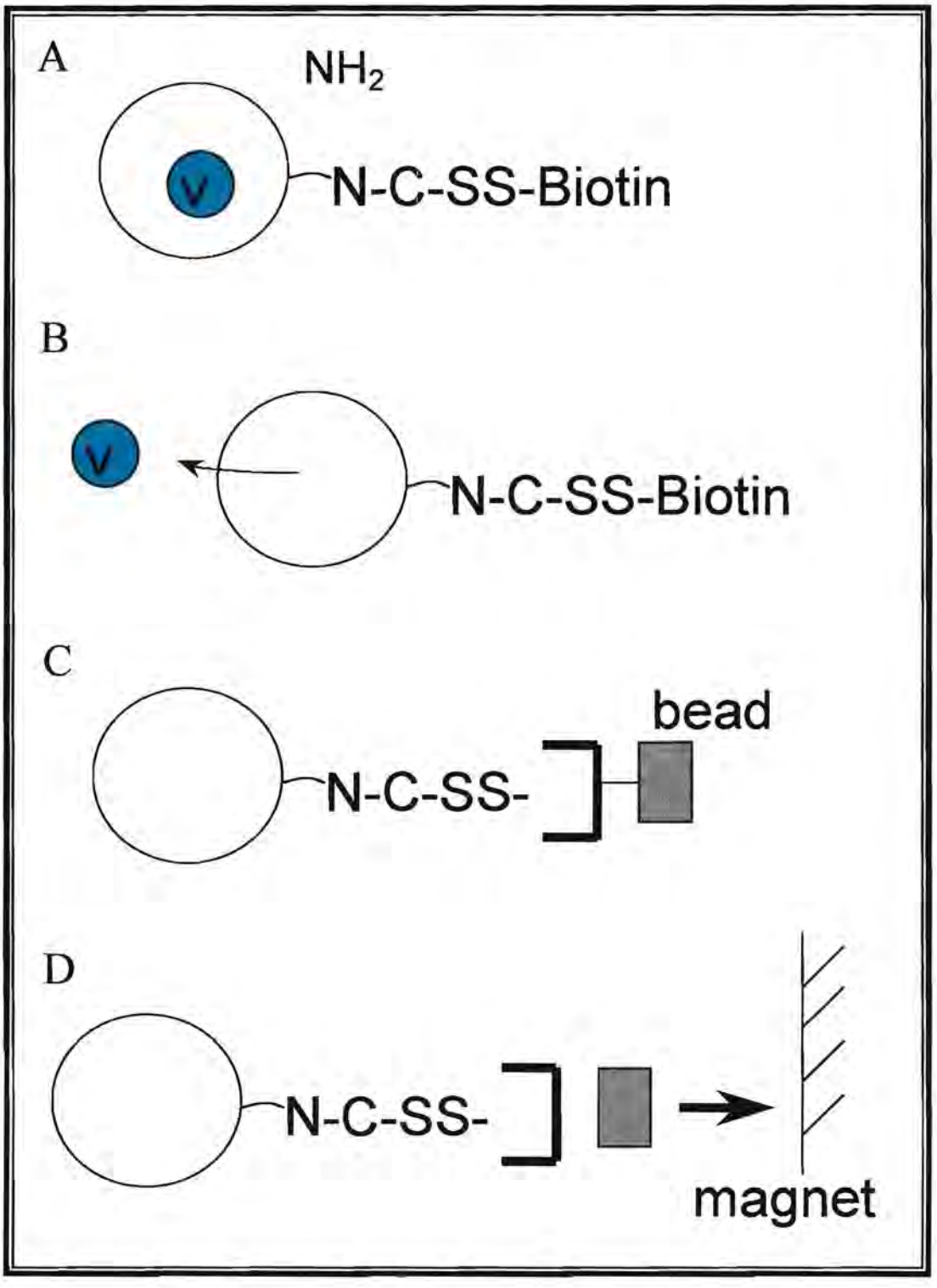

Figure 2: Isolation of *P. falciparum* plasma membrane vesicles. (A) Biotinylation of the trophozoite membrane with a reversible agent. (B) Disruption of the *P. falciparum* plasma membrane by nitrogen decompression. (C) Binding of the magnetic streptavidin beads to the biotinylated plasma membrane. (D) Recovery of the parasite plasma membrane using a magnet.

Table 1. Acetylcholine esterase activity of isolated erythrocyte ghosts and isolated *Plasmodium falciparum* trophozoites.

	Specific activities (μmol/min/mg protein)	
Sample	Vander Jagt *et al.* (1982)	This study
Erythrocyte ghosts	2.30	2.30 ± 0.15
Trophozoites[a]	0.08	-
Trophozoites[b]	0.0	-
Trophozoites[c]	-	0.14 ± 0.02
Trophozoites[d]	-	0.08 ± 0.04
Trophozoites[e]	-	0.05 ± 0.01

[a] Trophozoites isolated by mechanical rupturing of infected erythrocytes followed by differential centrifugation.
[b] Trophozoites isolated by free-flow electrophoresis.
[c] Trophozoites isolated by saponin lysis.
[d] Trophozoites isolated by sorbitol lysis followed by immunoaffinity.
[e] Trophozoites isolated by saponin lysis followed by immunoaffinity.
Values are means ± standard deviation of 3 separate experiments from separate trophozoite isolations or erythrocyte membrane preparation.

2.2.2.2 Parasite lactate dehydrogenase activity of *Plasmodium falciparum* trophozoites

Detailed methodology is described in section 7.6. Parasite lactate dehydrogenase (pLDH) activities of trophozoites isolated by saponin lysis was 0.19± 0.05 (n=3) whereas those further immunopurified displayed a pLDH activity of 0.14 ± 0.03 μmol/min/mg protein (n=3) (table 2). The pLDH activity in the trophozoites isolated by saponin lysis did not differ significantly from those further purified by immunoaffinity chromatography (p= 0.8153).

The pLDH activities of trophozoites were found to be lower in this study than those measured by Vander Jagt *et al.* (1982). These trophozoites were isolated by different methods and an explanation could be that the plasma membrane become leaky after saponin treatment. However, the fact that the trophozoites released by saponin

treatment do not concentrate trypan blue indicates that the integrity of the plasma membrane has been preserved.

Table 2. Parasite lactate dehydrogenase activity of isolated *Plasmodium falciparum* trophozoites.

	Specific activities (μmol/min/mg protein)	
Sample	Vander Jagt *et al.* (1982)	This study
Trophozoites[a]	4.4	-
Trophozoites[b]	3.9	-
Trophozoites[c]	-	0.19 ± 0.05
Trophozoites[d]	-	0.14 ± 0.03

[a] Trophozoites isolated by mechanical rupturing of infected erythrocytes followed by differential centrifugation.
[b] Trophozoites isolated by free-flow electrophoresis.
[c] Trophozoites isolated by saponin lysis.
[d] Trophozoites isolated by saponin lysis followed by immunoaffinity.
Values are means ± standard deviation of 3 separate experiments from separate trophozoite isolations.

2.2.2.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of *Plasmodium falciparum* trophozoites

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of erythrocyte ghosts, isolated trophozoites from RSA11 prepared by saponin lysis, sorbitol treatment followed by immunoaffinity purification and saponin treatment followed by immunoaffinity chromatography on a 10 % polyacrylamide gel stained with silver revealed no significant erythrocyte membrane protein contamination in the trophozoite preparations. The main indicator for erythrocyte contamination is the double band of 80-85 Kdaltons molecular weight. Although the pattern was complex, no erythrocyte-specific band could be detected in the various trophozoite preparations. Furthermore, certain bands present in the trophozoites isolated by sorbitol or saponin lysis were absent in the trophozoite preparation isolated by saponin lysis followed by immunoaffinity chromatography, suggesting that additional erythrocyte proteins had been removed in this preparation (plate 1).

2.2.2.4 Western blot analysis of isolated *Plasmodium falciparum* trophozoites

Isolated trophozoite proteins (20μg) from the *P. falciparum* strain RSA11 prepared either by saponin treatment followed by immunoaffinity chromatography were electrophoresed, transferred onto nitrocellulose and probed with an anti-erythrocyte antibody (α-erythrocyte antibody) (detailed methodology in section 7.15). Erythrocyte membranes were included in the gel as a control. Separation of erythrocyte constituents from the trophozoites was apparent by the visible reduction in proteins probed by the anti-erythrocyte antibody. No erythrocyte-specific band was detected in the trophozoite preparation isolated by saponin lysis followed by immunoaffinity purification (Plate 2).

2.2.3. Trophozoite integrity

The integrity of purified trophozoites was monitored by measuring the uptake of the trypan blue dye, into the parasite cytoplasm. Briefly, 10μl of trypan blue (10mg/ml in distilled water) was added to 100μl of trophozoite suspension in PBS and incubated for 5 minutes at room temperature. The proportion of parasites showing trypan blue accumulation was evaluated using a phase contrast microscope. It was shown that 98% of the trophozoites released by saponin lysis did not concentrate the dye, indicating an intact plasma membrane.

2.2.4 Recovery of *Plasmodium falciparum* trophozoites

An average of 12% ± 8% of trophozoites was recovered from saponin lysis of *Plasmodium falciparum* infected-erythrocytes followed by chromatography using an anti-erythrocyte antibody (Table 3).

Table 3. Recovery of trophozoites

Number of iRBC*	Number of trophozoites	% Recovery
7.4×10^8	101×10^6	14
2×10^8	10.5×10^6	5
1×10^8	6×10^6	6
2×10^8	44×10^6	22

* Infected red blood cells

2.2.5 *Plasmodium falciparum* plasma membrane purification

For a detailed description of the method, see section 7.3. Two different methods were used to prepare parasite plasma membranes. In the first method, the parasite plasma membrane of *P. falciparum* was isolated by fixation on a reversible covalent solid support prepared by covalent binding of dithiobis (succinimidylpropionate) onto amino-propyl glass beads. The resulting dithio-NHS support bound the trophozoites. The remaining sites were blocked by adding Tris-HCl. The contents of the trophozoites were released after lysis by nitrogen cavitation in presence of the protease inhibitor aprotinin and DnaseI with the plasma membrane vesicles being retained on the solid support. In the second method, the trophozoites were biotinylated with NHS-SS-biotin prior to nitrogen cavitation in presence of the protease inhibitor aprotinin and DnaseI. The resulting biotinylated membranes bound streptavidin-magnetic beads and were separated from the lysate by passage through a magnetic column. The membrane vesicles were collected by simply removing the magnet from the column. In both methods, membranes were eluted using dithiothreitol.

2.2.6 Parasite plasma membrane purity

2.2.6.1 Acetylcholine esterase activity of *Plasmodium falciparum* plasma membrane

Detailed methodology is described in section 7.5. There was no detectable AchE activity (less than 0.01μmol/min/mg protein) in the parasite plasma membrane preparations (n=3). By comparison, the specific activity of AchE was 2.30 ± 0.15 and 0.05 ± 0.03 μmol/min/mg protein in erythrocyte ghosts and isolated trophozoites (n=3) (Table 4).

Table 4.Acetylcholine esterase activity of isolated erythrocyte ghosts, isolated *Plasmodium falciparum* trophozoites and isolated parasite plasma membranes.

Samples	Specific activities (μmol/min/mg protein)
Erythrocyte ghosts	2.30 ± 0.15
Trophozoites	0.05 ± 0.03
Vesicles prepared by fixation onto dithiobis-coated beads	No activity
Vesicles prepared by fixation onto magnetic beads	No activity

Values are means ± standard deviation of 3 separate experiments from separate parasite plasma isolations, trophozoite isolations or erythrocyte membrane preparation.

2.2.6.2 Parasite lactate dehydrogenase activity of *Plasmodium falciparum* plasma membrane

To investigate whether the parasite plasma membranes were contaminated by cytosolic components, the pLDH activities of the various preparations were determined. Detailed methodology is described in section 7.6. There were no detectable pLDH activity (less than 0.01μmol/min/mg protein) in the parasite plasma membrane preparations (n=3). By comparison, the specific activity in isolated trophozoites was 0.14 ± 0.01 μmol/min/mg protein (n=3) (Table 5).

Table 5. Parasite lactate dehydrogenase activity of isolated *Plasmodium falciparum* trophozoites and isolated parasite plasma membranes.

Samples	Specific activities (μmol/min/mg protein)
Trophozoites	0.14 ± 0.01
Vesicles prepared by fixation onto dithiobis-coated beads	No activity
Vesicles prepared by fixation onto magnetic beads	No activity

Values are means ± standard deviation of 3 separate experiments from separate parasite plasma isolations or trophozoite isolations.

2.2.6.3 Microscopic examination of *Plasmodium falciparum* plasma membrane

Homogenous preparations of purified *Plasmodium falciparum* plasma membranes were examined using phase contrast microscopy (Plate 3) and transmission electron microscopy (Plate 4). No visible contamination by other membranes or organelles was observed, although some liberated hemozoin crystal could occasionally be seen. Similarly, no visible contamination of the trophozoites preparations was observed using phase contrast microscopy (Plate 5) and transmission electron microscopy. A transmission electron micrograph of an isolated *P. falciparum* trophozoite is shown in Plate 6. For comparison, a trophozoite-infected erythrocyte can be viewed using phase contrast microscopy (Plate 7) and transmission electron microscopy (Plate 8).

2.2.6.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of *Plasmodium falciparum* plasma membrane

Trophozoites, food vacuoles and parasite plasma membranes isolated from the *Plasmodium falciparum* strains D10 and RSA11 along with erythrocyte membranes as a control were examined by SDS-PAGE. No visible contamination by food vacuole or erythrocyte proteins was observed in the parasite plasma membrane preparations; bands present in the lane containing the food vacuoles or the erythrocytes were absent in the lane containing the parasite plasma membranes

(Plate 9). Specific band intensities were found to be quite different in the lanes containing the plasma membranes isolated from the *P. falciparum* strains D10 and RSA11 which could suggest a differential protein content in these 2 strains.

2.2.7 Parasite plasma membrane integrity

Purified *Plasmodium falciparum* plasma membranes examined using transmission electron microscopy were observed to form intact vesicles although some open membranes could be seen (Plate 4). These vesicles were found to have an average diameter of 500 nm.

2.2.8 Recovery of *Plasmodium falciparum* plasma membrane

An average of 8.2 % ± 6.1 (n=5) and 5.5 %± 1.3 (n=4) of trophozoite protein was recovered from nitrogen cavitation of isolated trophozoites linked to magnetic beads and dithiobis-coated beads, respectively (Table 6).

Table 6: Recovery of Plasmodium falciparum plasma membranes.

Samples	% Recovery
Vesicles prepared by fixation onto dithiobis-coated beads	5.5 ± 1.3 (n=4)
Vesicles prepared by fixation onto magnetic beads	8.2 ± 6.1 (n=5)

Recovery is expressed by protein determination and represent percentage of trophozoites. Values are means ± standard deviation of separate experiments from separate parasite plasma isolations.

2.3 Discussion

A procedure for parasite plasma isolation from *Plasmodium falciparum*-infected erythrocytes was developed. Membrane vesicle preparations contained 8.2% of the

total trophozoite protein. This result is consistent with the percent protein yield (8-10%) obtained for membrane vesicles prepared from cancer cells (Cornwell *et al.*, 1986). Interestingly, SDS-PAGE analysis revealed a differential protein content between plasma membranes isolated from the *P.falciparum* strains D10 and Fac8. However, these results should be confirmed on a larger number of strains in order to investigate an eventual correlation between specific band intensities and the resistant phenotype.

In this method, trophozoites were released from the erythrocytes by saponin lysis, the remaining erythrocytes membranes being removed by immunoaffinity chromatography using anti-erythrocyte antibodies. Previously, anti-erythrocyte monoclonal antibodies have been used to remove unlysed erythrocytes and ghost membranes (Hoppe *et al.*, 1992). However, in order to remove most of the erythrocyte ghosts by centrifugation, all the erythrocytes must be lysed by the detergent therefore allowing isolation of the trophozoites in a larger scale. Only parasitized erythrocytes were lysed with sorbitol while non-infected remained intact. On the other hand, saponin lysed uninfected and infected erythrocytes. For this reason, trophozoites released by sorbitol lysis were not retained in this study to further purify the plasma membranes. Futhermore, saponin-treatment of parasitised erythrocytes has been shown to permeabilise both the erythrocyte plasma membrane and the parasitophorous membrane (Ansorge *et al.*, 1996; 1997). However, contamination of the plasma membrane vesicle preparations by the parasitophorous membrane could not be discounted since there is no specific marker for this membrane to our knowledge.

Evidence for the membrane integrity of saponin-freed parasites came from studies measuring the rate of incorporation of [^{14}C]-isoleucine into protein (Saliba and Kirk, 1999) and the rate of phosphorylation of the pantothenic acid (Saliba *et al.*, 1998). In this study, experiments with the trypan blue showed that 98% of the trophozoites released by saponin lysis were capable of maintaining an intact plasma membrane.

Nitrogen decompression has been previously shown to be effective for the disruption of cells and was used in this study to disrupt the parasite plasma membranes (Cornwell *et al.*, 1986). When membranes bound to the dithiobis support were recovered by centrifugation, food vacuoles and hemozoin were found to pellet along with the membranes, making their purification difficult. Furthermore, fragments of glass were observed in the samples examined by transmission electron microscopy indicating that some of the glass beads had broken during the nitrogen cavitation step. Therefore, this method was abandoned as a means of membrane purification. Alternatively, biotinylated membranes bound to streptavidin-magnetic beads recovered using a magnet were shown to be free of food vacuole contamination. The fact that the membranes were bound to a support facilitated their purification from contaminants and their recovery. Another advantage of this technique is that only the plasma membrane vesicles formed the right way round will be recovered.

A cocktail of protease inhibitors (aprotinin, leupeptin and PMSF) was used to prevent the degradation of membrane proteins by the proteases released during the nitrogen cavitation step. The presence of DNA presents an additional problem, as the parasites adhere to the sticky DNA liberated during parasite lysis. Dnase I was, therefore, included in the isolation process to eliminate the DNA liberated during the nitrogen cavitation step.

Purified *Plasmodium falciparum* plasma membranes examined using transmission electron microscopy were observed to be intact and free of contaminating membranes and organelles even though small amount of liberated haemozoin could occasionally be seen in certain fields. The isolation of individual plasma membrane vesicles was difficult since these vesicles tended to be aggregated and formed sheets as shown in plate 4. The absence of parasite cytosolic and erythrocyte protein in the parasite plasma membranes preparation was confirmed by non-detectable parasite lactate dehydrogenase and erythrocyte acetylcholine esterase activities. Further evidence for the absence of erythrocyte membranes in parasite plasma membrane preparations came from SDS-PAGE analysis and western blot analysis with an anti-

erythrocyte antibody. Therefore, the method of parasite plasma membrane isolation presented in this thesis represents a major improvement of the Herwaldt et al. (1990) method used to prepare membrane vesicles from infected-erythrocytes.

2.4 Conclusion

A method has been developed for the preparation of *Plasmodium falciparum* plasma membrane vesicles. These isolated membranes were intact and isolated in a high enough yield to enable a characterisation of their ATPase activities and an investigation of their drug transport properties.

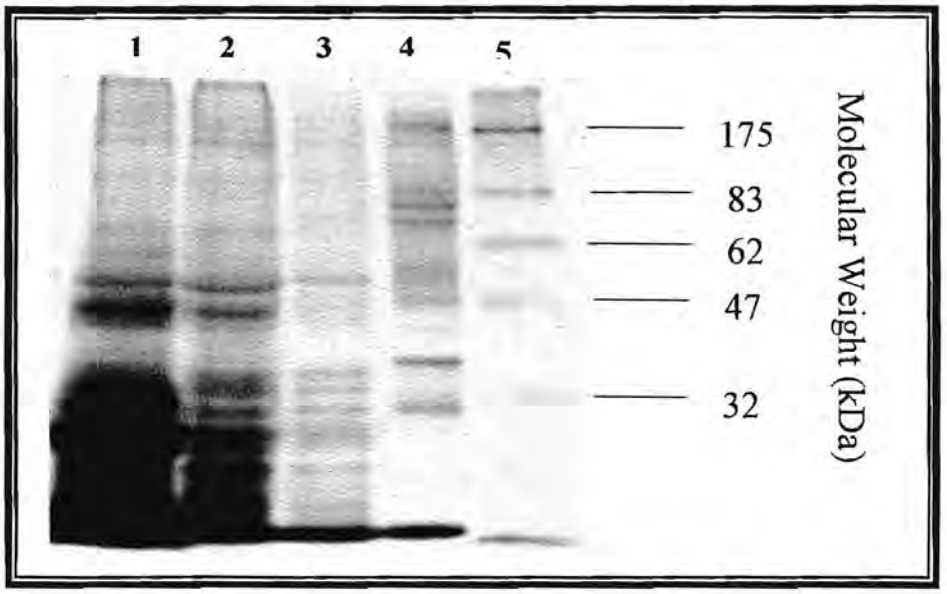

Plate 1: SDS-PAGE of 20μg isolated RSA11 trophozoite protein released by sorbitol lysis followed by immunoaffinity purification, saponin lysis and saponin lysis followed by immunoaffinity purification; lane 1, 2, 3, respectively, compared to 10μg of erythrocyte membrane protein, lane 4. Lane 5 contains protein markers with approximate molecular weight expressed in kDa.

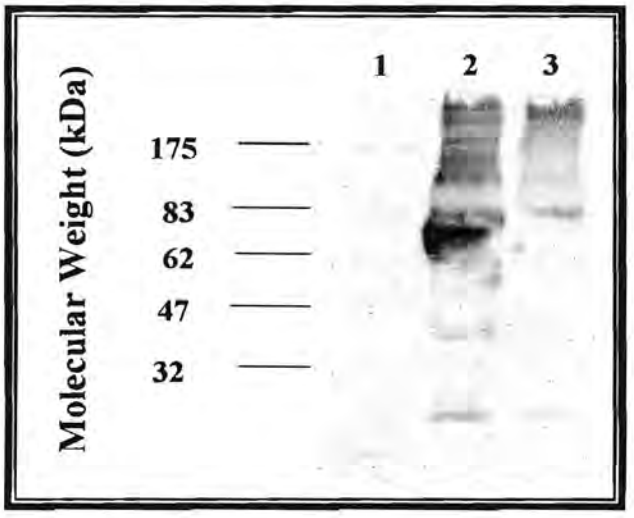

Plate 2: The protein of 20μg isolated RSA11 trophozoites released by saponin lysis followed by immunoaffinity purification (lane 3), and 10μg of erythrocyte membrane protein (lane 2) were separated by SDS-PAGE transferred to nitrocellulose, probed with α-erythrocyte antibodies and recognised bands detected colorimetrically. Lane 1 contains protein markers with approximate molecular weight expressed in kDa.

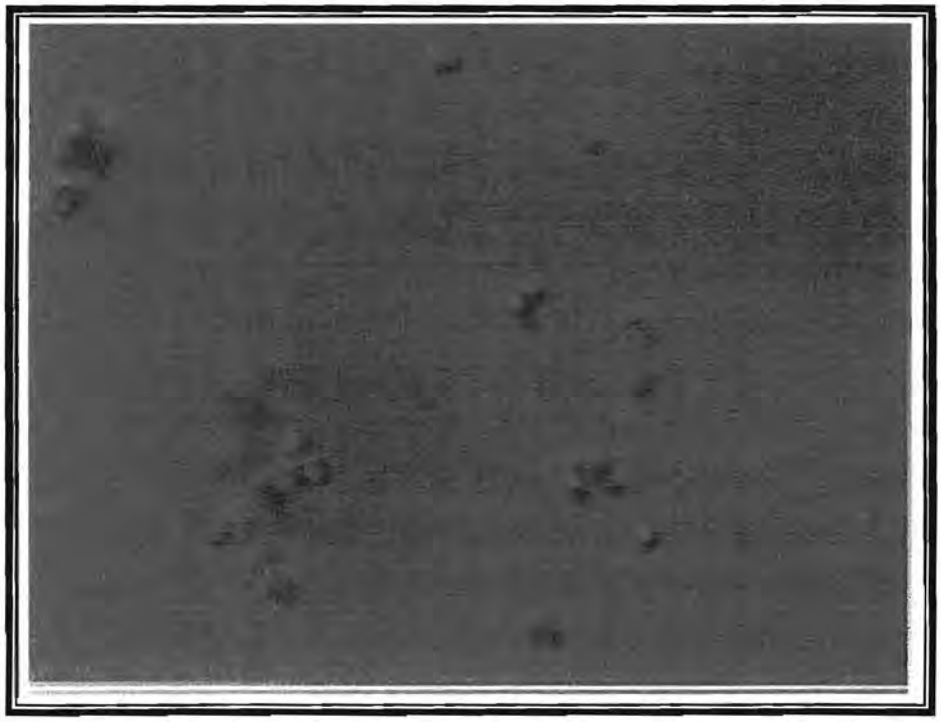

Plate 3: Phase contrast micrograph of purified *Plasmodium falciparum* plasma membranes

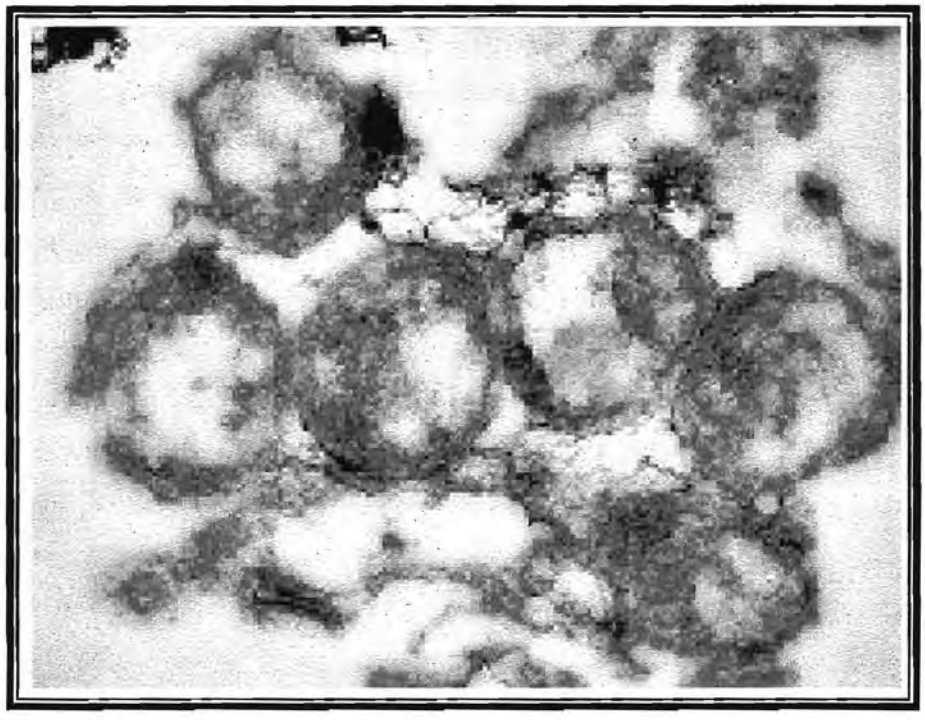

Plate 4: Transmission electron micrograph of purified *Plasmodium falciparum* plasma membranes (magnification x25,000).

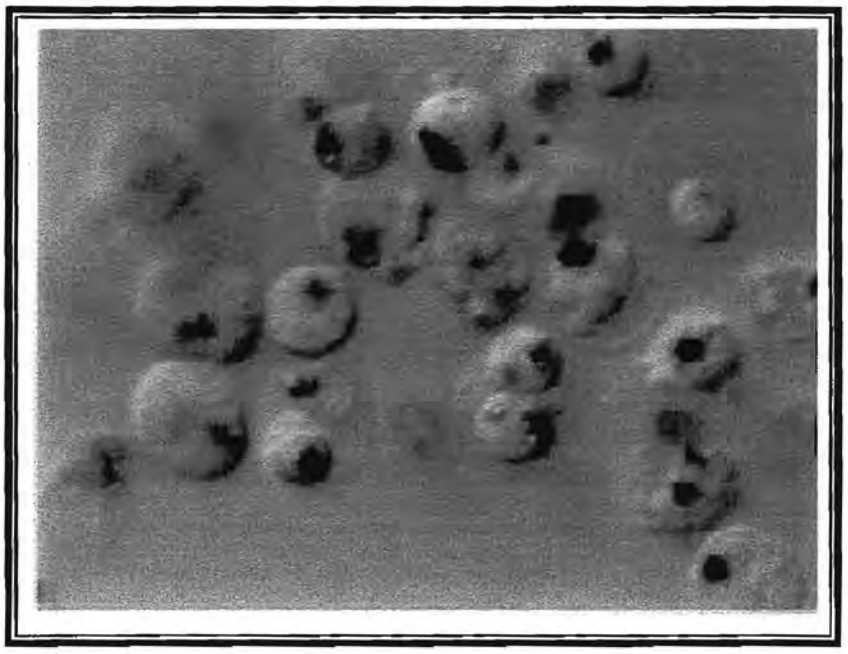

Plate 5: Phase contrast micrograph of purified isolated *Plasmodium falciparum* trophozoites

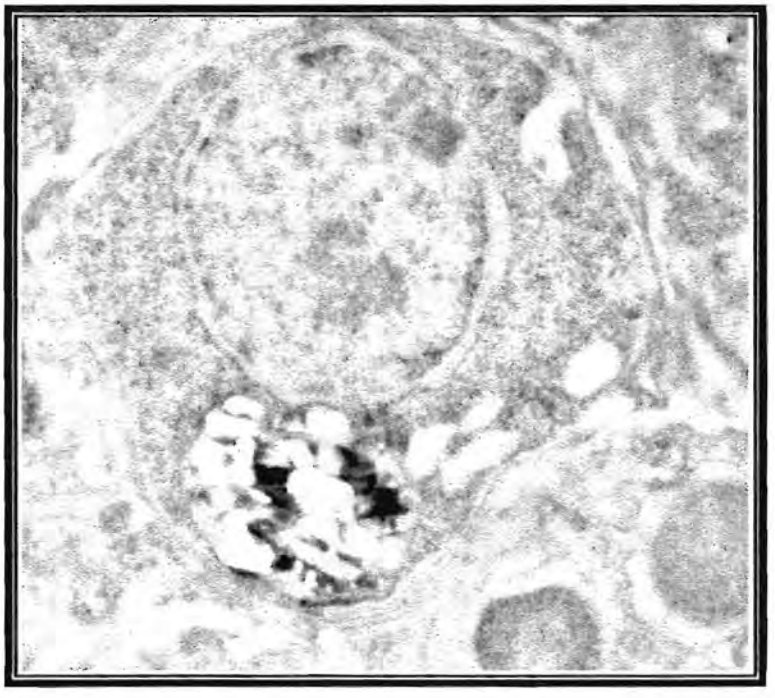

Plate 6: Transmission electron micrograph of isolated *Plasmodium falciparum* trophozoites (magnification x10000).

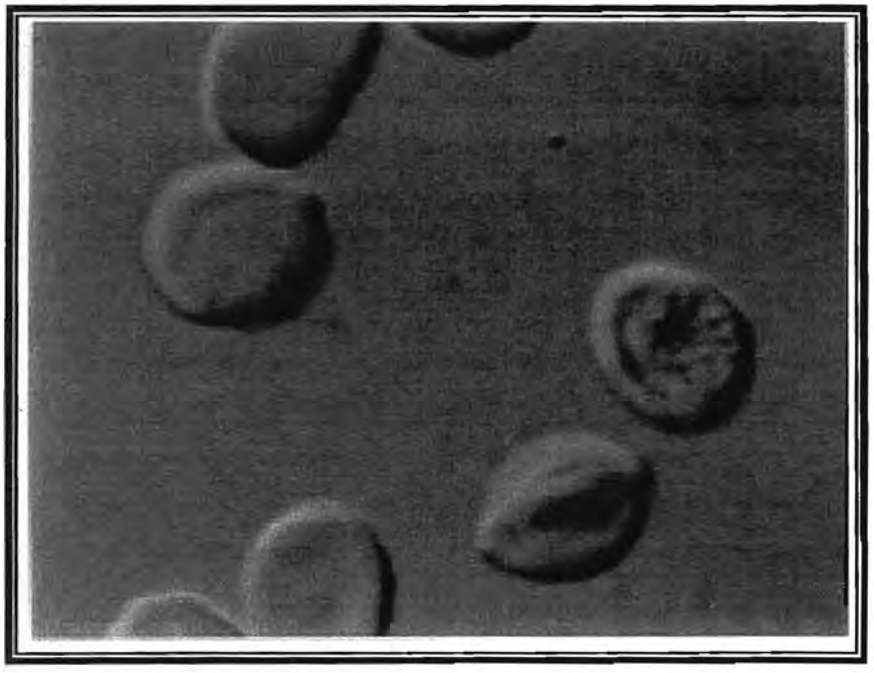

Plate 7: Phase contrast micrograph of erythrocytes infected with *Plasmodium falciparum* trophozoites.

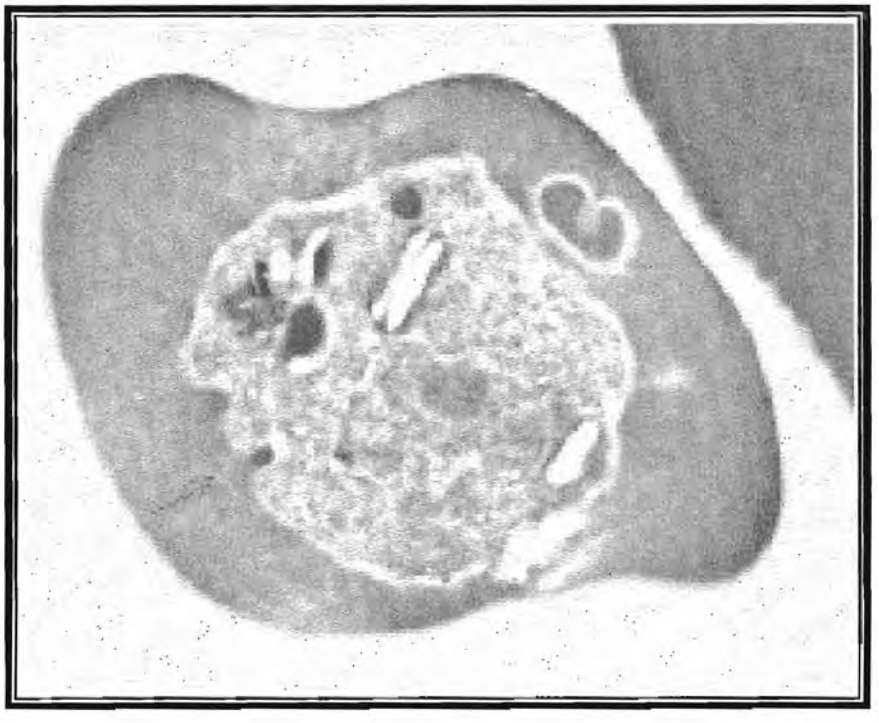

Plate 8: Transmission electron micrograph of erythrocytes infected with *Plasmodium falciparum* trophozoites (magnification x10,000).

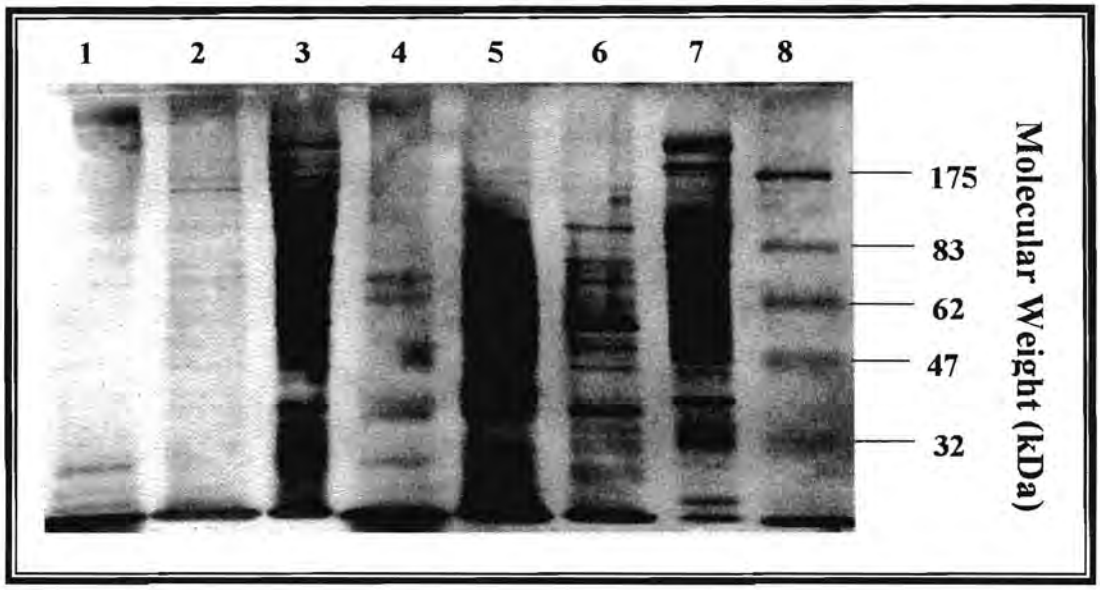

Plate 9: SDS-PAGE of 20μg of isolated RSA11 plasma membrane protein, food vacuole protein and trophozoite protein; lane 1, 2, 3 respectively; 50μg of isolated D10 plasma membrane protein, food vacuole protein and trophozoite protein; lane 4, 5, 6 respectively; and 10μg of erythrocyte membrane protein, lane 7. Lane 8 contains protein markers with approximate molecular weight expressed in kDa.

CHAPTER 3

Characterisation of *Plasmodium falciparum* plasma membrane ATPase activity

3.1 Introduction

The aminoacid sequence of Pgh1 indicates two consensus sequences for ATP binding (Foote *et al.*, 1989) and ATP has been shown by photoaffinity-labelling studies to bind to Pgh1 (Karcz *et al.*, 1993b), suggesting that Pgh1 may act as transport ATPase. In addition, the malaria parasite has been shown to posses a V-type ATPase (Bray *et al.*, 1992b) and two subunits of this ATPase have been cloned (Karcz *et al.*, 1993a; 1994). Indirect immunofluorescence studies revealed the presence of this ATPase over most of the parasite during the trophozoite and shizont stages (Karcz *et al.*, 1994). This heterogenous distribution lead to the proposal that the function of this V-ATPase is not confined to the regulation of the pH of digestive vacuoles.

A V-type ATPase (Karcz *et al.*, 1994) and a P-glycoprotein homologue (Pgh1) (Cowman *et al.*, 1991) have been reported to be located in the food vacuole and the plasma membrane of *Plasmodium falciparum* and have been identified as potential sites involved in the mechanism of chloroquine-resistance. Although the *Plasmodium falciparum* vacuolar ATPase activity have been characterised (Choi and Mego, 1988), the ATPase activity of the parasite plasma membrane has not yet been investigated. In cancer cells, the MDR phenotype is believed to be the result of the expression of a P-glycoprotein in the plasma membrane (Gottesman and pastan, 1993; Gottesman *et al.*, 1995) and this molecule has been shown to possess drug-stimulated ATPase activity (Chang et al., 1997). It is the aim of this chapter to

evaluate the dependence of *Plasmodium falciparum* plasma membrane ATPase activity on ATP and other nucleotides, divalent cations, time, ATP and Mg^{2+} concentrations, and to compare the ATPase activity of plasma membranes isolated from two chloroquine-sensitive strains and three chloroquine-resistant strains.

ATPases have been classified into three major groups, namely P-type ATPases which form phosphorylated intermediates during their reaction cycle, the V-type ATPases found in vacuoles and the F-type ATPases primarily used in ATP synthesis (Pedersen and Carafoli, 1987). These classes of ion-motive ATPases can be distinguished according to their relative sensibility to inhibitors. Vanadate is believed to inhibit P-type ATPases by preventing the formation of a phosphoaspartyl intermediate. Ouabain is a specific inhibitor of the Na^+/K^+-ATPases while the sulfhydryl reagent N-ethylmaleimide and oligomycin are known inhibitors of the proton pump and the mitochondrial membrane ATPases, respectively. However, the specificity of these inhibitors has been questioned since vanadate has been shown to inhibit non-P-type-membrane-associated ATPases (Prossnitz *et al.*, 1989) and NEM has been reported by Hamada and Tsuruo (1988) to inhibit P-glycoprotein ATPase activities. Nevertheless, bafilomycin A1 is believed to be a high-affinity inhibitor of V-ATPases (Bowman *et al.*, 1988). In order to identify the type of ATPase present in the parasite plasma membrane, the effect of these inhibitors on the *Plasmodium falciparum* plasma membranes will be studied. The concentrations of the V-type and P-type ATPase inhibitors tested in this study were similar to those chosen by Choi and Mego (1988) to investigate their effect on the plasmodial food vacuole ATPase activity.

Chloroquine resistance in *Plasmodium falciparum* can be reversed by a certain number of chemically distinct compounds (Martin *et al.*, 1987; Krogstad *et al.*, 1987a). It is difficult to predict how chemosensitisers might affect the ATPase activity of the parasite plasma membranes, since the mechanism by which they reverse chloroquine resistance is unknown. Moreover, their mode of action might vary depending the type of chemosensitiser. The three chemosensitisers chosen in this study belong to various classes of compound known to sensitise multidrug

resistance cells to chemotherapeutic agents in mammalian cancer cells (Georges *et al.*, 1990). These include the calcium channel blocker verapamil, the calmodulin inhibitor trifluoperazine and the steroid hormone progesterone.

A Na^+/H^+ exchanger (NHE) has been identified on *Plasmodium falciparum* plasma membrane (Bosia *et al.*, 1993) and was initially believed to regulate the parasite's cytosolic pH. Latter studies suggested that the plasmodial NHE facilitates the import of chloroquine (Sanchez *et al.*, 1997) and that an altered NHE is responsible for the phenomenon of chloroquine resistance (Wunsh *et al.*, 1998). These proposals were based on studies using the amiloride and its derivatives known as specific inhibitors of the NHE and have since been challenged (Bray *et al.*, 1999; Saliba and Kirk, 1999). In this study, the effect of amiloride, and its derivatives dimethylamiloride (DMA) and 5-(N-ethyl-isopropyl) amiloride (EIPA) on the ATPase activity of *Plasmodium falciparum* plasma membranes isolated from a chloroquine-sensitive and a chloroquine-resistant strains will be observed.

Finally, the effect of the antimalarials quinine, mefloquine and artemisinin on the *Plasmodium falciparum* plasma membrane ATPase activity will be examined to determine whether an ATP-dependent transport of these drug occurs at the plasma membrane level and to investigate an eventual regulation of the ATPase activity by the transported substrate in a chloroquine-sensitive and in a chloroquine-resistant strain.

3.2 Results

3.2.1 Time-dependence of the ATPase activity in *Plasmodium falciparum* plasma membranes

For a detailed description of the assay, see section 7.10. Time dependence of the ATPase activity of plasma membranes isolated from the *Plasmodium falciparum* strain D10 was evaluated over a time period of 45 minutes. A linear relationship of

time versus ATPase activity was found with a correlation coefficient of 0.717 (n=3)(Figure 3).

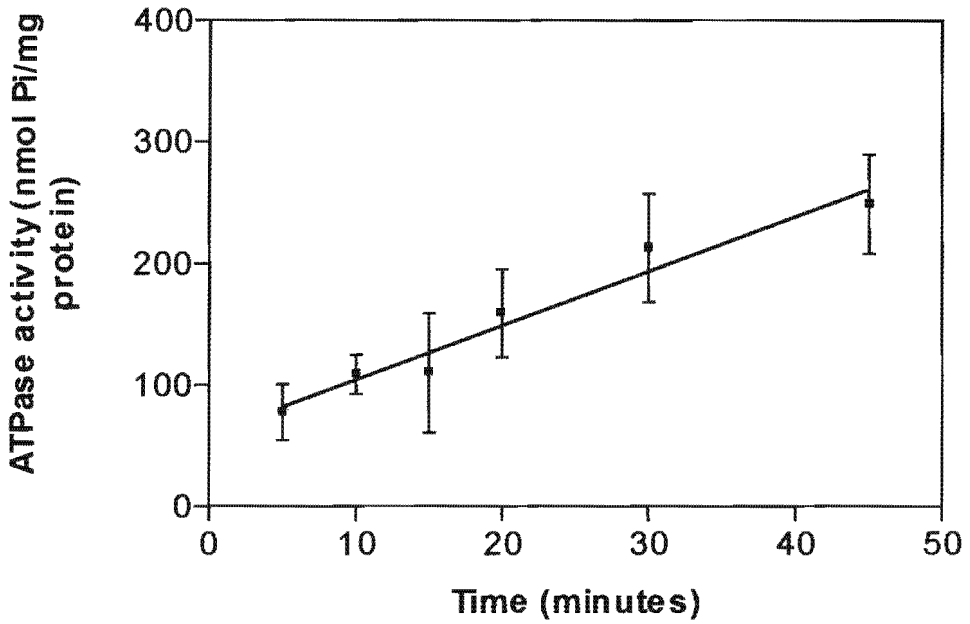

Figure 3: Time course of D10 plasma membranes ATPase activity (nmol Pi/mg protein) over a time period of 45 minutes. ATPase activity of parasite plasma membranes tested without ATP was subtracted. Errors bars represent standard deviations from means of 3 separate experiments, each performed in quadruplicate.

3.2.2 Nucleotide-dependence of the ATPase activity in *Plasmodium falciparum* plasma membranes

The ability of 2mM concentrations of ATP and other nucleotides to support the ATPase activity of plasma membranes isolated from the *Plasmodium falciparum* strain D10 was investigated. Other nucleoside triphosphates tested, namely guanosine triphosphate (GTP), cytosine triphosphate (CTP), and uridine triphosphate (UTP) were hydrolysed as effectively as ATP while 72.6±16.7% and 55.4±17.1% of the nucleoside diphosphates, adenosine diphosphate (ADP) and guanosine diphosphate (GDP) were hydrolysed. Only adenosine monophosphate (AMP) was not hydrolysed by the D10 plasma membranes (Table 7).

Table 7: ATPase activity of isolated D10 plasma membranes in the presence of 2mM concentrations of various nucleotides.

	ATPase activity (% of control)		
Nucleotide	Triphosphate	Diphosphate	Monophosphate
Adenosine	100	72.6±16.7	-9.2±9.3
Guanosine	120.8±19.6	55.4±17.1	N.D.
Cytosine	64.2±7.6	N.D.	N.D.
Uridine	101.4±55.0	N.D.	N.D.

Values are means of 3 separate experiments, each performed in quadruplicate ± standard deviations and are expressed as % of control in presence of ATP. ATPase activity tested without nucleotide and absorbance due to the nucleotide hydrolysis was subtracted. N.D.: not determined.

3.2.3 Effect of ATP concentration on the ATPase activity in *Plasmodium falciparum* plasma membranes

The ATPase activity of D10 plasma membranes was evaluated over a range of increasing ATP concentrations from 0.5 to 5mM, at a fixed Mg^{2+} concentration of 2mM. The ATPase activity was 3.96±0.84nmol Pi/min/mg protein in presence of 2mM ATP and no appreciable increase was observed with higher ATP concentrations (Figure 4).

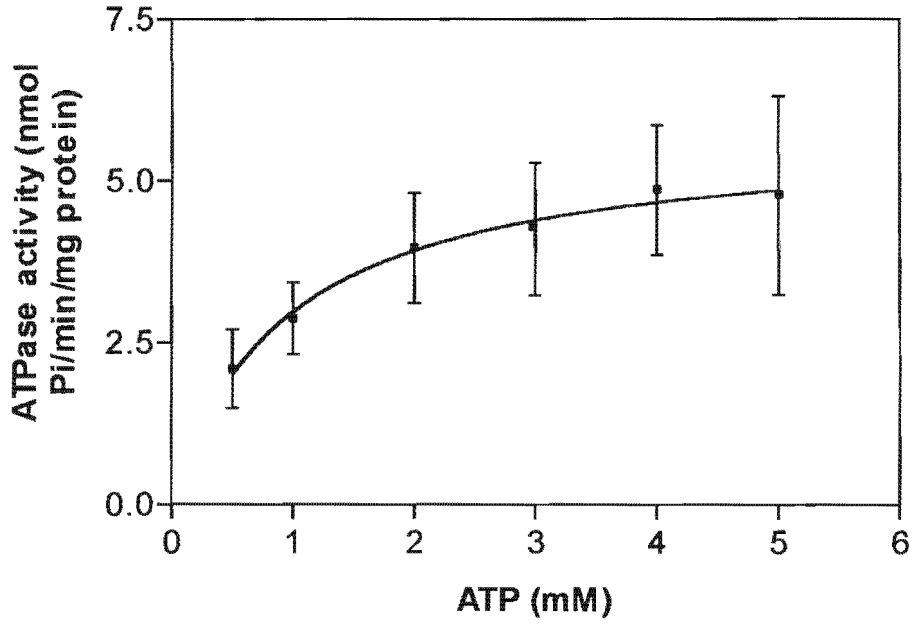

Figure 4: Dependence of ATPase activity on ATP concentration. ATPase activity of freshly purified D10 plasma membranes expressed as nmol Pi/min/mg protein was measured over ATP concentrations ranging from 0.5 to 5mM. ATPase activity of parasite plasma membranes tested without ATP and absorbance due to ATP hydrolysis was subtracted. Data for Michaelis Menten curve was fitted using GraphPad Prism (GrapPad Software) and the substrate concentration at which half the velocity occurs (Km=1mM) and maximal velocity (Vm=5nmol Pi/min/mg) was determined from the curve. Errors bars represent standard deviations from means of 3 separate experiments, each performed in quadruplicate.

3.2.4 Divalent cation-dependence of the ATPase activity in *Plasmodium falciparum* plasma membranes

The ability of 2mM concentration of Mg^{2+} and the other divalent cations Mn^{2+} and Ca^{2+} to support the ATPase activity of purified plasma membranes isolated from the *Plasmodium falciparum* strain D10 was evaluated, along with no divalent cation addition as a control. Maximal ATPase activity was obtained in presence of 2mM Mg^{2+}. A significant activity (87.8±33.1%) was measured when Mg^{2+} was replaced with Mn^{2+}. When Ca^{2+} was substituted for Mg^{2+}, only 20.9±19.2% of the ATPase activity was retained (Table 8).

Table 8: Effect of divalent cations on purified D10 plasma membranes ATPase activity. ATPase activity was determined using 2mM concentration of Mg^{2+}, Mn^{2+} and Ca^{2+}. ATPase activities are expressed as percentages of control.

Cations	Concentration (mM)	ATPase activity (% of control)
Mg^{2+} (control)	2	100
Mn^{2+}	2	87.8±33.1
Ca^{2+}	2	20.9±19.2

Plasma membrane ATPase activity in the absence of divalent cation was subtracted. Errors bars represent standard deviations from means of 3 separate experiments, each performed in quadruplicate.

3.2.5 Effect of Mg^{2+} concentration on the ATPase activity in *Plasmodium falciparum* plasma membranes

The ATPase activity of D10 plasma membranes was evaluated over a range from 0 to 5mM of Mg^{2+} concentrations. An optimal ATPase activity of 3.46±1.59nmol Pi/min/mg protein was achieved with 2mM Mg^{2+} (Figure 5).

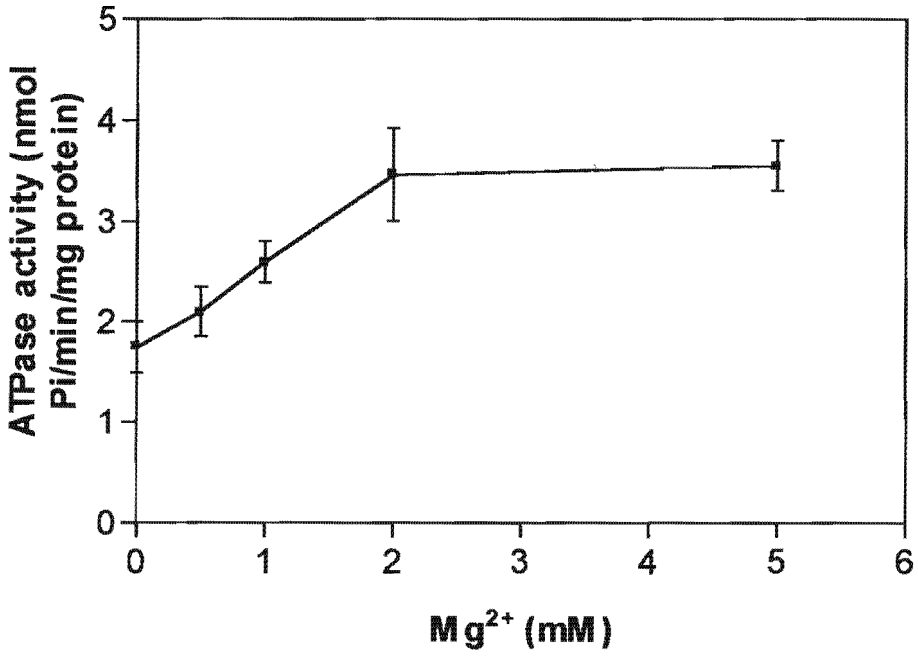

Figure 5: Dependence of ATPase activity on Mg^{2+} concentration. ATPase activity of freshly purified D10 plasma membranes expressed as nmol Pi/min/mg protein

was measured over Mg^{2+} concentrations ranging from 0 to 5mM. ATPase activity of parasite plasma membranes tested without ATP and absorbance due to ATP hydrolysis was subtracted. Errors bars represent standard deviations from means of 3 separate experiments, each performed in quadruplicate.

3.2.6 ATPase activity of parasite plasma membranes isolated from chloroquine-resistant and -sensitive strains of *Plasmodium falciparum*

The ATPase activity of parasite plasma membranes isolated from different strains of *Plasmodium falciparum* exhibiting various degrees of chloroquine resistance was evaluated. Purified parasite plasma membranes isolated from the sensitive strains D10 and RSA3 displayed an ATPase activity of 3.25±1.75 and 4.17±2.09nmol Pi/min/mg protein, respectively, while those isolated from the chloroquine-resistant strains Fac8, RSA11 and K1 displayed an activity of 11.90±1.46, 10.05±0.79 and 5.81±0.66nmol Pi/min/mg protein, respectively (Figure 6). ATPase activities of

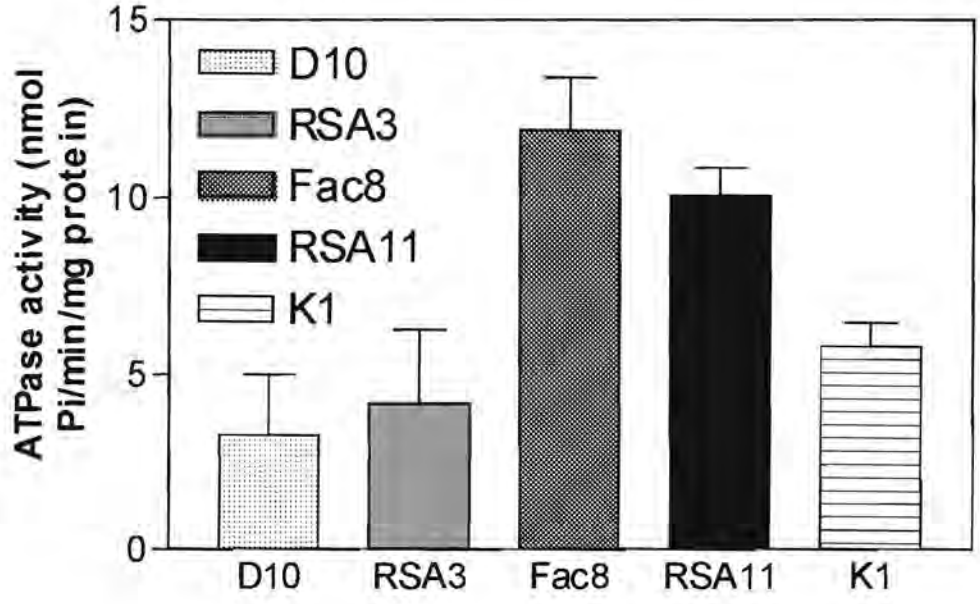

Figure 6: ATPase activity (nmol Pi/min/mg protein) of parasite plasma membranes isolated from the *Plasmodium falciparum* strains D10, RSA3, Fac8, RSA11 and K1. ATPase activity of freshly purified parasite plasma membranes was determined using 2mM ATP and 2mM Mg^{2+}. ATPase activity of parasite plasma membranes tested without ATP and absorbance due to ATP hydrolysis was subtracted. Errors bars represent standard deviations from means of 3 separate experiments, each performed in quadruplicate.

parasite plasma membranes isolated from the chloroquine-sensitive strains D10 and RSA3 were found to be significantly different to those of the membranes isolated from the resistant strains Fac8, RSA11 and K1 with p values <0.0001 for all the sensitive and resistant strains compared, except for RSA3 and K1 (p=0.0172).

3.2.7 Effect of P-type ATPase inhibitors on *Plasmodium falciparum* plasma membranes ATPase activity

Ouabain (1mM), a Na^+/K^+-ATPase inhibitor had no significant effect on the D10 and Fac8 plasma membrane ATPase activities with p values of 0.3019 and 0.2930, respectively. Oligomycin (50μg/ml) inhibited the ATPase activity of membranes purified from the *P. falciparum* strains Fac8 by 42±13 (p=0.0126), and had little effect on D10 plasma membrane ATPase activity (p=0.0462), while vanadate (0.1mM) inhibited by 41±23 (p=0.0337) and 29±14% (p=0.0499) the ATPase activity of purified plasma membranes from the *P. falciparum* strains D10 and Fac8, respectively (Figure 7). There is no statistically significant difference between the ATPase activity of membranes purified from the *P. falciparum* strains Fac8 and D10 in the presence of vanadate (p=0.4606) or oligomycin (p=0.0563).

The presence of dimethylsulfoxide (DMSO), used to solubilise ouabain and oligomycin at a final concentration of 0.5 and 0.2%, respectively, had no effect on the parasite plasma membrane ATPase activity (data not shown).

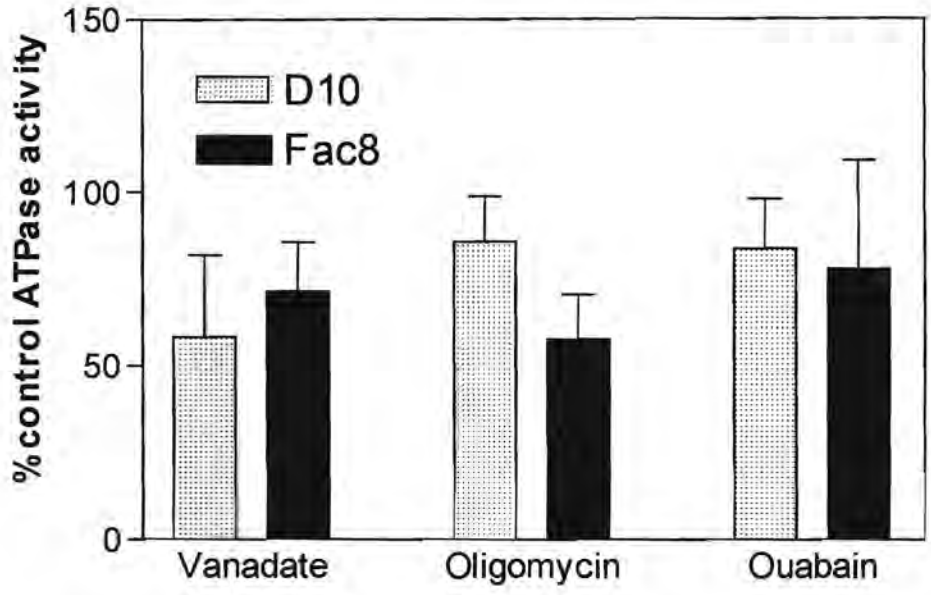

Figure 7: Effect of the P-type ATPase inhibitors vanadate (0.1mM), oligomycin (50μg/ml) and ouabain (1mM) on the ATPase activity of parasite plasma membranes isolated from the *Plasmodium falciparum* strains D10 and Fac8. ATPase inhibitors were added to the assay 15-min. prior to the addition of ATP. Values are expressed as percentages of controls without drugs and error bars represent standard deviations from means of 3 separate experiments, each performed in quadruplicate.

3.2.8 Effect of V-type ATPase inhibitors on *Plasmodium falciparum* plasma membranes ATPase activity

Bafilomycin A1 (1μM), a high-affinity inhibitor of V-ATPases and N-ethylmaleimide (NEM) (2mM) inhibited D10 plasma membrane ATPase activities by 47±22 (p=0.0101)and 32±22% (p=0.3040), respectively, while these inhibitors decreased Fac8 plasma membrane ATPase activities by 17± 14 (p=0.1637) and 14± 20% (p=0.3063), respectively (Figure 8). There is no statistically significant difference between the ATPase activity of membranes purified from the *P. falciparum* strains Fac8 and D10 in the presence of Bafilomycin A1 (p=0.0970) or N-ethylmaleimide (p=0.3691).The presence of dimethylsulfoxide (DMSO), used to solubilise bafilomycin A1 at a final concentration of 0.1% had no effect on the parasite plasma membrane ATPase activity (data not shown).

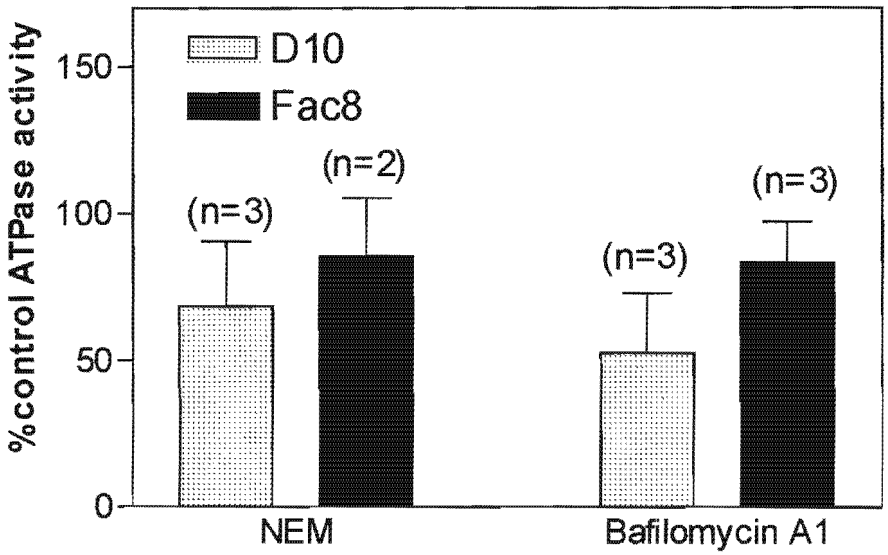

Figure 8: Effect of the V-type ATPase inhibitors Bafilomycin A1 (1μM), and N-ethylmaleimide (NEM) (2mM) on the ATPase activity of parasite plasma membranes isolated from the *Plasmodium falciparum* strains D10 and Fac8. ATPase inhibitors were added to the assay 15-min. prior to the addition of ATP. Values are expressed as percentages of controls in the absence of drugs and error bars represent standard deviations from means of separate experiments, each performed in quadruplicate. Number of experiments is indicated above the bars.

3.2.9 Effect of Na^+/H^+-exchanger inhibitors on *Plasmodium falciparum* plasma membranes ATPase activity

Amiloride and its derivatives have been shown to inhibit the activity of eukaryotic Na^+/H^+-exchangers at micromolar concentrations and Sanchez et *al.* (1997) demonstrated that the half-maximal inhibition of chloroquine uptake was achieved at an EIPA concentration of 6.2μM for HB3 and 62μM for Dd2. Therefore, the effect of the Na^+/H^+-exchanger inhibitors amiloride, dimethylamiloride (DMA) and 5-(N-ethyl-isopropyl) amiloride (EIPA) on *P. falciparum* plasma membrane activity was determined at a 100μM concentration. Amiloride (100μM) stimulated the plasma membrane ATPase activity of D10 and Fac8 to 317±62% and 169±36%, respectively while its derivative DMA (100μM) had a stimulatory effect of 317±63% and 147±32% on D10 and Fac8 plasma membranes, respectively. The

more potent NHE inhibitor, EIPA (100μM) increased the ATPase activity of D10 and Fac8 plasma membranes by 209±40% and 123±21%, respectively (figure 9). The effect of the Na^+/H^+-exchanger inhibitors on D10 and Fac8 plasma membrane ATPase activity was found to statistically significant with p values <0.0001 for the effect of amiloride and DMA on both strains while p=0.0013 and 0.0050 for the effect of EIPA on D10 and Fac8, respectively. There is a statistically significant difference between the ATPase activities of parasite plasma membranes isolated from the chloroquine-sensitive and -resistant strains of *P.falciparum* measured in the presence of all the Na^+/H^+-exchanger inhibitors tested with p values <0.0001.

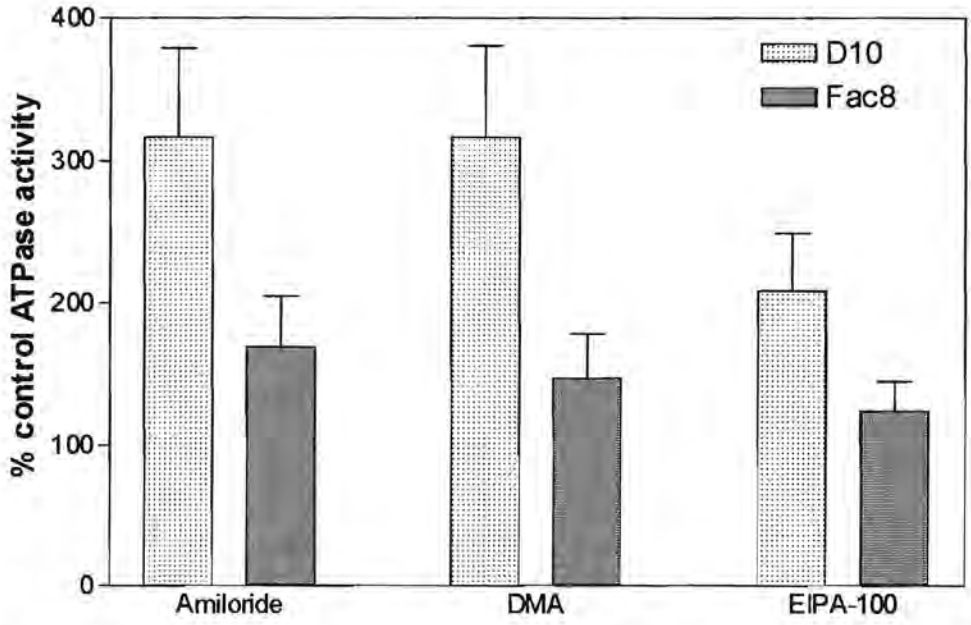

Figure 9: Effect of the NHE inhibitors amiloride (100μM), DMA (100μM) and EIPA (100μM) on the ATPase activity of plasma membranes isolated from the *P. falciparum* strains D10 and Fac8. NHE inhibitors were added to the assay 15 min. prior to the addition of ATP. Values are expressed as percentages of controls without drugs and error bars represent standard deviations from means of 3 separate experiments, each performed in quadruplicate.

3.2.10 Effect of chemosensitisers on *Plasmodium falciparum* plasma membranes ATPase activity

Verapamil (10μM), trifluoperazine (10μM) and progesterone (100μM) had little or no effect on the parasite plasma membrane ATPase activities. Of the chemosensitisers tested, trifluoperazine (10μM) and progesterone (100μM) inhibited D10 plasma membrane ATPase activities by 29±13 and 31±24%, respectively (Figure 10). There were no statistically significant effect of the chemosensitisers tested on *P. falciparum* plasma membranes ATPase activities with p values >0.05. The presence of ethanol used to solubilise progesterone at a final concentration of 0.25% had no effect on the parasite plasma membrane ATPase activity (data not shown).

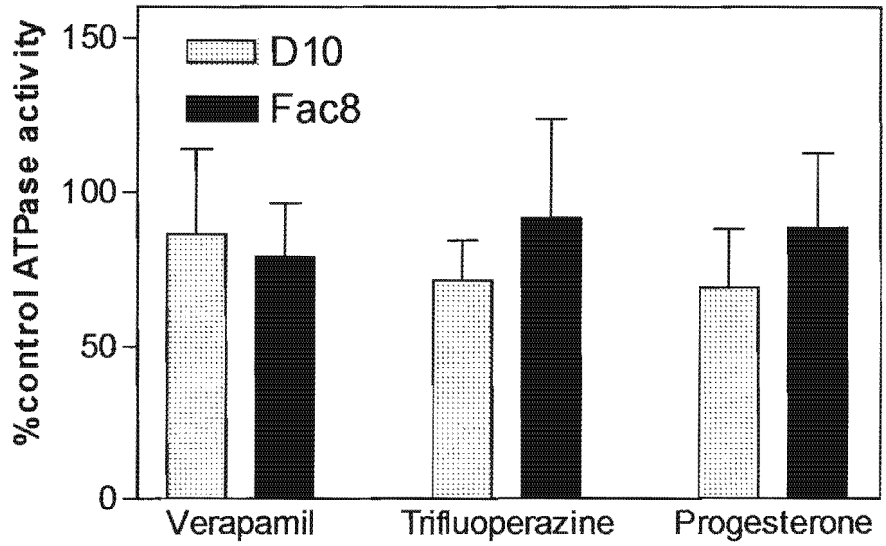

Figure 10: Effect of the chemosensitisers verapamil(10μM), trifluoperazine (10μM) and progesterone (100μM) on the ATPase activity of parasite plasma membranes isolated from the *Plasmodium falciparum* strains D10 and Fac8. Chemosensitisers were added to the assay 15 min. prior to the addition of ATP. Values are expressed as percentages of controls without drugs and error bars represent standard deviations from means of 3 separate experiments, each performed in quadruplicate.

3.2.11 Effect of anti-malarials on *Plasmodium falciparum* plasma membranes ATPase activity

The antimalarial mefloquine (1mM) inhibited the ATPase activity of membranes purified from the *Plasmodium falciparum* strains D10 and Fac8 by 21±17 ($p=0.0438$) and 39±12% ($p<0.0001$), respectively, while quinine (0.1mM) inhibited by 16±14 ($p=0.0806$) and 29±20% ($p<0.0001$) the ATPase activity of purified plasma membranes from the *Plasmodium falciparum* strains D10 and Fac8, respectively. Artemisinin (1mM) inhibited by 11 ±6 % ($p=0.0890$) and 4 ±24 % ($p=0.5577$) the ATPase activity of purified plasma membranes from the *Plasmodium falciparum* strains D10 and Fac8, respectively (Figure 11). There were no statistically significant difference between the ATPase activities of parasite plasma membranes isolated from the chloroquine-sensitive and –resistant strains of *P.falciparum* measured in the presence of quinine and artemisinin with p values of 0.0743 and 0.3219, respectively, while the effect of mefloquine was found to be statistically significant with a p value of 0.0055.

The presence of dimethylsulfoxide (DMSO), used to solubilise mefloquine at a final concentration of 0.1% had no effect on the parasite plasma membrane ATPase activity (data not shown). The antimalarial chloroquine available in our laboratory was a diphosphate salt and could not therefore be included in this assay which is based on colorimetric determination of phosphate.

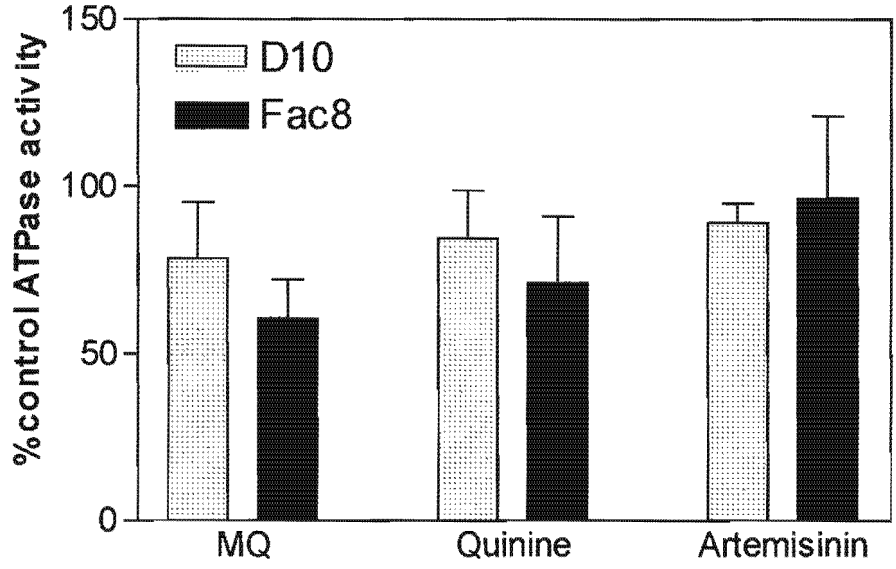

Figure 11: Effect of the antimalarials mefloquine(1mM), quinine (0.1mM) and artemisinin (1mM) on the ATPase activity of parasite plasma membranes isolated from the *Plasmodium falciparum* strains D10 and Fac8. Antimalarials were added to the assay 15 min. prior to the addition of ATP. Values are expressed as percentages of controls in the absence of the drugs and error bars represent standard deviations from means of 3 separate experiments, each performed in quadruplicate.

3.3 Discussion

Conditions required for optimal *Plasmodium falciparum* plasma membrane ATPase activity were determined using the chloroquine-sensitive strain D10. Parasite plasma membranes showed optimal ATPase activity at 2mM Mg^{2+} and 2mM ATP. Other divalent cations are able to substitute for Mg^{2+} and maintain the function of a variety of different ATPases (Doige *et al.*, 1992). In this study, it was found that Ca^{2+} could not substitute for Mg^{2+} ATPase activity in parasite plasma membranes, whereas activity was preserved when Mn^{2+} was substituted for Mg^{2+}. Therefore, the parasite plasma membrane ATPase activity was found to be similar to the vacuolar membrane ATPase activity (Adams, 1999) However, This contradicts Choi and Mego (1988) study showing that Ca^{2+} could support the *Plasmodium falciparum* vacuolar ATPase activity in the absence of Mg^{2+}.

Table 9: Chloroquine sensitivity and ATPase activity (nmol Pi/min/mg protein) of parasite plasma membranes isolated from the *Plasmodium falciparum* strains D10, RSA3, Fac8, RSA11 and K1.

Strain	IC50 (nM)	ATPase activity (nmol Pi/min/mg protein)
D10	28.32±3.53	3.25±1.75
RSA3	52.46±17.01	4.17±2.09
Fac8	157.50±55.98	11.90±1.46
RSA11	252.53±20.79	10.05±0.79
K1	379.10±34.96	5.81±0.66

Values are means of 3 separate experiments ± standard deviation.

Other nucleoside triphosphates tested were hydrolysed as effectively as ATP, suggesting that the parasite plasma membrane contains a nucleotide triphosphate hydrolase, rather than a specific ATPase. However, the nucleoside monophosphate AMP was not hydrolysed suggesting that the parasite plasma membrane ATPase activity is distinguishable from membrane-associated alkaline and acid phosphatases (Ketcham *et al.*, 1985). Although no linear relationship was observed between the level of ATP activity and chloroquine sensitivity, plasma membranes isolated from the chloroquine-sensitive strains of *P. falciparum* presented a lower level of ATPase activity than their resistant counterparts (Table 9). This result could be consistent with the presence of an overexpressed ATP-driven drug exporter in the plasma membrane of resistant strains of *P. falciparum*. The lack of correlation observed between the ATPase activity in the parasite plasma membranes and the IC_{50} of the *P.falciparum* strains tested could be due to the use of strains as opposed clones and it would therefore be interesting to confirm this result on a larger number of clones.

The ATPase activity of parasite plasma membranes was found to be of similar magnitude to that of cancer cells. By comparison, the specific ATPase activity of plasma membranes prepared from multidrug-resistant cancer cells is 1.15μmol/min/mg membrane protein (Rebbeor *et al.*, 1998). Since the parasite plasma membranes were isolated in the form of intact vesicles the right-side-out, the

ATP hydrolysing regions of the ATPase proteins should be enclosed within the vesicles arising the question whether all of the compound tested are gaining access to the relevant part of the protein, and whether the differences between the ATPase activities measured in the presence of the different agents might reflect, at least to some extent, the different ability of these agents to gain access to the interior of the vesicles. Therefore, it could not be excluded that the saturable dependence of ATPase activity on the concentration of ATP in the medium could reflect the saturable transport of ATP into the vesicles, rather than the saturation of the ATPase itself. Similarly, the differential ability of divalent cations to support ATPase activity could result of a differential transport of these cations into the vesicles.

Pgh1, a P-type ATPase was identified on the *P. falciparum* plasma and vacuolar membrane and was believed to play a role in both the mechanism of chloroquine accumulation and chloroquine-resistance (Cowman *et al.*, 1991). Whether Pgh1 transports chloroquine or controls its uptake through regulation of the intravacuolar pH by a chloride channel activity is still subject of controversy. In this study, the effect of P-type ATPase inhibitors on the ATPase activity of plasma membranes was observed in order to determine their participation in the ATPase activity measured. Vanadate reduced the plasma membrane ATPase activity of the *P. falciparum* strain D10 and Fac8 by 41% and 28%, respectively, therefore implicating a P-type component in the ATPase activity observed since the V-ATPases are not sensitive to vanadate (Mellman *et al.*, 1986). Furthermore, the inhibition of Fac8 ATPase activity by oligomycin would be consistent with possible higher levels of Pgh1 in Fac8 (Cowman *et al.*, 1991) since this inhibitor has been shown to inhibit P-gp homologues (Mc Kinney and Hosford, 1993; Decottignies *et al.*, 1994). Ouabain had no effect on the plasmodial plasma membrane ATPase activity indicating that the *P. falciparum* plasma membrane does not contain an ouabain-sensitive Na+/K+-ATPase.

Many compounds that reverse MDR in cultured cells have been identified. These chemosensitisers were shown to have a stimulatory effect on P-glycoprotein ATPase activity in plasma membranes (Rao *et al.*, 1994; Sarkadi *et al.*, 1994; Scarborough,

1995), in Pgp reconstituted in liposomes (Rebbeor and Senior, 1998) and purified P-glycoprotein (Doige *et al.*, 1992). Similarly, chloroquine resistance can be reversed by chemosensitisers such as verapamil (Martin *et al.*, 1987). In this study, none of the chemosensitisers tested had an effect on the ATPase activity of the parasite plasma membranes. If the chemosensitisers act directly on a plasma membrane efflux pump, it would indicate that this activity has not been stimulated. However, the target of these chemosensitisers has not yet been identified in *P. falciparum* and these results would suggest either that the target of chemosensitiser is absent from the parasite plasma membrane or their mode of action does not involve an ATP-dependent mechanism. Moreover, a similar study conducted in our laboratory failed to show any effect of verapamil, progesterone and trifluoperazine on *P. falciparum* vacuolar ATPase activity (Adams, 1999). Nevertheless, it would be interesting to test the effect of these chemosensitisers on the ATPase activity over a range of concentrations, since it has been shown that drugs like trifluoperazine or verapamil have a biphasic effect on P-glycoprotein ATPase activity, producing a stimulation of activity a low concentrations, followed by inhibition at higher concentrations (Doige *et al.*, 1992; Garrigos *et al.*, 1993).

Chloroquine accumulation in resistant *P. falciparum* parasites was observed to be more sensitive to the V-type ATPase inhibitor bafilomycin A1 than its sensitive counterparts which lead to the proposal that a weakened vacuolar proton pumping activity could be responsible for reduced chloroquine accumulation (Bray *et al.*, 1992a). However, chloroquine-sensitive and –resistant parasites were shown to display similar vacuolar ATPase in the presence of bafilomycin A1 and thus failed to provide evidence for an weakened proton pump activity in purified food vacuoles from chloroquine-resistant *P. falciparum* (Adams, 1999). In contrast, this study showed that plasma membranes isolated from chloroquine-sensitive *P. falciparum* were more sensitive to bafilomycin A1 than their resistant counterparts. Bafilomycin A1 (1μM) reduced the D10 plasma membrane ATPase activity by 47%, while this inhibitor had no effect on Fac8 plasma membrane ATPase activity suggesting the presence of a V-type ATPase in D10 plasma membrane which is underexpressed or altered in the plasma membrane of the chloroquine-resistant Fac8. However, the

difference observed between the ATPase activities of plasma membranes isolated from D10 and Fac8 in the presence of bafilomycin A1 was not statistically significant (p=0.097) while N-ethylmaleimide (2mM) had no significant effect on the parasite plasma membrane activity. These results are consistent with the reported subcellular distribution of the V–type-H^+-ATPase B-subunit within the parasite, shown by indirect immunofluorescence to be located in both the plasma and vacuolar membrane (Karcz *et al.*, 1994). It has been argued that the plasma membrane V-type ATPase is the major mechanism accounting for the H^+-efflux (Saliba and Kirk, 1999). Although the genes encoding the A and B subunits of the *P. falciparum* proton pump have been sequenced, no mutations or overexpression of these genes which could explain chloroquine resistance has yet been observed (Karcz *et al.*, 1993a; Karcz *et al*, 1994).

Sanchez *et al.* (1997) demonstrated that amiloride and its derivatives inhibit chloroquine uptake in infected erythrocytes. Further studies indicated that the NHE of chloroquine-sensitive parasites was stimulated by chloroquine whereas the NHE of chloroquine-resistant parasites was constitutively activated and therefore insensitive to further stimulation by chloroquine (Wunsh *et al.*, 1998). Surprisingly, D10 plasma membrane ATPase activity was considerably stimulated by amiloride and its derivatives tested in this study while its resistant counterpart was shown to be stimulated to a lesser extend by the NHE inhibitors amiloride, DMA and EIPA at a concentration of 100μM. Interestingly, a study conducted in our laboratory indicated that EIPA at low concentration was able to stimulate chloroquine accumulation in K1 and D10 infected erythrocytes. However, the specificity of amiloride and its derivatives can be challenged since amiloride has been shown to inhibit the generation of a H^+ gradient by a V-type H^+-ATPase in the plasma membrane of insect cells (Wieczorek, *et al.*, 1991). Moreover, Saliba and Kirk (1999) argued that amiloride and EIPA effects on pHi are not related to an effect on an NHE but rather consistent with amiloride acting as an inhibitor of the V-type H^+-ATPase. This proposal was supported by the finding that the effect of amiloride and bafilomycin A1 was not additive. Furthermore, Bray *et al.* (1999) argued that than the effect of amiloride and its derivatives on chloroquine uptake is due to chloroquine binding

interactions within the parasite. The ability to stimulate the plasma membrane ATPase activity found in this study indicates that amiloride and its derivatives have properties other than that of a NHE inhibitor. Since D10 plasma membrane ATPase activity was stimulated to a greater extent than its resistant counterpart, the ATPase composition of these 2 membranes appeared to be different.

Mefloquine, quinine and artemisinin were reported to inhibit the vacuolar ATPase activity (Choi et Mego, 1988). In this study, we found that mefloquine (1mM) and quinine (0.1mM) inhibit the ATPase activity of membranes isolated from the chloroquine-resistant strain Fac8 by 40 and 29%, respectively, while these antimalarials had little effect on the ATPase activity of membrane purified from the chloroquine-sensitive strain D10. The differential sensitivity of the ATPase from plasma membranes isolated from chloroquine-sensitive and –resistant strains towards these antimalarials suggests the possible presence of a component in the parasite plasma membrane involved in the mechanism of mefloquine-resistance. In contrast, artemisinin had no or little effect on the ATPase activity of purified plasma membranes from the chloroquine-resistant and –sensitive strain, respectively. This differential effect of mefloquine and quinine, in one hand, and artemisinin, on the other hand on the plasma membrane ATPase activity indicates that these antimalarials might not share the same target in the plasma membrane.

3.4 Conclusion

Optimal conditions for *P. falciparum* plasma membrane ATPase activity were determined using the D10 strain. Plasma membranes isolated from chloroquine-sensitive parasites appeared to display a lower ATPase activity than their resistant counterparts. This chapter support the view of an ATPase activity associated with a V-type ATPase in the plasma membranes isolated from the chloroquine-sensitive strain of *P. falciparum*, as well as differential activities for mefloquine and quinine compared to artemisinin.

CHAPTER 4

Characterisation of chloroquine accumulation in isolated *Plasmodium falciparum* plasma membrane vesicles

4.1 Introduction

The mechanism responsible for chloroquine-resistance is still controversial. Drug transport is believed to be of crucial importance for understanding the mechanism of chloroquine resistance as it is clear that chloroquine-resistant parasites accumulate less chloroquine than resistant parasites (Bray and Ward, 1998). This lack of chloroquine accumulation was initially attributed to an efflux mechanism but latter studies suggested rather the presence of an uptake mechanism (Ridley, 1998). Chloroquine accumulates into parasitised erythrocytes several hundred folds when compared to uninfected erythrocytes (Geary *et al.*, 1986). However, chloroquine is concentrated in the food vacuoles of *P. falciparum* 600- to 800-fold more than can be accounted for by its properties as a diprotic weak base (Krogstad, and Schlesinger 1987b). The role of the parasite plasma membrane in accumulating chloroquine in the parasitized erythrocyte is still unknown. Chloroquine accumulation studies in the plasmodial food vacuoles failed to fully explain the mechanism of chloroquine resistance and could suggest a possible involvement of the parasite plasma membrane in chloroquine transport mechanism. Therefore to obtain purified parasite plasma membranes is of particular interest to study its role in the mechanism of chloroquine accumulation.

This chapter will determine the chloroquine sensitivity of all the strains used in this study. Chloroquine accumulation in infected-erythrocytes and in plasma membranes isolated from chloroquine-sensitive and –resistant parasites will be characterised.

The specificity of [^{3}H]-chloroquine accumulation in plasma membranes will be examined using an excess of cold chloroquine. Chloroquine accumulation in parasitised erythrocytes has been shown to be energy-dependent and saturable (Fitch *et al.*, 1974; Chou *et al.*, 1980). Therefore, the ATP-dependence of chloroquine accumulation in plasma membranes will be investigated to determine whether an ATP-driven drug transport occurs at the plasma membrane level. To further characterise the chloroquine accumulation capabilities of plasma membranes, the effect of external chloroquine concentration on chloroquine accumulation in plasma membranes isolated from 2 chloroquine-sensitive and 3 chloroquine-resistant *P. falciparum* strains will be examined.

Observations of reduced chloroquine accumulation in chloroquine-resistant *P. falciparum* and the ability of verapamil and a number of other unrelated agents to reverse chloroquine resistance prompted mechanistic comparison of chloroquine resistance in the parasite with multidrug resistance in mammalian cancer cell lines (Martin *et al.*, 1987). The P-glycoprotein is proposed to function as an ATP-driven drug exporter in the plasma membrane of cancer cells and chemosensitisers are thought to either bind directly to P-pg or act as inhibitors of drug transport via indirect biophysical effects on plasma membrane fluidity (Wadkins and Houghton, 1993). Despite compelling evidence for common resistance reversal mechanism in the 2 cell types, this model has been questioned by several investigators (Sanchez *et al.*, 1997; Bray *et al.*, 1999). To determine whether the chemosensitisers act at the plasma membrane level, their effect on chloroquine accumulation will be examined.

Pgh1 was identified on the *P. falciparum* plasma and vacuolar membrane as a potential component of chloroquine uptake and its resistance mechanism (Cowman *et al.*, 1991). P-type ATPases, such as Pgh1, are characterised by the presence of a phosphorylated intermediate and can be inhibited by vanadate, a transition state analogue of phosphate. Oligomycin, an inhibitor of the mitochondrial membrane ATPase by preventing phosphoryl group transfer has been shown to inhibit P-gp mediated drug transport (Hollo *et al.*, 1994; Homolya *et al.*, 1993). It would therefore be interesting to study the effect of ATPase inhibitors on the chloroquine

accumulation capabilities of isolated plasma membranes in order to establish the role played by P-type and V-type ATPases in chloroquine accumulation.

4.2 Results

4.2.1 Chloroquine susceptibility of *Plasmodium falciparum* strains

Susceptibility of the parasites to chloroquine was evaluated using the method described by Makler et al (1993b). Chloroquine dose-response relationships of the 6 strains used in this study were determined after a 48h-drug exposure using the parasite lactate dehydrogenase activity as an indicator of parasite viability. The recommended chloroquine plasma concentration of 30 µg/l required for the treatment of susceptible *P. falciparum* (Wernsdorfer and McGregor, 1988, Katzung, 1995) was taken as the threshold for chloroquine sensitivity. D10 and RSA3 were found to be chloroquine-sensitive with IC_{50} values of 28.32±3.53 and 52.46± 17.01nM (n=3), respectively. Fac8, RSA11, RSA15 and K1 were chloroquine-resistant with IC_{50} values of 157.50± 55.98, 252.53± 20.79, 294.77± 31.86 and 379.10± 34.96nM (n=3), respectively (figure 12).

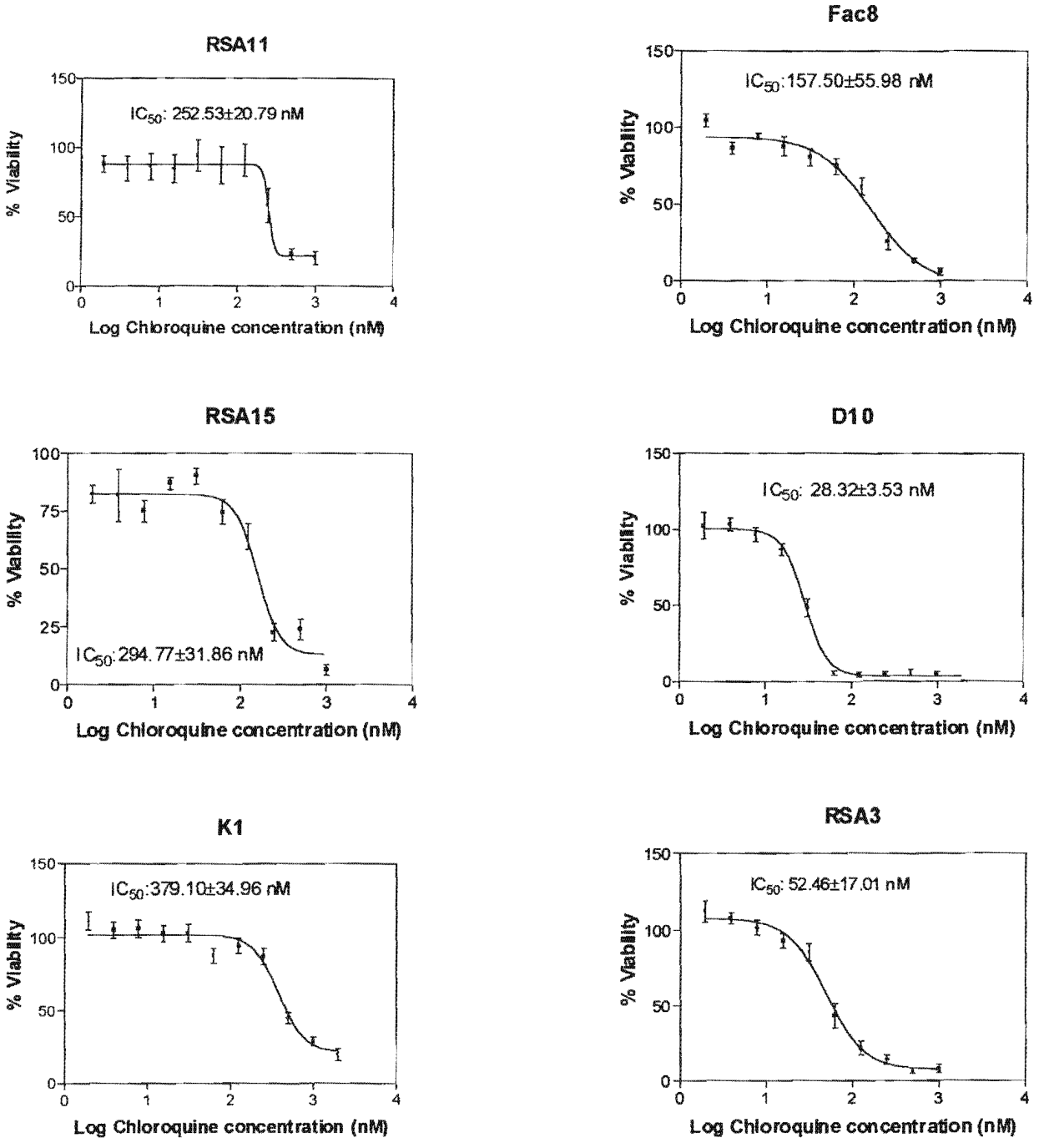

Figure 12: Chloroquine dose-response curves for the *Plasmodium falciparum* strains D10, RSA3, Fac8, RSA11, RSA15 and K1. The range of chloroquine concentration was either 1.95 to 1000 nM or 3.90 to 2000nM. Each point represents the means of 3 separate experiments. Each experiment was performed in quadruplicate. Curves were fitted using the GraphPad Prism version 2.01 software.

4.2.2 Chloroquine accumulation in erythrocytes parasitised with chloroquine-resistant or –sensitive *Plasmodium falciparum*

Chloroquine accumulation in parasitised erythrocytes was evaluated over a 1 to 500nM external chloroquine concentration range (see section 7.8). The chloroquine-sensitive strain D10 accumulated more chloroquine than the chloroquine-resistant strains Fac8 and K1 at all the concentrations tested (Figure 13).

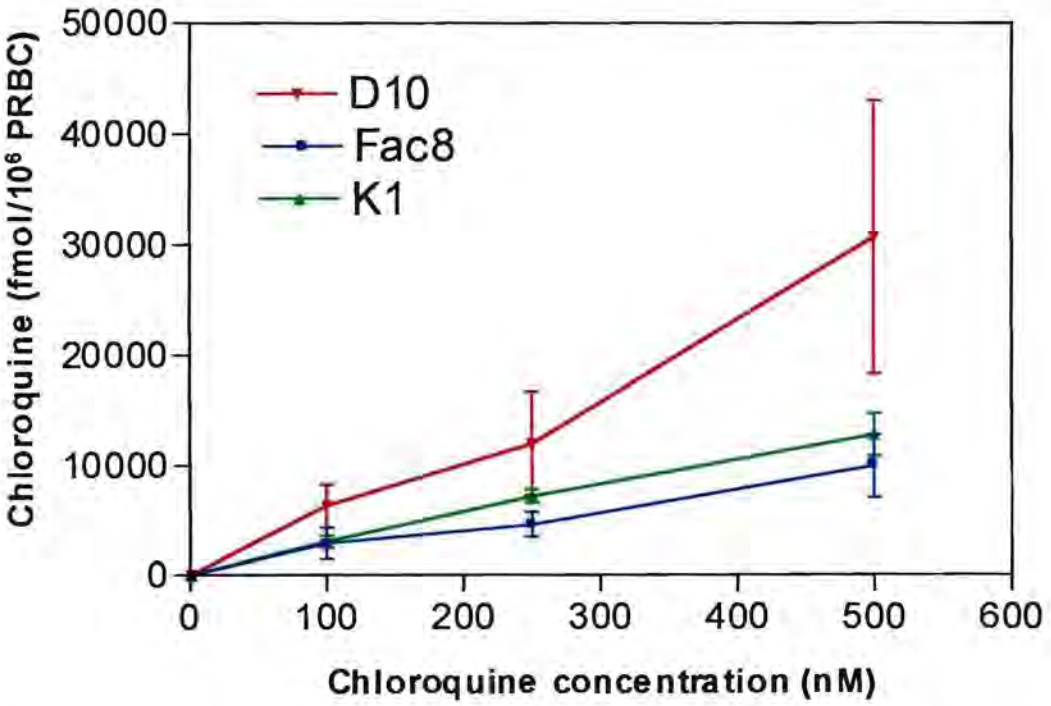

Figure 13: Chloroquine accumulation in erythrocytes parasitised with the *Plasmodium falciparum* strains D10, Fac8 and K1 over a range of external chloroquine concentrations from 1 to 500nM. Chloroquine accumulation in unparasitised erythrocytes was subtracted. Chloroquine accumulation is expressed as fmol/10^6 parasitised erythrocytes. Error bars represent standard deviations from means of 3 separate experiments, each performed in duplicate.

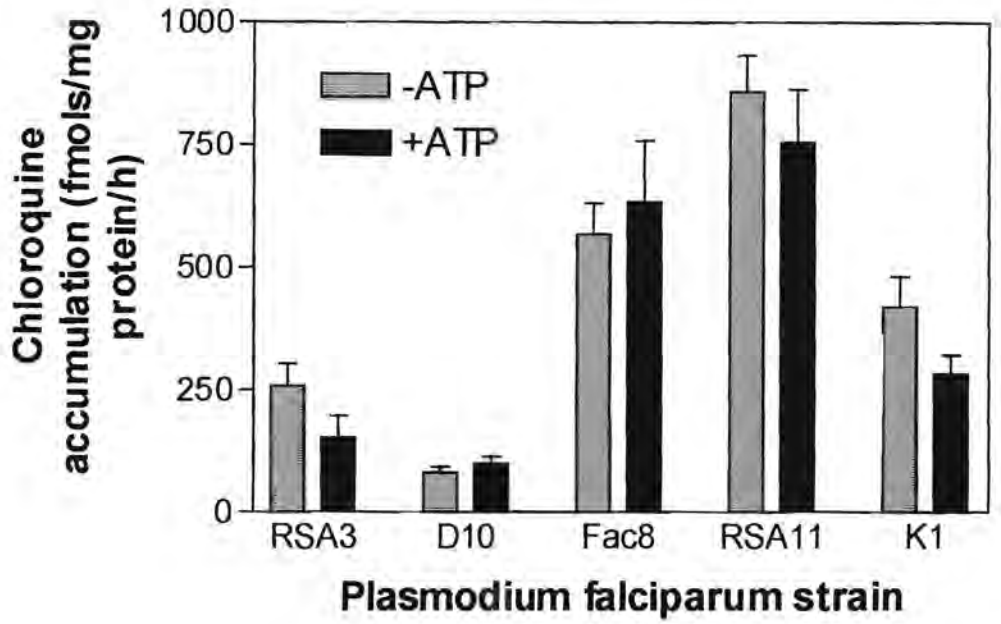

Figure 14: Chloroquine accumulation in *Plasmodium falciparum* plasma membranes isolated from the *Plasmodium falciparum* strains D10, RSA3, RSA11, Fac8 and K1 in the presence or absence of 2mM ATP. Chloroquine accumulation is expressed as fmol/mg protein/h. Error bars represent standard deviations from means of 3 separate experiments, each performed in duplicate.

4.2.5 Effect of external chloroquine concentration on chloroquine accumulation in parasite plasma membranes isolated from chloroquine-resistant and -sensitive strains of *Plasmodium falciparum*

Chloroquine accumulation by parasite plasma membranes isolated from different strains of *Plasmodium falciparum* exhibiting various degrees of chloroquine resistance was evaluated at 1, 100, 250 and 500nM of external chloroquine concentrations in the presence of 2mM ATP. Parasite plasma membranes isolated from the chloroquine-sensitive strains D10 and RSA3 accumulated significantly ($p \leq 0.05$) less chloroquine than the chloroquine-resistant strains RSA11, Fac8 and K1 at all the concentrations tested (Figure 15).

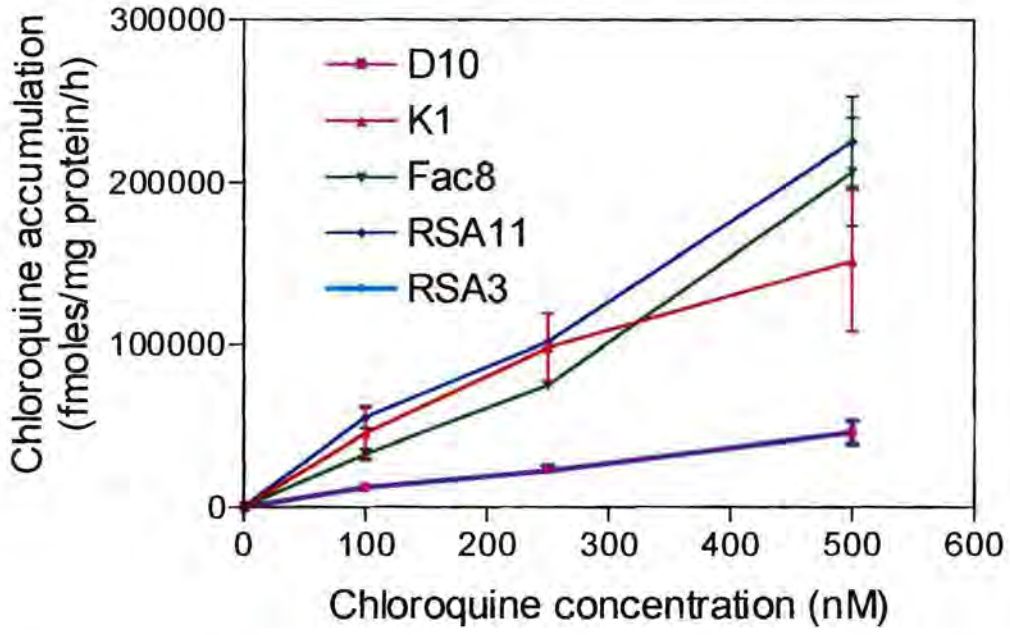

Figure 15: Chloroquine accumulation by parasite plasma membranes isolated from the *Plasmodium falciparum* strains D10, RSA3, RSA11, Fac8 and K1 at external chloroquine concentrations of 1, 100, 250 and 500nM. Chloroquine accumulation is expressed as fmol/mg protein/h. Error bars represent standard deviations from means of 3 separate experiments, each performed in duplicate.

4.2.6 Effect of chemosensitisers on chloroquine accumulation in *Plasmodium falciparum* plasma membranes

Verapamil (10μM), trifluoperazine (10μM) and progesterone (100μM) had little or no significant effect on the accumulation of [^{3}H]-chloroquine by plasma membrane purified from the *P. falciparum* strain Fac8 (Figure 16) with p values of 0.2963, 0.0455 and 0.2878, respectively. Ethanol (0.25%) used to solubilise progesterone had no effect on chloroquine accumulation by the membranes (data not shown).

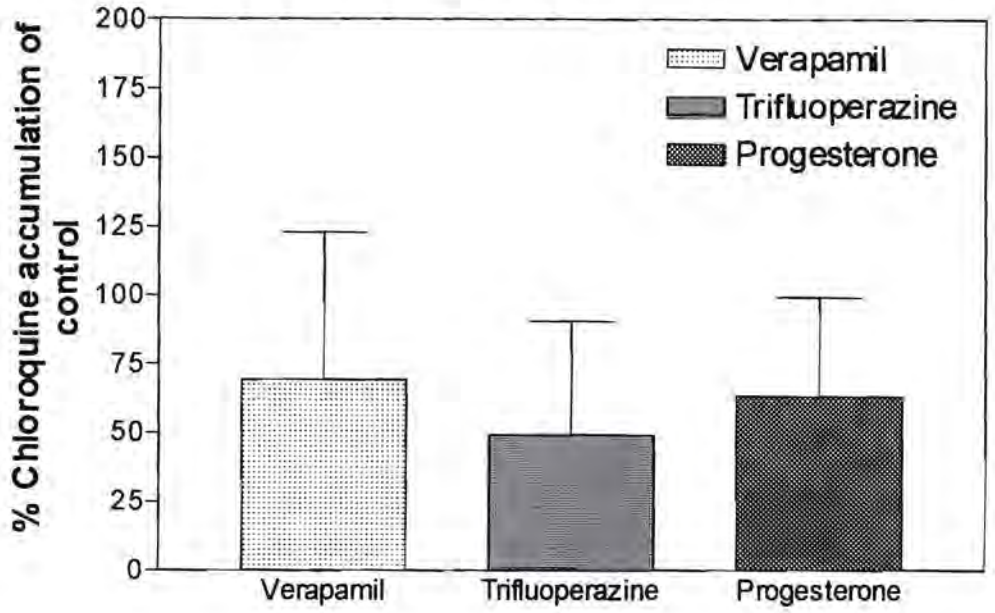

Figure 16: Effect of the chemosensitisers verapamil(10μM), trifluoperazine (10μM) and progesterone (100μM) on the accumulation of [^{3}H]-chloroquine by plasma membrane purified from the *P. falciparum* strain Fac8. Chemosensitisers were added to the assay 15-min. prior to the addition of [^{3}H]-chloroquine. Values are expressed as percentages of controls without drugs and error bars represent standard deviations from means of 3 separate experiments, each performed in duplicate.

4.2.7 Effect of ATPase inhibitors on chloroquine accumulation in *Plasmodium falciparum* plasma membranes

The absence of ATP-dependence for chloroquine accumulation into any of the isolated membrane vesicles tested suggested that the membrane ATPases were not involved in the uptake process. In order to confirm this, chloroquine uptake was measured in the presence of a number of ATPase inhibitors. Surprisingly, several inhibitors were found to reduce chloroquine uptake in the *P. falciparum* plasma membrane vesicles. The V-type ATPase inhibitors N-ethylmaleimide (NEM) (2mM) and bafilomycin A1 (1μM) reduced chloroquine accumulation in D10 plasma membranes by 57.3% and 61.3%, respectively. Ouabain (1mM), a classic inhibitor of the mammalian Na+/K+-ATPase, and an inhibitor of cation transport (Dawson, 1984) inhibited chloroquine accumulation by 53.2% while oligomycin (10μg/ml)

decreased chloroquine accumulation by 64.3%. In contrast, vanadate (100μM) did not affect chloroquine accumulation in D10 plasma membranes (figure 17).

The effect of most of the ATPase inhibitors tested, namely oligomycin, ouabain bafilomycin A1 and NEM on chloroquine accumulation in plasma membranes isolated from the *P. falciparum* strain Fac8 was found to be statistically significant with p values of 0.0014, 0.0065, 0.0023 and 0.0095, respectively. Only vanadate was shown to not have a statistically significant effect on chloroquine accumulation in Fac8 plasma membranes (p=0.4870). The presence of dimethylsulfoxide (DMSO), used to solubilise ouabain, oligomycin and bafilomycin A1 at a final concentration of 0.5, 0.2 and 0.1% respectively, had no effect on the accumulation of chloroquine in purified parasite plasma membranes (data not shown).

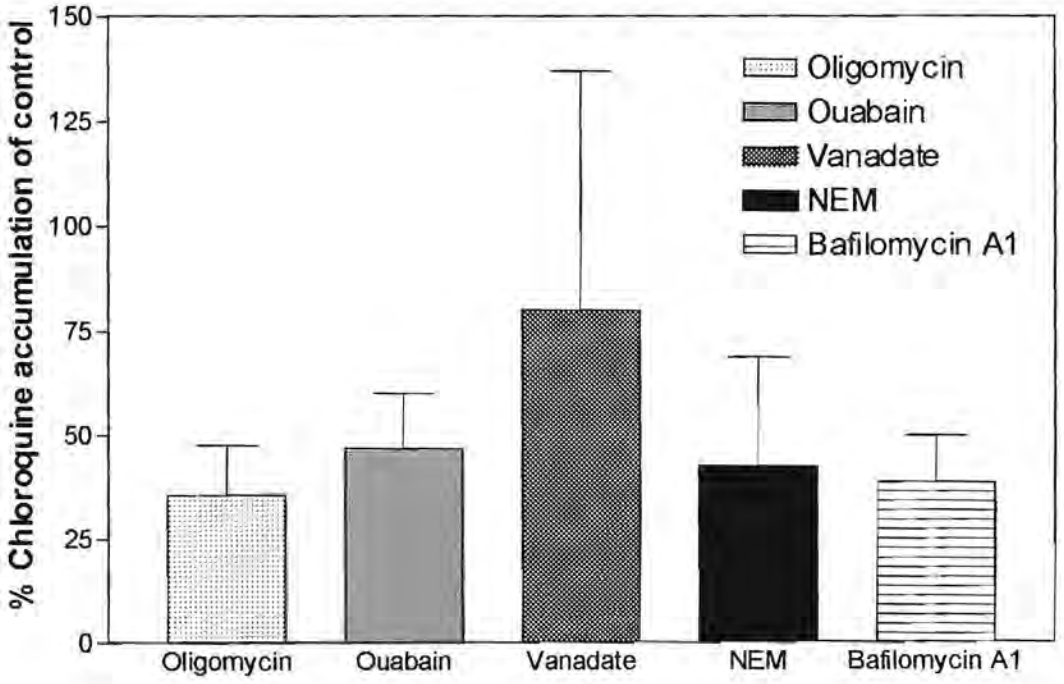

Figure 17: Effect of the ATPase inhibitors vanadate (0.1mM), oligomycin (50μg/ml), ouabain (1mM), bafilomycin A1 (1μM), and N-ethylmaleimide (NEM) (2mM) on the accumulation of [^{3}H]-chloroquine by plasma membrane purified from the *P. falciparum* strain D10. ATPase inhibitors were added to the assay 15-min. prior to the addition of [^{3}H]-chloroquine. Values are expressed as percentages of controls without drugs and error bars represent standard deviations from means of 3 separate experiments, each performed in duplicate.

3.3 Discussion

Of the *P. falciparum* strains used, D10 and RSA3 had IC_{50} values consistent with chloroquine-sensitive strains, while Fac8, RSA11, RSA15 and K1 had IC_{50} values consistent with chloroquine-resistant strains. The strains exhibited a 13-fold difference in chloroquine sensitivity, which made them suitable for an investigation of chloroquine resistance. RSA15 was discarded in this study, as its IC_{50} is similar to that of RSA11.

Chloroquine accumulation patterns of erythrocytes infected with D10, Fac8 and K1 trophozoites exposed to increasing concentration of external chloroquine were found to correlate with published data (Fitch, 1970; Bray *et al.*, 1992a; Bray and Ward, 1993). These results confirmed the observation that chloroquine-sensitive parasites accumulate chloroquine at a higher level than the chloroquine-resistant parasites. The significant decline in chloroquine accumulation between infected erythrocytes and their purified vacuoles (Saliba *et al.*, 1998) indicates a possible role for the plasma membranes in accumulating chloroquine.

[^{3}H]-Chloroquine accumulation by parasite plasma membrane vesicles isolated from the *Plasmodium falciparum* strain D10 was demonstrated to be specific as it was effectively inhibited by excess unlabelled chloroquine. Chloroquine accumulating capabilities of plasma membranes isolated from *P. falciparum* strains exhibiting various degree of chloroquine sensitivity was evaluated at external chloroquine concentrations of 1, 100, 250 and 500nM. Herwaldt *et al.* (1990) found similar chloroquine accumulation in membrane preparation from either a chloroquine-sensitive or –resistant parasites. In contrast, this study showed different chloroquine accumulating capabilities of membranes isolated from different strains. Parasite plasma membranes isolated from D10 and RSA3 accumulated significantly less chloroquine than those isolated from Fac8, K1 and RSA11 at all the concentrations tested. These results are difficult to interpret since the vesicles isolated from CQR parasites would have been expected to accumulate less chloroquine than their sensitive counterparts. Nevertheless, it is interesting to notice that the degree of

chloroquine accumulation appears to correlate with the level of ATPase activity measured in plasma membranes isolated from the various strains tested (see section 3.2.6). An explanation could be that these vesicles became leaky after saponin treatment. However, the fact that the trophozoites do not concentrate the dye trypan blue indicates that the integrity of the plasma membranes has been preserved. Another explanation for these findings could be that an ATPase is involved, either directly or indirectly in the mechanism of chloroquine accumulation into these vesicles. However, while Herwaldt *et al.* (1990) found an ATP-dependent chloroquine accumulation in membrane preparations from *P. falciparum* infected erythrocytes, this study failed to show an ATP-dependent mechanism of chloroquine accumulation in the purified plasma membranes. Chloroquine accumulation in plasma membranes was similar in presence or in absence of ATP. Increased permeability to chloroquine or H^+ in plasma membrane vesicles isolated from the CQS strains could explain the difference in the level of chloroquine accumulation observed. Increased permeability to H^+ ions could lead to lower pH in CQR vesicles, resulting to increased chloroquine accumulation. However, it is unlikely that the vesicles are able to maintain a transmembrane pH gradient in the absence of ATP. Another likely explanation could be that the vesicles are not primarily right-side-out but, instead, inside-out in which case the difference in chloroquine accumulation observed between the membranes isolated from the chloroquine-sensitive and –resistant parasites could be attributed to an active pumping of chloroquine. However, the finding that chloroquine accumulation is independent of ATP would argue against a drug-pumping phenomenon and would suggests that the accumulation maybe a consequence of trapping of free chloroquine within the vesicles or binding of the chloroquine to a component of the membranes rather than transport. Considering the significant difference in chloroquine accumulation at all the concentrations tested of *Plasmodium falciparum* plasma membranes isolated from D10 and Fac8, these 2 strains were chosen to further characterise their chloroquine accumulation capabilities in this study.

Vanadate has been previously shown to be a potent inhibitor of Pgp ATPase activity (Shapiro and Ling, 1994; Urbatsch *et al.*, 1994) and can inhibit Pgp mediated

transport in membrane vesicles (Horio *et al.*, 1988). In this study, vanadate did not affect chloroquine accumulation in D10 plasma membranes, suggesting that a P-type ATPase is not involved in the mechanism of chloroquine accumulation at the plasma membrane level. Herwaldt *et al.* (1990) also failed to show inhibition of chloroquine accumulation by vanadate in membrane preparations from *P. falciparum* infected erythrocytes. Moreover, chloroquine accumulation was reduced in the presence of oligomycin and NEM. Similarly this study found an inhibitory effect of these two ATPase inhibitors on plasma membrane chloroquine accumulation. The ATPase for chloroquine accumulation is similar to the mammalian vacuolar ATPase in its sensitivity to the V-ATPase inhibitors, NEM and bafilomycin A1. In addition, oligomycin and ouabain inhibited chloroquine accumulation in the plasma membranes. However as chloroquine accumulation was not dependent on the presence of ATP, one has to strongly mention the possibility of a non-specific effect of these inhibitors. It is difficult to reconciliate the inhibitory effect of ouabain with the absence of a Na^+/K^+-ATPase.

Verapamil and other unrelated compound has been shown to reverse chloroquine resistance by increasing chloroquine accumulation in parasitised erythrocytes (Martin *et al.*, 1987; Krogstad *et al.*, 1987a; Bray *et al.*, 1994). None of the chemosensitisers tested in this study, namely verapamil, trifluoperazine and progesterone had an effect on chloroquine accumulation in plasma membranes. This is consistent with the results obtained by Herwaldt *et al.* (1990) indicating that the drug tested (verapamil, vinblastine, desipramine and dialtiazem) inhibited chloroquine accumulation from resistant parasites only partially. Since these chemosensitisers are known reversers of chloroquine resistance, these results suggest that resistance reversal does not occur at the plasma membrane level.

4.4 Conclusion

This chapter determined the chloroquine sensitivity of the various *P. falciparum* strains used in this study and confirmed that parasitised erythrocytes isolated from

chloroquine-sensitive parasites accumulate more chloroquine than their resistant counterpart. The specificity of chloroquine accumulation by parasite plasma membrane was demonstrated and the chloroquine accumulation profile of plasma membranes isolated from chloroquine-resistant and –sensitive parasites was determined over several external chloroquine concentrations. Plasma membranes isolated from CQS parasites were found to accumulate less chloroquine than those isolated from CQR parasites. The observation that the degree of chloroquine accumulation correlates with the level of ATPase activity measured from various strains of *P. falciparum* lead us to the proposal that the parasite plasma membrane might play a role in determining the global level of chloroquine accumulation and hence chloroquine-resistance. However, the effect of ATPase inhibitors on chloroquine accumulation was inconclusive since chloroquine accumulation was found to be ATP-independent, suggesting that the measured accumulation might be due to binding rather than transport. This study failed to provide evidence that the chemosensitisers act at the plasma membrane level.

CHAPTER 5

Role of P-Glycoprotein Homologue 1 in Chloroquine accumulation by *Plasmodium falciparum* plasma membrane

5.1 Introduction

Even though it has been extensively investigated, the role of Pgh1 in the mechanism of chloroquine resistance has not yet been elucidated. Chloroquine resistance shows similarities with multidrug resistance seen in mammalian tumor cells, since resistance can be reversed in vitro by verapamil and other unrelated compounds (Martin *et al.*, 1987). Multidrug resistant tumor cells are able to expel a large range of chemically distinct anti-tumor drugs (Skovsgaard, 1978). The multidrug resistance phenotype is believed to result from the overexpression of an ATP-dependent drug exporter (Riordan *et al.*, 1985) and can be inhibited by several drugs including the calcium channel blockers (Tsuruo *et al.*, 1984). A 170-kDa protein located on the plasma membrane has been identified as a likely candidate accounting for the phenomenon of multidrug resistance (Endicott and Ling, 1989). This P-glycoprotein can be photoaffinity labelled by both drugs (Safa, 1992; Cornwell *et al.*, 1986) and ATP (Cornwell *et al.*, 1987b; Georges *et al.*, 1991).

Plasmodium falciparum has 2 genes (*pfmdr1* and *pfmdr2*) that are homologous to the *mdr* genes in mammals (Foote *et al.*, 1989; Wilson *et al.*, 1989). Of these 2 genes, *pfmdr1* has been found to be amplified in some chloroquine-resistant isolates of *Plasmodium falciparum* (Foote *et al.*, 1989). The *pfmdr1* product, Pgh1 is mainly localised on the membrane of the plasmodial digestive vacuole (Cowman *et al.*,

1991). Pgh1 contain 2 homologous ATP-binding cassette regions and has been shown to bind nucleotides by photoaffinity labelling (Karcz *et al.*, 1993b), which is consistent with a role in drug transport. However, Further studies indicated that the level of chloroquine resistance in a number of field isolates did not correlate with the level of Pgh1 expression (Cowman *et al.*, 1994). It has then been suggested that several alleles of *pfmdr1* are closely linked to chloroquine resistance (Foote *et al.*, 1989). In contrast, another study demonstrated that chloroquine resistance segregated independently of the *pfmdr1* and *pfmdr2* genes in a genetic cross between a chloroquine-sensitive and a chloroquine-resistant strain (Wellems *et al.*, 1990, 1991). Using a heterologous expression system for *pfmdr1* gene to express Pgh1 in CHO cells, Van Es *et al.* (1994a) found that wild-type Pgh1 could mediate chloroquine accumulation while chloroquine-resistant associated mutated forms could not. However, chloroquine failed to photoaffinity label Pgh1 (Foley *et al.*, 1994). In view of these conflicting data, no consistent molecular explanation which could account for a role of Pgh1 in chloroquine-resistance has yet been found.

The results presented in chapter 3 showed elevated ATPase activities in plasma membranes isolated from the CQR strains compared to the CQS strains. Although overall levels of Pgh1 protein in the whole parasites have not been correlated with the chloroquine resistance phenotype, it is possible that Pgh1 protein is overexpressed in the plasma membrane of CQR parasites. This chapter aim to investigate this hypothesis. For this purpose, Pgh1 was identified in plasma membranes isolated from CQR and CQS strains of *P. falciparum* and the subcellular localisation of Pgh1 in infected-erythrocytes was examined. The effect of anti-Pgh1 antibodies on chloroquine accumulation was also investigated.

5.2 Results

5.2.1. Preparation of anti-Pgh1 antibodies

5.2.1.1. Restriction enzymatic digestion of the pGEX vectors

Plasmids pGEX-3X encoding for GST-Pgh1 fusion proteins (donated by Dr. A.F. Cowman from the Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) were transformed into *E. coli*. The presence of the correct recombinant was confirmed by restriction digestion using Hinc II, Hind III and Pst I digestions and gel analysis (Plate 10 and 11).

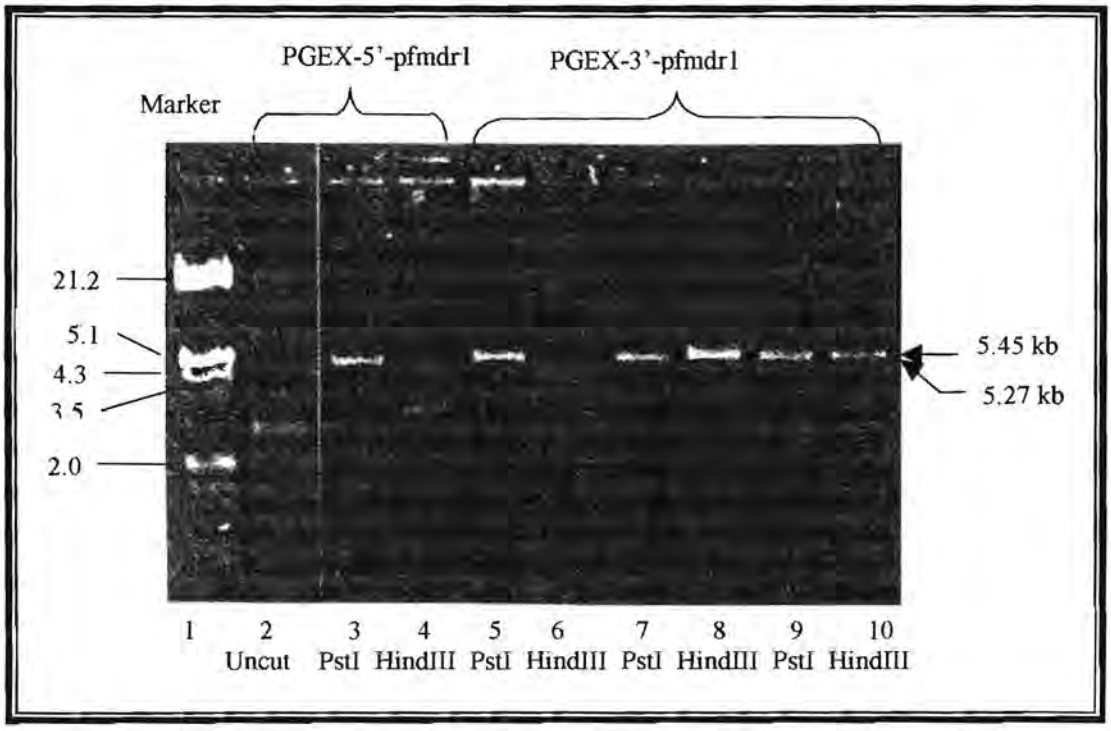

Plate 10: Agarose gel electrophoresis of pGEX-5'-*pfmdr1* and pGEX-3'-*pfmdr1* digested with PstI and HindIII. Lane 1 contains DNA markers with approximate size expressed in kb. Lane 2 contains pGEX-5'-*pfmdr1* uncut. Lane 3 and 4 contain of pGEX-5'-*pfmdr1* digested with PstI and HindIII, respectively. Lane 5, 7 and 9 contain pGEX-3'-*pfmdr1* digested with PstI while pGEX-3'-*pfmdr1* is to be found lane 6, 8 and 10.

The pGEX-3X vector presents 1 restriction site for Pst1 at position 1905, 3 restriction sites for Hinc II at positions 184, 1605 and 4157 and none for Hind III. One restriction site for Hind III is to be found within the 3'-*pfmdr1* insert at position 4428 (figure 18). Therefore, the predicted restriction pattern for pGEX-3'-*pfmdr1* is a single band of 5459 bp for Pst I or Hind III digestions and 3 DNA bands of 979, 1928 and 2552 bp for Hinc II digestion. Similarly, pGEX-5'-*pfmdr1* digested with Pst I should present one single band of 5267pb, 3 DNA bands of 979, 1736 and 2552 pb for Hinc II digestion while this plasmid should stay uncut with Hind III. Electrophoresis of the digested plasmids on 1% agarose gels showed the predicted fragments (plate 10 and 11).

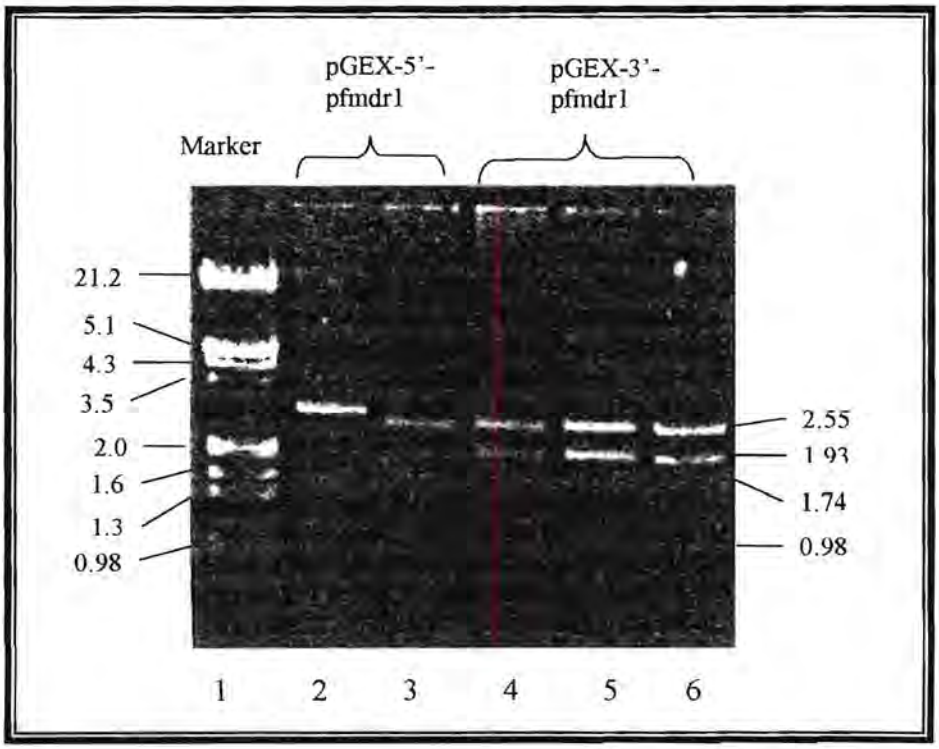

Plate 11: Agarose gel electrophoresis of pGEX-5'-*pfmdr1* (lane3) and pGEX-3'-*pfmdr1* (lane4, 5, 6) digested with HincII. Lane 1 contains DNA markers with approximate size expressed in kb. Lane 2 contains pGEX-5'-pfmdr1 uncut.

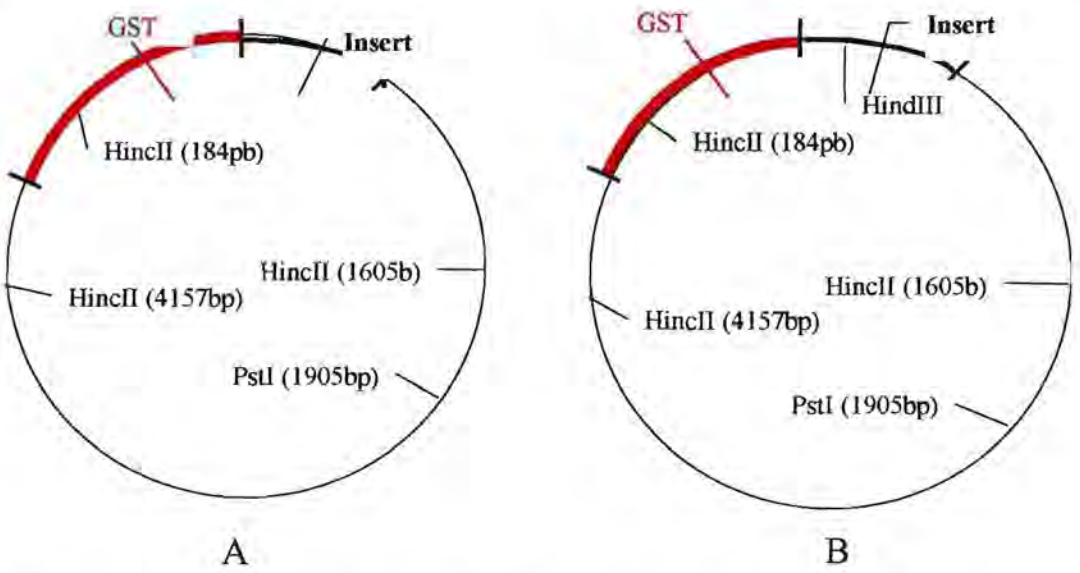

Figure 18: restriction map of pGEX-3X vector containing the 315 bp 5'-*pfmdr1* (A) or the 507 bp 3'-*pfmdr1* (B) insert.

5.2.1.2. Sequencing of the *Pfmdr1* inserts of the pGEX-3X vector

The sequence of *pfmdr1* inserts within the plasmids pGEX-3'-*pfmdr1* and pGEX-*5'pfmdr1* were confirmed by sequencing (see section 7.12.5). Figure 19 shows the 5'-DNA sequence of the 2024-2339 pb *pfmdr1* gene fragment encoding for the N-terminal-ATP binding site (Foote *et al.*, 1989; Gene bank accession number M29154). One base mismatch was found at position 249; however, this mismatch, a G instead of an A, does not alter the amino acid sequence.

Similarly, the sequence of the 4252-4758 pb *pfmdr1* gene fragment encoding for the C-terminal Pgh1 protein is represented figure 20 (Gene bank accession number M29154).

```
5' ccc cCG AAT TCT ATG ACA TCA AAT GAA TTA TTA GAA ATG AAA AAA  45
  P  P   N   S   M   T   S   N   E   L   L   E   M   K   K

 GAA  TAT CAA ACT ATT AAA GAT TCT GAT GTT GTT GAT GTG TCC AAA  90
 E    Y   Q   T   I   K   D   S   D   V   V   D   V   S   K

 AAA  GTA CTT ATA CAT GAT TTT GTA TCA TCA TTA CCA GAT AAA TAT 135
 K    V   L   I   H   D   F   V   S   S   L   P   D   K   Y

 GAT  ACC TTA GTA GGT TCC AAT GCA TCC AAA TTA TCA GGT GGA CAA 180
 D    T   L   V   G   S   N   A   S   K   L   S   G   G   Q

 AAA  CAA AGA ATA TCC ATT GCA AGA GCA ATT ATG AGA AAT CCT AAA225
 K    Q   R   I   S   I   A   R   A   I   M   R   N   P   K

 ATT  CTA ATT CTT GAT GAA GCT ACG TCT TCT TTA GAT AAT AAA TCT 270
 I    L   I   L   D   E   A   T   S   S   L   D   N   K   S

GAG  TAT TTA GTA CAA AAA ACA ATT AAT AAT TTG AAA GGA AAT GAA 315
E    Y   L   V   Q   K   T   I   N   N   L   K   G   N   E

 AAT  AGg gaa ttc atc gtg act gac tga 3'                     342
 N    R   E   F   I   V   T   D   *
```

Figure 19: Nucleotide sequence and deduced amino acid sequence of the insert of pGEX-5'-*pfmdr1* vector. At the 5'-end, 4 nucleotides (lower case) from the cloning vector encoding the amino acids PP are indicated. The 3'-end of the *pfmdr1* insert ends with AG at position 320. The following nucleotides (lower case) are part of the multiple cloning site of the pGEX-3X vector (gene bank accession number U13852). The mismatch in *pfmdr1* sequence is shaded.

```
5' TCA  ATA GTT AGT CAA GAA CCC ATG TTA TTT AAT ATG TCC ATA TAT 45
  S    I   V   S   Q   E   P   M   L   F   N   M   S   I   Y

  GAA  AAT ATC AAA TTT GGA AGA GAA GAT GCA ACA TTG GAA GAT GTT 90
  E    N   I   K   F   G   R   E   D   A   T   L   E   D   V

  AAA  CGT GTT AGT AAG TTT GCT GCT ATA GAT GAA TTT ATC GAA TCA 135
  K    R   V   S   K   F   A   A   I   D   E   F   I   E   S

  TTA  CCA AAT AAA TAT GAT ACA AAT GTT GGA CCA TAT GGT AAA AGC 180
  L    P   N   K   Y   D   T   N   V   G   P   Y   G   K   S

  TTA  TCA GGT GGA CAA AAA CAG AGA ATA GCT ATA GCT AGA GCA TTA 225
  L    S   G   G   Q   K   Q   R   I   A   I   A   R   A   L

  TTA  AGA GAA CCT AAA ATA TTA TTA TTA GAT GAA GCA ACA TCA TCA 270
  L    R   E   P   K   I   L   L   L   D   E   A   T   S   S

  CTT  GAT TCC AAT TCT GAG AAA TTA ATT GAA AAA ACT ATT GTA GAT 315
  L    D   S   N   S   E   K   L   I   E   K   T   I   V   D

  ATT  AAA GAT AAA GCT GAC AAA ACT ATT ATT ACT ATT GCC CAC AGA 360
  I    K   D   K   A   D   K   T   I   I   T   I   A   H   R

  ATT  GCA TCT ATA AAA CGA TCA GAC AAA ATT GTG GTA TTT AAT AAC 405
  I    A   S   I   K   R   S   D   K   I   V   V   F   N   N

  CCT  GAT CGA AAT GGA ACC TTT GTA CAG TCA CAT GGA ACA CAC GAT 450
  P    D   R   N   G   T   F   V   Q   S   H   G   T   H   D
```

```
GAA TTA TTA TCA GCA CAA GAT GGA ATA TAT AAA AAA TAT GTA AAA  495
E   L   L   S   A   Q   D   G   I   Y   K   K   Y   V   K

TTA GCT AAA tga                                             507
L   A   K   *
```

Figure 20: Nucleotide sequence and deduced amino acid sequence of the insert of pGEX-3'-*pfmdr1* vector.

5.2.1.3. Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis of purified fusion protein

The GST-fusion proteins were purified by affinity chromatography. The yield of recovery expressed by protein determination of the N-terminal ATP-GST fusion protein was 1.5-2.0 and 0.1-0.4mg/l of bacterial culture, respectively.

Purified fusion proteins were examined using SDS-PAGE along with bacterial extract after induction of the GST-fusion protein by IPTG. Purification of the proteins was shown by the presence of a band of molecular weight of 38kD and 45 kD corresponding to the N-terminal-ATP-GST and the C-terminal-GST fusion protein, respectively (plate 12). However, approximately 60% of the N-terminal-ATP-GST fusion protein was degraded during the purification steps.

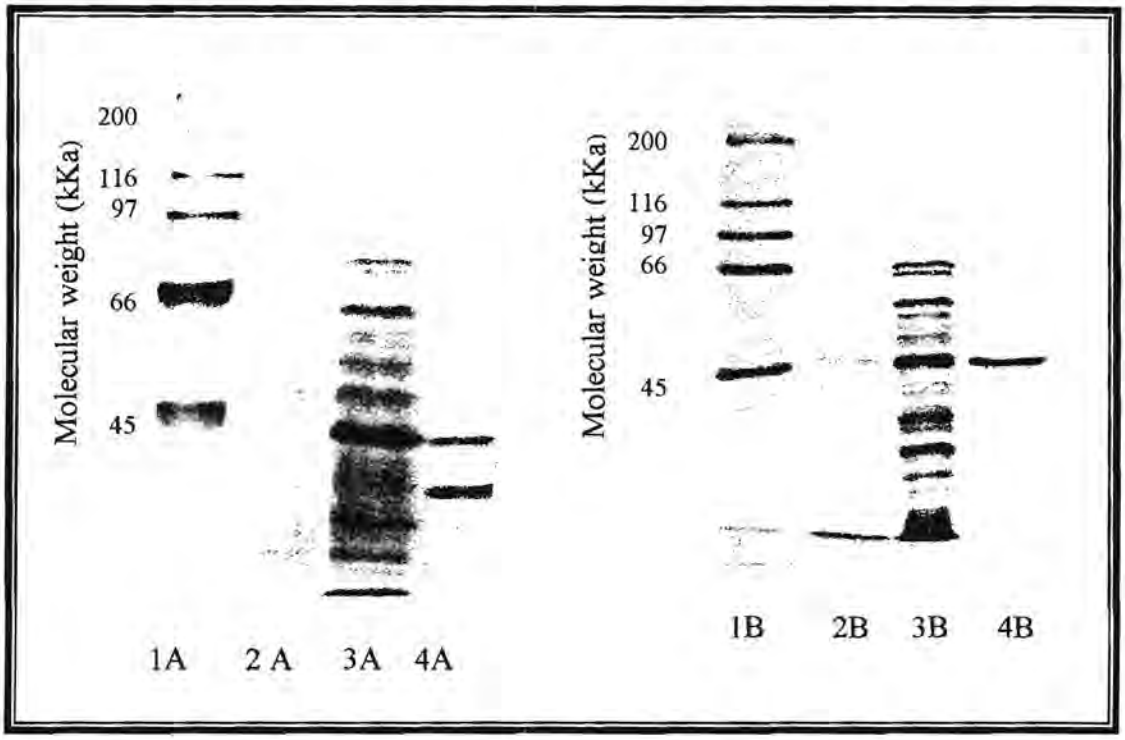

Plate 12: SDS-PAGE of purified ATP-GST fusion protein (lane 4A) and C-terminal GST fusion protein (lane 4B) compared to the bacterial extract after induction of the N-terminal ATP-GST (lane3A) and C-terminus-GST (lane3B) expression. Lane 2A and 2B contain the bacterial extract of the transformed cells before induction. Lane 1A and 1B contain protein markers with approximate molecular weight expressed in kDa.

5.2.1.4. Recognition of Pgh1 in trophozoites by rabbit serum directed against the N-terminal ATP-binding site and the C-terminus of Pgh1

Western blots of K1 trophozoites revealed with rabbit serum against Pgh1 were performed to determine whether these antibodies recognised Pgh1. 10µg of purified K1 trophozoite protein were electrophoresed, transferred on PVDF membranes, and probed with rabbit serum raised against the N-terminal ATP-binding site or the C-terminus of Pgh1, at a dilution of 1:1000. Rabbit pre-immune serum was included as a control. Visualisation was performed with goat anti-rabbit antibodies conjugated to horseradish peroxydase. For a detailed description of the method see section 7.15.

Antibodies raised against the N-terminal ATP-binding site and the C-terminus of Pgh1 did recognise a band of approximate molecular weight of 160kDa corresponding to Pgh1 protein. A lower band of 75kDa was also recognised by the antibodies raised against the N-terminal ATP-binding site. None of these bands was recognised by the pre-immune sera (plate 13).

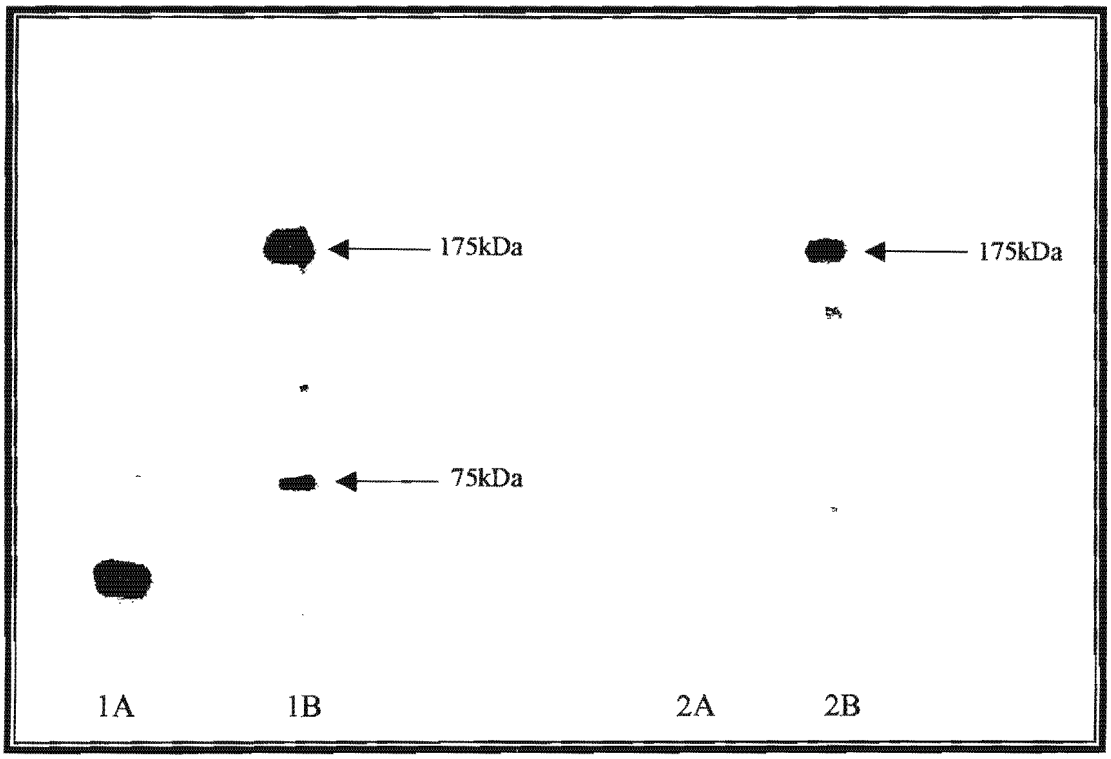

Plate 13: Purified K1 trophozoites protein (10μg) were electrophoresed, transferred to PVDF membranes and probed with either the rabbit serum raised against the N-terminal ATP-binding site (lane 1B) or the C-terminus of Pgh1 (lane 2B), at a dilution of 1:1000. Pre-immune sera were included as a control (lane 1A and 2A). Recognised bands were detected using a chemiluminescent system. Approximate molecular weights of the recognised bands are indicated.

5.2.2 Identification of Pgh1 in parasite plasma membranes isolated from *Plasmodium falciparum*

Equal amount of protein (10μg) of Fac8 food vacuoles, D10, RSA3, K1, Fac8 and RSA11 plasma membranes were electrophoresed, transferred onto nitrocellulose and probed with the anti-Pgh1 antibodies raised against the C-terminus fragment of the protein. In the lane containing Fac8 food vacuoles, a double band in the 70-75 kDa region was detected in addition to the major band at 175 kDa. In all lanes containing

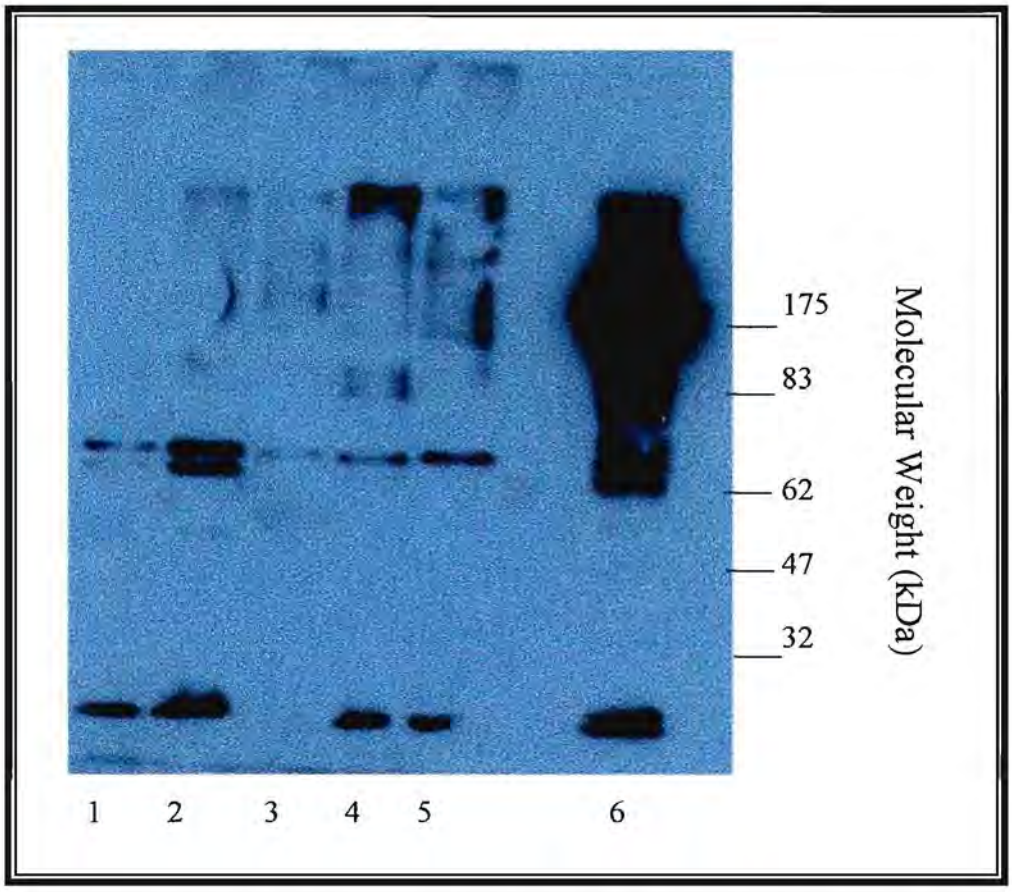

Plate 14: Equal amount of protein (10μg) of Fac8 food vacuoles (lane 6), D10 (lane 2), RSA3 (lane 1), K1 (lane 3), Fac8 (lane 4) and RSA11 (lane 5) plasma membranes were electrophoresed, transferred onto nitrocellulose and probed with the anti-Pgh1 antibodies raised against the C-terminus fragment of the protein. Recognised band were detected using chemiluminescence. Approximate molecular weights expressed in kDa are indicated

plasma membranes proteins, a band of approximate molecular weight of 75 kDa was detected while no band was found at 175 kDa. The intensity of the 75 kDa band was similar for RSA3, Fac8 and RSA11 plasma membranes proteins while D10 plasma membrane proteins produced the strongest signal and K1 plasma membranes protein, the weakest (Plate 14). These bands of lower molecular weight might result from breakdown products of Pgh1. The very strong band in lane 6 confirms the reported high concentration of Pgh1 protein present in Fac8 food vacuoles (Plate 14).

5.2.3 Localisation and membrane recognition with anti-Pgh1 antibodies by immunoelectron microscopy

The subcellular localisation of Pgh1 was investigated using immunoelectron microscopy by labelling section of RSA11 trophozoite-infected erythrocytes using antibodies against $Pgh1_{1-18}$ peptide prepared in our laboratory (see section 7.12.9) and 10nm gold particles. Briefly, infected-erythrocytes were fixed with glutaraldehyde/formaldehyde and embedded in LR White resin. Then, 1μm-thick sections of the sample embedded in resin were put onto a copper grid and stained with uranyl acetate. The rabbit anti-$Pgh1_{1-18}$ was added and then anti-rabbit antibodies labelled with 10nm gold particles. As a control, rabbit pre-immune serum was used. For a detailed description of the method see section 7.17.

No binding of gold was observed using a rabbit pre-immune serum (plate 18). The black dots show that Pgh1 is distributed all over the trophozoites but there is no Pgh1 in the erythrocyte itself (plate 15). Labeling was weak over the parasite plasma membrane indicating a very low amount of Pgh1 present in this membrane (plate 15, 16, 17).

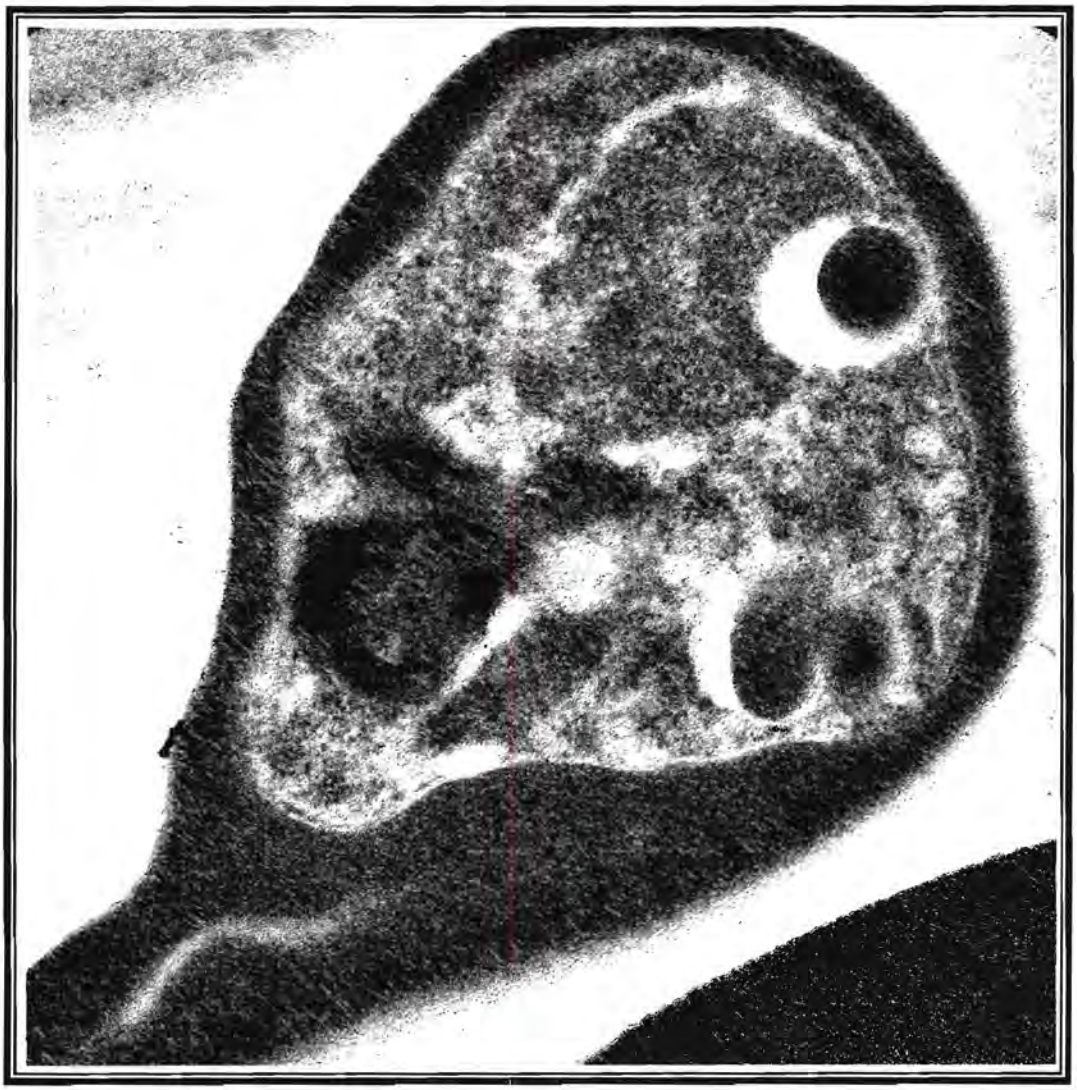

Plate 15: Immunogold electron microscope localisation of Pgh1 in RSA11 trophozoites infected-erythrocytes (Magnification x25,000). The antibody used was the anti-Pgh1$_{1-18}$ followed by anti-rabbit labelled with 10nm gold particles.

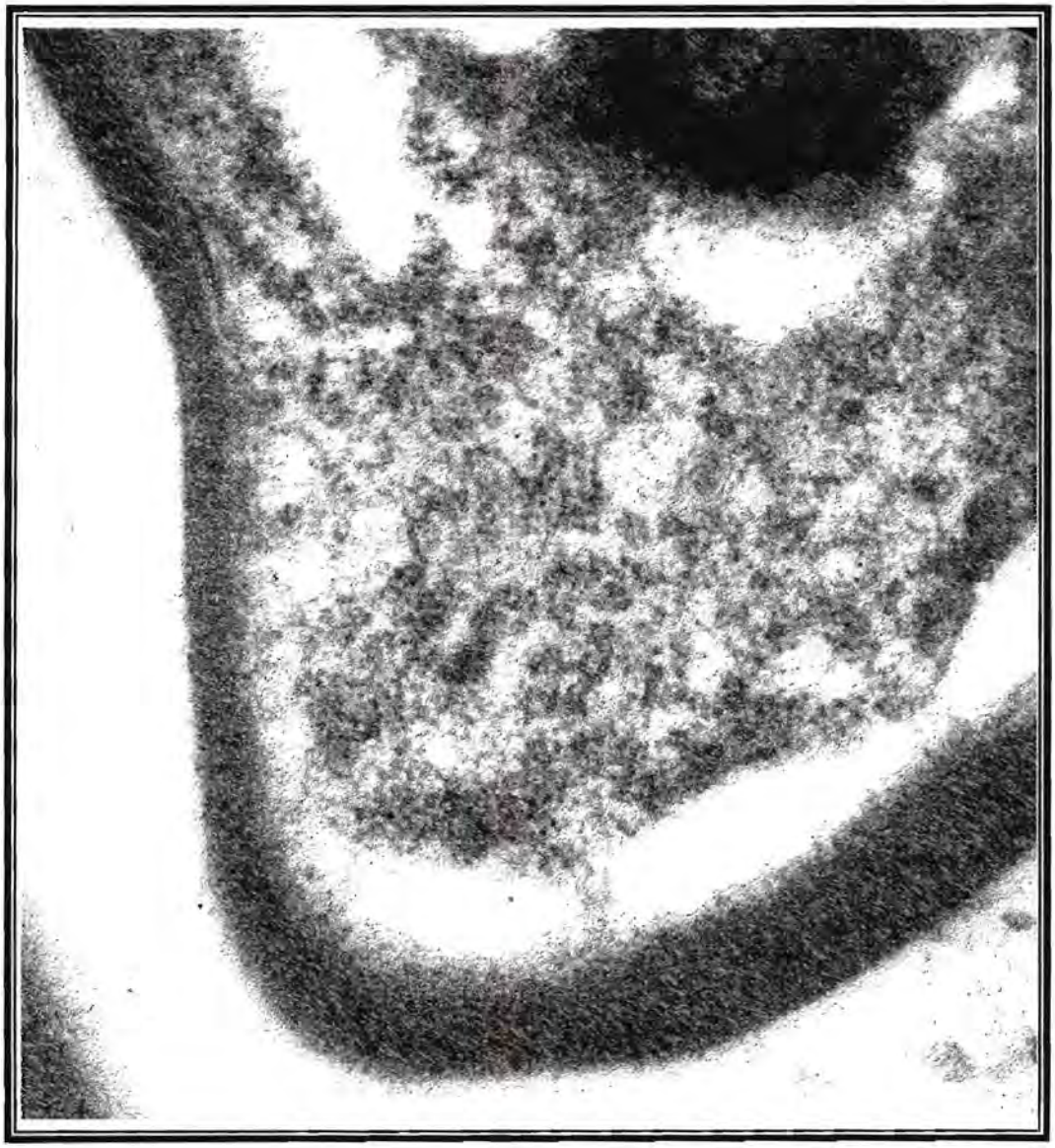

Plate 16: Immunogold electron microscope localisation of Pgh1 in RSA11 trophozoites infected-erythrocytes (Magnification x40,000). The antibody used was the anti-Pgh1$_{1-18}$ followed by anti-rabbit labelled with 10nm gold particles.

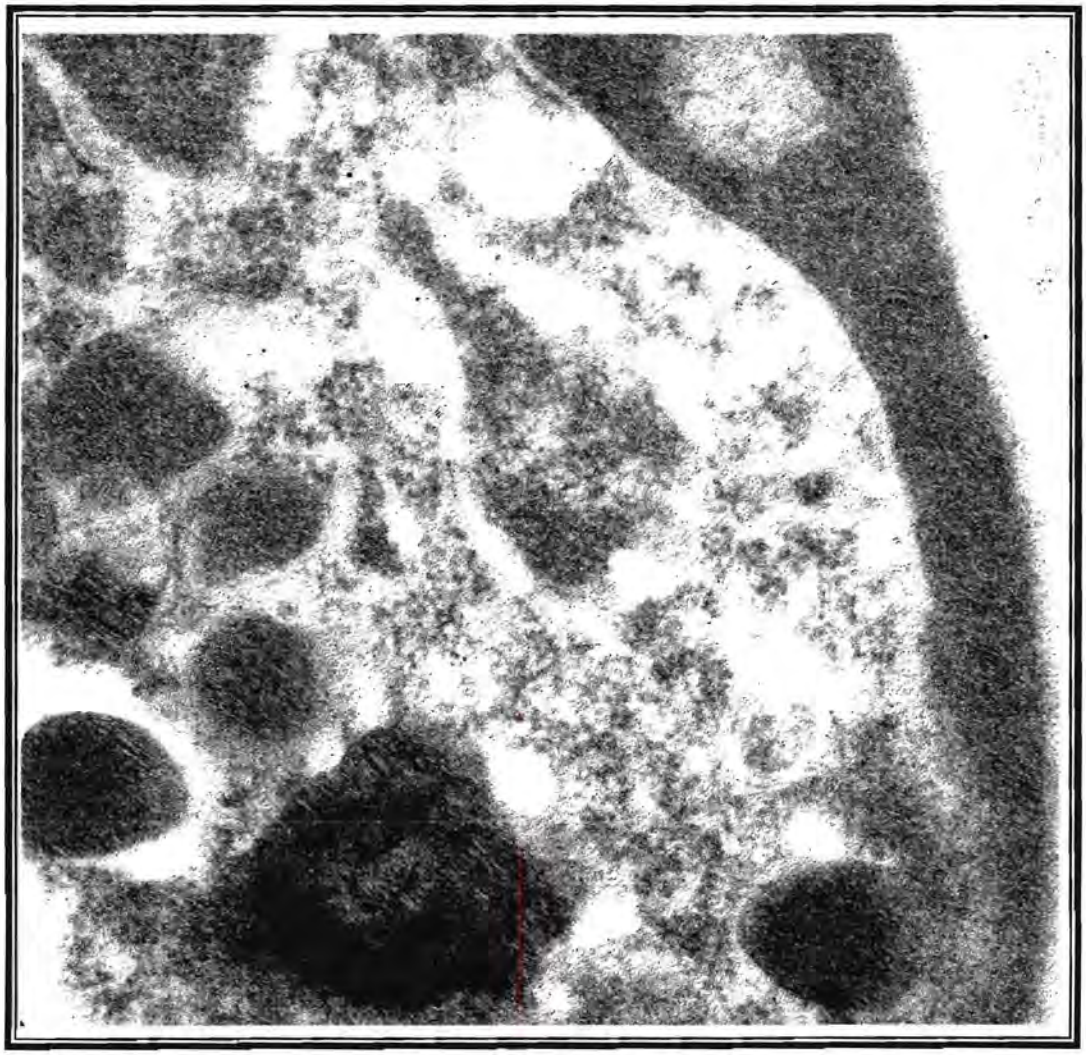

Plate 17: Immunogold electron microscope localisation of Pgh1 in RSA11 trophozoites infected-erythrocytes (Magnification x40,000). The antibody used was the anti-$Pgh1_{1-18}$ followed by anti-rabbit labelled with 10nm gold particles.

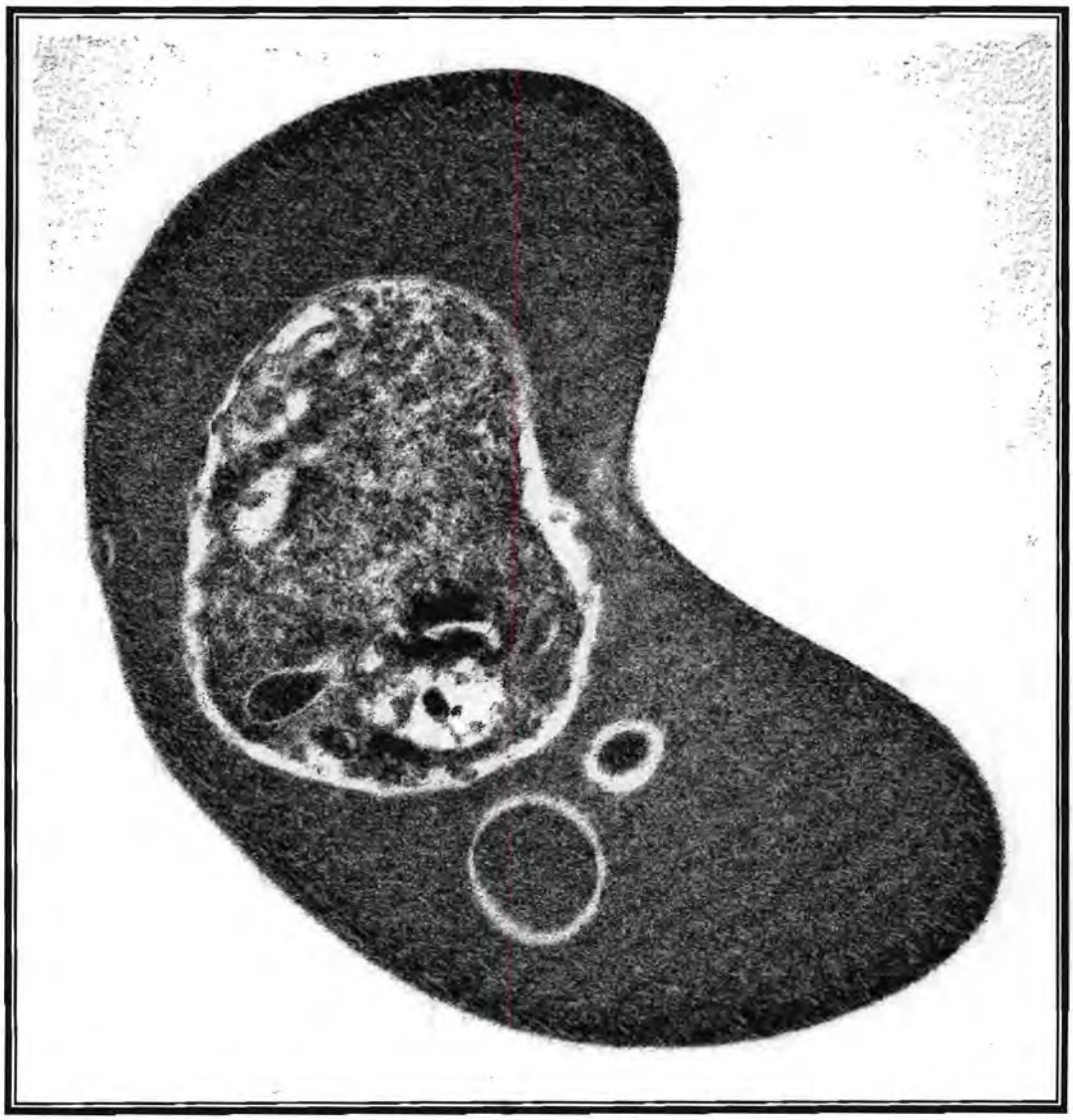

Plate 18 : Electron micrograph of RSA11 trophozoite infected-erythrocytes immunogold labelled using rabbit pre-immune serum (control) (Magnification x10,000).

5.2.4 Effect of anti-Pgh1 antibody on chloroquine accumulation in isolated *Plasmodium falciparum* plasma membranes

Antibodies to 2 fragments of *pfmdr1* encoding for the N-terminus ATP-binding site and the C-terminus of Pgh1 diluted 1:20 in vesicle buffer II were incubated with 40μg protein of purified D10 plasma membranes for 15 minutes prior to the addition of 1nM [^{3}H]-chloroquine (see methods section 7.9). Plasma membranes were also exposed to the rabbit pre-immune sera as a control. None of the antibodies tested had a significant effect on chloroquine accumulation by plasma membranes (Figure 21)

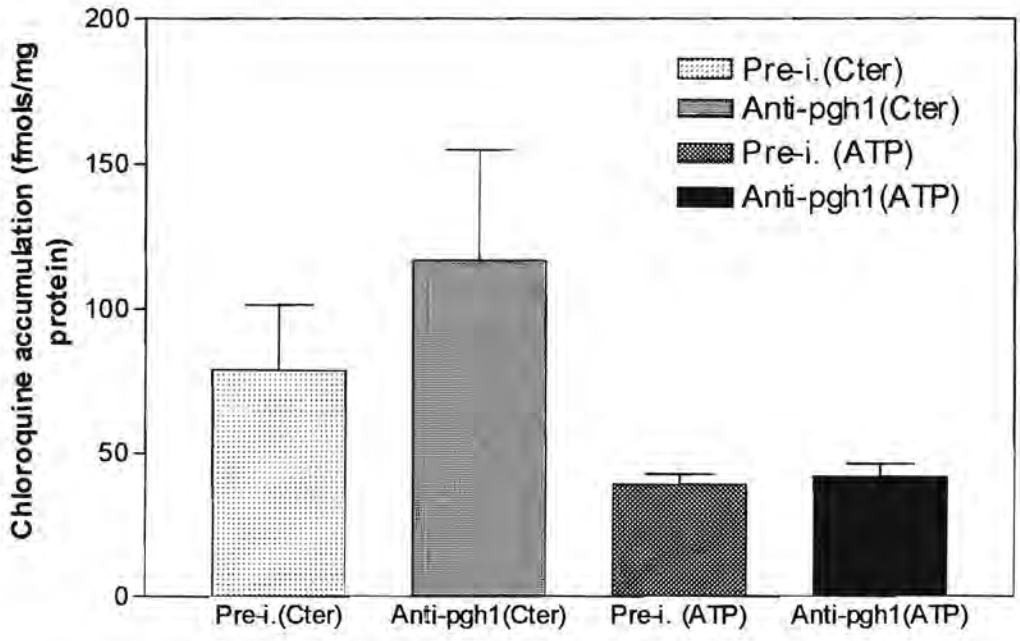

Figure 21: Effect of anti-Pgh1 antibodies raised against the C-terminus and the N-terminal ATP-binding site on chloroquine accumulation by D10 plasma membranes. As a control, chloroquine accumulation by D10 plasma membranes in presence of the rabbit pre-immune sera was determined. Chloroquine accumulation is expressed as fmol/mg protein/h. Error bars represent standard deviations from means of 3 separate experiments, each performed in duplicate.

5.3 Discussion

5.3.1. Preparation of antibodies to 2 fragments of *pfmdr1* encoding for the N-terminal ATP-binding site and the C-terminus of Pgh1

Competent cells were transformed with two plasmid expression vector constructs encoding for the N-terminal ATP-binding site and the C-terminus of Pgh1. The presence of the correct recombinant was confirmed by restriction digestion using HincII, HindII and PstI digestions and gel analysis as well as sequence analysis of the inserts. More than 50% of the N-terminal ATP-GST fusion protein was degraded during the purification steps. Using milder sonication conditions or adding protease inhibitors during the purification of the protein did not improve this yield. In contrast, the C-terminal-GST fusion protein did not show any degradation during its purification. However, the yield of recovery was very low, probably due to the expression of the protein as an inclusion body. Antibodies raised against these two fusion proteins were shown to recognise Pgh1 in trophozoites and food vacuoles in western blotting experiments, as well as a protein of approximate molecular weight of 75kDa. Western blot of the *P. falciparum* plasma membrane failed to detect a band of 175kDa. The *pfmdr1* gene product pgh1 is reported to have an approximate molecular weight of 175kDa. This suggests that either the band recognised by the antibodies in the serum is not pgh1, or else in the presence of reducing agents the protein degrades into a 75kDa protein. Using a chemiluminescent detection system, Van Es *et al.* (1994a) detected a band with an apparent weight of 75kDa in addition to the band at 160kDa in the mitochondrial-lysosomal fractions of CHO cells expressing *pfmdr1*. It is possible that under the condition of the experiment, Pgh1 present in the plasma membrane was degraded and that only the breakdown products were detected by western blot. However, it is likely that the 75kDa band detected in western blotting experiments is the result of a non-specific interaction with a parasite's protein.

5.3.2 Identification of Pgh1 in parasite plasma membranes isolated from *Plasmodium falciparum*

The MDR phenotype in mammalian tumour cells is linked to the level of expression of a P-glycoprotein present in the plasma membranes of these cells (Endicott and Ling, 1989). By analogy, a similar mechanism was thought to occur in *P. falciparum* chloroquine-resistant strains. Cowman *et al.* (1991) demonstrated that Pgh1 protein of *P. falciparum* is expressed at equivalent levels in trophozoites and food vacuoles isolated from chloroquine-sensitive and most of the chloroquine–resistant parasites. However, its expression in the plasma membrane of CQR and CQS strains of *P. falciparum* has not been shown. To determine if Pgh1 expression in the plasma membrane correlates with the resistant phenotype, we examined the level of expression in plasma membranes isolated from chloroquine-sensitive and –resistant parasites. This study failed to detect Pgh1 in plasma membranes isolated from CQR and CQS parasites and indicates that the level of Pgh1 protein expression in the plasma membrane is not linked to the resistant phenotype. Interestingly, Cowman et al (1991) found that the CQR cloned line Fac8 displayed a higher expression of Pgh1 protein in trophozoites and food vacuoles than any other isolates they tested. Since the level of resistance of Fac8 was not greater than other isolates showing no increased Pgh1 expression, it was argued that no direct correlation could be established between overexpression of Pgh1 protein and CQR phenotype. The data presented in this study suggest that Pgh1 is not present in the parasite plasma membrane or at such a low level that it could not be detected by western blot. Even if we consider that the 75kDa band detected is a breakdown product of Pgh1, the intensity of this band was considerably low compared to the 175kDa band detected in the food vacuoles therefore invalidating any correlation between an overexpression of Pgh1 protein in the plasma membrane and the resistant phenotype. Since CQR phenotype has not been linked to either a mutation of *pfmdr1* or an overexpression of Pgh1 protein, it is unlikely that Pgh1 acts as a direct transporter of chloroquine. Further evidence that chloroquine does not directly interact with Pgh1 came from photoaffinity studies showing that a photoaffinity analogue of chloroquine failed to label Pgh1 (Foley *et al.*, 1994).

5.3.3 Localisation and membrane recognition with anti-Pgh1 antibodies by immunoelectron microscopy

Cowman *et al.* (1991) demonstrated by immunoelectron microscopy that Pgh1 protein is present mainly in the food vacuoles of mature parasites. In this study, examination of the subcellular localisation of Pgh1 in trophozoite infected-erythrocytes by immunoelectron microscopy revealed that a very low amount of Pgh1 protein is present on the plasma membrane of the trophozoites. These results are not entirely consistent with Cowman *et al.* (1991) results showing that most of the Pgh1 protein is localised on the food vacuole. The data shown in this study indicates that Pgh1 protein is distributed all over the trophozoites, most probably in the endoplasmic reticulum. The difference between the two experiments is that Cowman *et al.* (1991) used antibodies against the COOH-terminal 168 amino acids of the protein whereas the antibodies used in this study were raised against the N-terminal 18 amino acids of Pgh1. The fact that we detected the N-terminal region whereas Cowman *et al.* (1991) detected the C-terminal region could account for the difference observed. It is therefore proposed that Pgh1 might be present in the membranous tubules and vesicles network of *P. falciparum*. However, it cannot be excluded that the antibodies raised against the N-terminal of Pgh1 interact non-specifically with some of the parasite's proteins.

5.3.4 Effect of anti-Pgh1 antibody on chloroquine accumulation in isolated *Plasmodium falciparum* plasma membranes

Antibodies directed at Pgp have been used to study drug accumulation properties of the protein. Several groups had demonstrated the ability of anti-Pgp to inhibit drug efflux in mammalian cancer cells (Hamada and Tsuruo, 1986; Meyers *et al.*, 1989; Mechetner and Robinson, 1992). In this study, none of the antibodies used, namely the antibodies directed at the N-terminal ATP-binding site and the C-terminus of Pgh1, had an effect on chloroquine accumulation by the parasite plasma membranes confirming that Pgh1 is not directly involved in the mechanism of chloroquine

accumulation at the plasma membrane level. Another study performed in our laboratory showed that chloroquine accumulation in food vacuoles was not affected by the presence of anti-Pgh1 antibodies (Saliba, 1997). However, since the N-terminal ATP binding site and the C-terminal, against which the antibodies are raised, would normally be exposed to the parasite cytosol, it is possible that the absence of effect on chloroquine accumulation in the parasite plasma membrane vesicles reflects the lack of accessibility of these antibodies to their binding site on Pgh1.

5.4 Conclusion

If Pgh1 play a role in chloroquine resistance, it is not linked to a differential expression of this protein in the plasma membrane. Subcellular localisation of Pgh1 failed to find evidence of its presence in the *P. falciparum* plasma membrane. The lack of inhibition of chloroquine accumulation by anti-Pgh1 antibodies and the absence of correlation between the level of Pgh1 expression in the plasma membrane and the chloroquine-resistant phenotype suggest that Pgh1 is not involved as a chloroquine transporter in the plasma membrane of *Plasmodium falciparum*.

CHAPTER 6

DISCUSSION AND CONCLUSION

6.1. Discussion

An early study from Aikawa (1972) demonstrated that chloroquine concentrates within *P. falciparum* food vacuoles. Since then, it has been well established that CQS parasites accumulate more drug than their resistant counterparts (Ginsburg *et al.*, 1991; Bray *et al.*, 1992a; Bray *et al.*, 1994; Martiney *et al.*, 1995; Sanchez et al., 1997; Bray *et al.*, 1998; Wunsh *et al.*, 1998). However, a study conducted by Saliba *et al.* (1998) demonstrated that chloroquine accumulates about 20-fold less in the isolated food vacuole of *P. falciparum* than in the same number of infected-erythrocytes. Since chloroquine has to cross several membranes to reach the food vacuoles where it concentrates, each of these could participate in the mechanism of drug accumulation. A facilitated transport or efflux mechanism within the parasite plasma membrane could therefore account for the decline in chloroquine accumulation observed between the infected erythrocytes and their food vacuoles. Furthermore, chloroquine accumulation has been shown to consist of a saturable and non-saturable component (Fitch *et al.*, 1970; 1974) and chloroquine sensitivity of the parasite has been correlated to the apparent affinity of the saturable uptake process (Raynes *et al.*, 1999). Membranes other than that of the food vacuole could be implicated in either of these processes.

To elucidate the possible role played by the *P. falciparum* plasma membrane in the mechanism of chloroquine accumulation and resistance, a method was developed for the preparation of parasite plasma membrane vesicles. One of the criteria for this

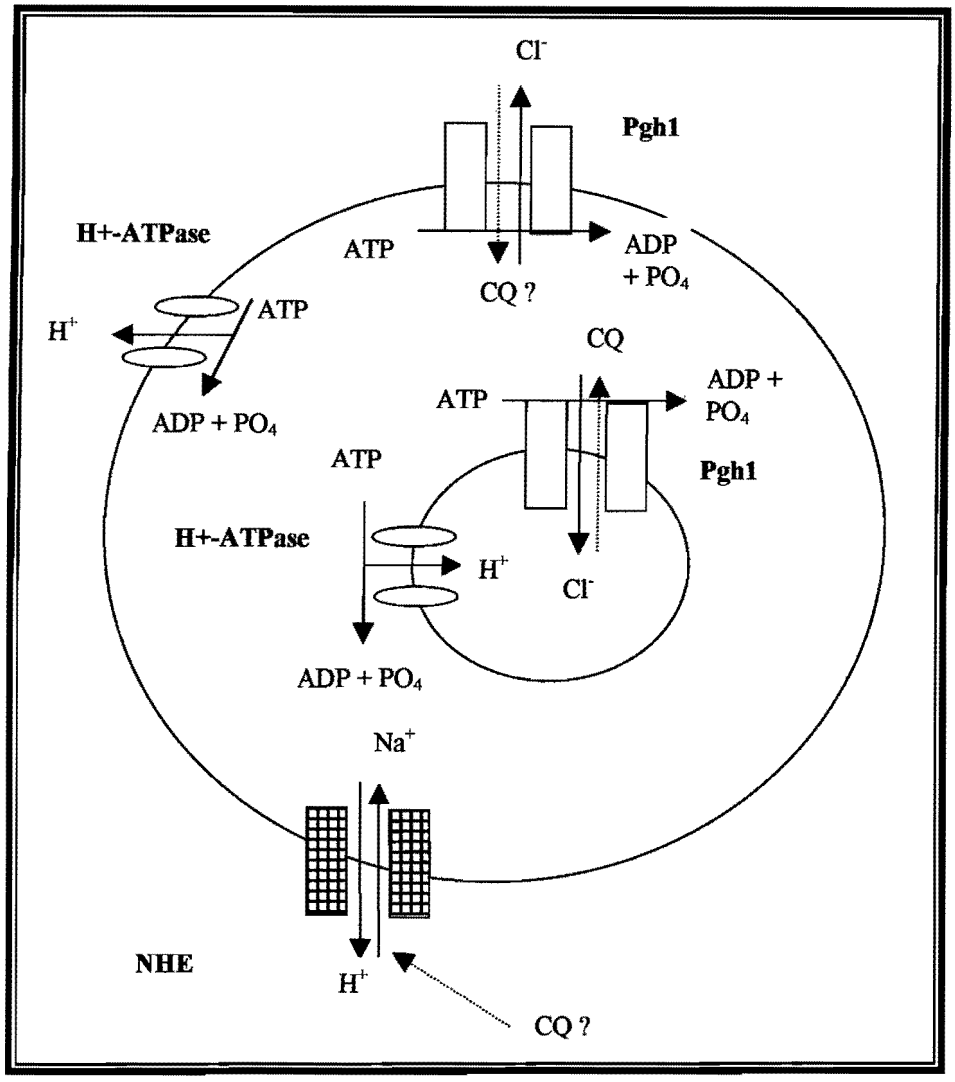

Figure 22: Schematic representation of various model describing chloroquine uptake and possible sites of chloroquine resistance in the *P. falciparum* trophozoites.

study was the obtention of the plasma membrane in the form of vesicles in order to study their drug transport capabilities. For the same reason, the vesicles recovered should be the right way around and not inside-out. Finally, another prime consideration was to ensure that these plasma membrane vesicles were intact and free of contaminating organelles and membranes. The method described in chapter II of this thesis meets these requirements and therefore allowed us to characterise the

ATPase activity and the drug transport properties of isolated *P. falciparum* plasma membranes.

Conditions were determined in chapter III for optimum ATPase activity of *P. falciparum* plasma membranes. No linear relationship was observed between plasma membrane ATPase activities and chloroquine sensitivity of *P. falciparum*. However, plasma membranes isolated from the CQS parasites were found to display a lower ATPase activity than their resistant counterparts. When the ATPase activity of the plasma membranes isolated from various strains of *P. falciparum* was compared to their chloroquine accumulation properties, it was striking to observe that they display a similar profile. This finding would suggest a possible link between the level of chloroquine uptake in these vesicles with the level of plasma membrane ATPase activity. However, the lack of dependence of chloroquine uptake on ATP complicates interpretation of this data. Chloroquine uptake in plasma membrane vesicles isolated from the CQR *P. falciparum* could result from (1) a decreased efflux of H^+ due to a defective H^+-ATPase, (2) a NHE facilitating the transport of chloroquine, (3) a P-type ATPase such as Pgh1 acting either as a chloroquine transporter or a chloride channel, (4) binding of chloroquine to a component of the parasite plasma membrane. The plasma membrane ATPase activities of a chloroquine-sensitive and a –resistant *P. falciparum* were selectively inhibited to determine any correlations between P-type and V-type ATPase activities and either the chloroquine uptake capabilities or chloroquine sensitivity of a strain.

Genes encoding the A and B subunits of a V-type H^+-ATPase have been identified in *P. falciparum* (Karcz *et al.*, 1993a; 1994) and chloroquine accumulation in resistant *P. falciparum* was found to be more sensitive to the V-type ATPase inhibitor bafilomycin A1 (Bray *et al.*, 1992a). However, a study conducted in our laboratory (Adams, 1999) showed that bafilomycin A1 (1μM) and NEM (10μM) reduced the vacuolar ATPase activity of D10 and Fac8 by 50% and 38%, respectively. Similar results were obtained by Choi and Mego (1988) using concentrations of NEM and NBD-Cl between 0.5mM and 1mM. In this study, it was found that bafilomycin A1 at

similar concentration inhibited D10 plasma membrane ATPase activity by 47% and had little effect on Fac8 plasma membrane ATPase activity, therefore indicating that the CQS strain D10 have a higher level of plasma membrane V-type ATPase activity than the CQR strain Fac8. The CQR parasite would therefore be expected to display a higher intracellular pH than the CQS parasites. This is consistent with Wunsch *et al.* (1998) study demonstrating by in vivo pH measurements that CQR parasites have an elevated cytoplasmic pH compared to that of chloroquine-sensitive parasites.

It was initially thought that the parasite maintains its cytosolic pH through a P-type ATPase similar to those that operate in the plasma membrane of yeast and plant cells (Mikkelsen *et al.*, 1982; 1986). This hypothesis has since been questioned and later studies suggested either that the parasites extrude H^+ by means of a NHE (Bosia *et al.*, 1993) or a V-type H^+-ATPase (Saliba and Kirk, 1999). It is possible that chloroquine uptake observed in the plasma membrane is partially pH-driven via a H^+-ATPase. This would be consistent with the presence of a V-type H^+-ATPase on D10 plasma membranes since chloroquine was taken up to a lesser extent into D10 vesicles compared to those from the resistant strain. It is also possible that the intracellular pH is maintained by a combination of proton pump activity and Pgh1 acting as a chloride channel. It would therefore be interesting to measure the intracellular pH of the vesicles in presence/absence of V-type and P-type ATPase inhibitors.

Chloroquine (pKa1 8.1 and pKa2 10.2) and its structural analogues mefloquine and quinine (pKa 8.6) and quinine (pKa 8.4) are weak bases driven to accumulate in acidic compartments by a pH gradient maintained by proton pump activity. The pKa values of chloroquine, mefloquine and quinine indicates that only a small fraction of the weak base should be uncharged at a physiological pH of 7.4. In some cell systems, a small pH change is compensated for by a decline in proton leakage via the NHE (Nelson, 1991) or a V-type H^+-ATPase (Moreno et al., 1998). This study showed that mefloquine (1mM) and quinine (0.1mM) inhibited Fac8 plasma membranes ATPase activity by 39% and 29%, respectively, while artemisinin had no

effect. An explanation could be that the weak base antimalarials inhibited a H^+-ATPase to compensate the pH increase due to the accumulation of the weak base in Fac8 plasma membrane vesicles. The pH change in D10 plasma membrane vesicles due to the weak base might be too small to induce inhibition of the H^+-ATPase since chloroquine has been shown to accumulate in these vesicles to a lesser extent.

Chloroquine uptake in membrane preparations from *P. falciparum* was reduced by 64% using oligomycin (10μg/ml) (Herwaldt *et al.*, 1990) while vanadate (100μM) completely inhibited the ATPase activity of purified P-glycoprotein from Chinese hamster ovary cells (Urbatsch *et al.*, 1994). Choi and Mego (1988) reduced the ATPase activity of *P. falciparum* vacuolar membranes by 15% using 100μM vanadate while oligomycin (2μg/ml) was completely ineffective. In this study, vanadate (0.1mM) inhibited by 41% and 28% the ATPase activity of purified plasma membranes from the strain D10 and Fac8, respectively. However, the specificity of vanadate can be questioned since it has been shown to inhibit non-P-type-membrane-associated ATPases (Prossnitz *et al.*, 1989). Therefore, the inhibition observed in this study could be non-specific and possibly result from the inhibition of a V-type ATPase or another ATPase present in the plasma membrane of *P. falciparum*. To combine the effect of bafilomycin A1 and vanadate on the plasma membrane activity would determine whether a P-type ATPase contribute in the effect observed with vanadate or result from a non-specific inhibition of a V-type ATPase. Interestingly, oligomycin (50μg/ml) inhibited Fac8 plasma membrane ATPase activity by 42% and had little effect on D10, suggesting the presence of a P-type component in the Fac8 plasma membrane. Furthermore, Herwaldt *et al.* (1990) showed that chloroquine uptake in vesicles prepared from *P. falciparum* was more sensitive to oligomycin than vanadate. These results would imply that Pgh1 does not play a role in regulating chloroquine uptake in *P. falciparum* plasma membrane.

Pgh1, a P-glycoprotein homologue, has been identified on the plasma and vacuolar membrane of *P. falciparum* and was initially believed to transport chloroquine (Cowman *et al.*, 1991). Based on the finding that chloroquine uptake in Chinese

hamster ovary cells transfected with pfmdr1 was inhibited by the proton pump inhibitor bafilomycin A1 and the pH dissipator ammonium chloride, Van ES *et al.* (1994b) argued that Pgh1 acts as a chloride channel, enhancing chloroquine accumulation by modulating the *P. falciparum* vacuolar pH rather than a specific carrier for chloroquine. In this study, the lack of correlation between Pgh1 expression and chloroquine sensitivity of *P. falciparum* together with the finding that antibodies directed against Pgh1 failed to inhibit chloroquine uptake in the *P. falciparum* plasma membrane vesicles indicates that Pgh1 is most probably not involved in the mechanism of chloroquine accumulation in the parasite plasma membrane.

Chloroquine resistance in *P. falciparum* can be reversed by chemosensitisers such as verapamil (Martin et al., 1987) and stimulation of the ATPase activity of P-glycoprotein using chemosensitisers is well documented (Sharom et al., 1995; Urbatsch et al., 1995; Garrigos et al., 1993; Shapiro and Ling, 1995). In this study none of the chemosensitisers tested had an effect either on the ATPase activity or chloroquine uptake by purified *P. falciparum* plasma membrane vesicles. It is therefore unlikely that the site of action of these chemosensitisers lies within the parasite plasma membrane. Since chemosensitisers which reverse multidrug resistance of tumor cells are also capable of modulating chloroquine resistance, these agents were proposed to function in a similar manner, and were therefore expected to stimulate a P-glycoprotein activity. However, verapamil (10μM), progesterone (5μg/ml) and trifluoperazine (10μM) had no significant effect either on the vacuolar ATPase activity of *P. falciparum* (Adams, 1999). It was also proposed that chemosensitisers increase the affinity of chloroquine binding for the haem molecule if accumulation is driven by this mechanism (Chou *et al.*, 1980; Bray *et al.*, 1998; 1999). It is therefore more likely that these chemosensitisers act at sites other than the plasma membrane of *P. falciparum*.

NHE has been identified in *P. falciparum* (Bosia *et al.*, 1993) and amiloride and its derivatives have been shown to inhibit chloroquine uptake in infected erythrocytes (Sanchez *et al.*, 1997). An increase in cytoplasmic sodium concentration stimulated

by chloroquine was observed in CQS parasites. Since this effect was inhibited by EIPA, it was argued that NHE is implicated in the mechanism of chloroquine uptake and probably resistance in *P. falciparum*. However, whether NHE play a role in chloroquine uptake was questioned as the process was shown to not require sodium-hydrogen exchange since EIPA competitively inhibited chloroquine binding to FPIX in erythrocyte ghosts while chloroquine uptake in *P. falciparum* was unaffected by removing sodium ions from the reaction buffer (Bray et al., 1998; 1999). Moreover, based on the finding that the effect of amiloride and bafilomycin A1 on pHi was not additive, Saliba and Kirk argued that these effects are not related to NHE but rather with amiloride acting as a V-type H^+-ATPase inhibitor. In this study, amiloride and its derivatives DMA and EIPA at a concentration of 100μM considerably stimulated D10 plasma membrane ATPase activity while its resistant counterpart was stimulated to a lesser extent. These results would suggest that amiloride and its derivatives are capable of stimulating an ATPase activity located in the plasma membrane of *P. falciparum* which would indicate that the effect observed with these inhibitors are not related to an effect on NHE.

6.2. Conclusion

Despite efforts to eradicate malaria in the 1950s, this disease remains a major health problem in tropical and subtropical regions of the world. The emergence and the spread of drug resistance in the malarial parasite *Plasmodium falciparum*, especially resistance to chloroquine, has proved to be a major obstacle to control this disease. Compared to other drugs, such as the antifolate pyrimethamine, resistance to chloroquine has been relatively slow to develop. Two foci of chloroquine resistance initially emerged in Southeast Asia and South America in the 1960s, and resistance has since spread to nearly every country where malaria is endemic. Resistance of *Plasmodium falciparum* to all the major antimalarials used is now widespread with the only exception of the artemisinin derivatives. Therefore, the need for the judicious use of alternative antimalarials as well as preventing drug-resistance to new drugs

from emerging has emphasised the importance of understanding the molecular mechanism involved in the development of drug resistance.

This thesis has focused on the development of a *P. falciparum* plasma membrane isolation which constitutes a suitable model for examining the mechanism involved in the accumulation of chloroquine and its resistance. Many sites, such as the parasitised-erythrocyte membrane, the parasitophorous membrane, the parasite plasma membrane and the food vacuole membrane can influence chloroquine accumulation in the malaria parasite. To date, elucidation of the role of the plasma membrane in the mechanism of chloroquine resistance has been impeded by the presence of other membranes in the membrane preparations. Therefore to obtain parasite plasma membranes purified from *Plasmodium falciparum* is of particular interest to study the mechanism of chloroquine accumulation. The availability of purified *Plasmodium falciparum* plasma membrane will be of value not only in the understanding of drug transport in this organelle but also to investigate processes such as nutrient uptake/efflux, pH regulation and ion balance.

Parasite plasma membranes were obtained by saponin lysis of the erythrocytes and disruption of the trophozoites by nitrogen decompression. Trophozoites were purified of erythrocyte membranes by immunoaffinity chromatography using anti-erythrocyte antibodies while the purification of the plasma membranes from contaminants was achieved using a magnetic cell sorting system. Enzyme marker assays, gel electrophoresis and transmission electron microscopy demonstrated the purity of plasma membranes from contaminating membranes and organelles. Furthermore, the degradation of membrane proteins by the proteases was prevented by the use of protease inhibitors. DNAse I was as well included in the purification process.

Chloroquine sensitivity of 6 strains of *P. falciparum* and chloroquine accumulating capabilities of erythrocytes parasitised with two resistant and one sensitive strain were confirmed. The ATPase activity displayed by *P. falciparum* plasma membrane

was found to be time-, ATP- and cation-dependent. Other nucleoside triphosphates were hydrolysed as efficiently as ATP, but not AMP. The divalent cation Mn^{2+} was shown to support the parasite plasma membrane ATPase activity while Ca^{2+} did not. This thesis also provides evidence of a V-type ATPase present in the *P. falciparum* plasma membrane of the chloroquine-sensitive strain D10.

[^{3}H]-chloroquine accumulation by parasite plasma membranes was demonstrated to be specific. Chloroquine accumulation capabilities of plasma membranes isolated from 5 strains of *P. falciparum* were evaluated over 4 external chloroquine concentrations. Plasma membranes isolated from two chloroquine-sensitive strains accumulated significantly less chloroquine than those isolated from two of the three chloroquine-resistant strains tested, at all the concentrations tested. These results were unexpected since it is well established that erythrocytes infected with CQS parasites accumulate chloroquine at a higher level than their resistant counterparts. The difference in the level of chloroquine accumulation observed between these membrane vesicles could result from a difference in permeability of plasma membranes isolated from the CQR and CQS strains or in a binding phenomenon to a component of the membranes. These results provide evidence for a possible role played by the plasma membrane in accumulating chloroquine in the parasite.

Results presented in this thesis indicate that the parasite plasma membrane is not the site of reversal of chloroquine resistance by agents such as verapamil. It has been proposed that two distinct mechanisms for chloroquine resistance exist, one that is verapamil reversible and one that is not. Therefore, a verapamil insensitive site of chloroquine accumulation, the parasite plasma membrane, and a verapamil sensitive one, probably the food vacuole contributes to the global chloroquine accumulation in the parasite.

Studies implicating Pgh1 in the mechanism of chloroquine resistance have been contradictory. Data presented in this study indicate that Pgh1 is not involved in the mechanism of chloroquine accumulation by the *P. falciparum* plasma membrane.

Subcellular localisation of Pgh1 indicated that this protein is seldom present in *Plasmodium falciparum* plasma membrane and antibodies directed at Pgh1 failed to inhibit chloroquine accumulation in these membranes. Furthermore, no overexpression of Pgh1 could be associated with chloroquine sensitivity of the *plasmodium falciparum* strains tested. Therefore, Pgh1 does not appear to play a role in the mechanism of chloroquine resistance, at least at the plasma membrane level.

In conclusion, a method for the isolation and purification of the *P. falciparum* plasma membrane was developed. This thesis provides evidence for a verapamil-insensitive site of chloroquine accumulation in the plasma membrane and demonstrated that Pgh1 is not involved in the mechanism of chloroquine resistance at the plasma membrane level. Finally evidence that a V-type ATPase is present in the parasite plasma membrane was demonstrated.

CHAPTER 7

METHODS

7.1. Culture of *Plasmodium falciparum*

The chloroquine sensitive cloned D10 (derived from FCQ-27 from Papua New Guinea) (Ekong *et al.*, 1993) and the chloroquine resistant FAC8 (derived from the Brazilian cloned line ITG2F6) (Biggs *et al.*, 1989) strains of *Plasmodium falciparum* were provided by Dr A. Cowman, Walter and Elisa Hall Institute of Medical Research in Melbourne, Australia. The chloroquine sensitive South African isolates RSA3 (North Eastern Transvaal) and the chloroquine resistant RSA11 and RSA15 (Kwazulu Natal) were obtained from Dr J. Freese, Medical Research Council of South Africa in Durban. The chloroquine resistant cloned isolates K1 (Thailand) (Thaithong and Beale, 1981) were donated by C. Weiss, School of Hygiene and Tropical Medicine, London.

Plasmodium falciparum strains were cultured essentially as described by Trager and Jensen (1976). Parasites were maintained in continuous culture at a 5% haematocrit in RPMI-1640 with glutamine (Sigma) supplemented with hypoxanthine (44mg/l), HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethansulphonic acid] (6g/l), glucose (4g/l), $NaHCO_3$ (2.1 g/l), gentamycin (50mg/l), and 10% A^+ human serum (Western Province Blood transfusion Service and Haematology Department, Groote Schuur Hospital, Cape Town, South Africa). Parasites were grown in O-positive red cells, which has been washed twice with 10 volumes of culture medium without serum to remove the buffy coat. The culture medium was changed daily and the parasitemia was kept between 10-15 %. Parasite cultures were incubated at 37°C in dessicator cabinets under an atmosphere of 93% nitrogen, 4% carbon dioxide, 3% oxygen.

Parasite proliferation was assessed by light microscopical examination of Giemsa stained thin smears of cultured blood. Thin smears were fixed with methanol and stained with 10% Giemsa (Sigma) in phosphate buffered saline (P.B.S.) for 10 minutes at room temperature. The slides were washed with water, dried and viewed under a Leitz laborlux 12 microscope with a 100 x oil (1,250 centistokes) (Sigma) immersion objective.

Synchronisation to ring forms was achieved by sorbitol treatment (Lambros and Vanderberg, 1979). Cultured parasites containing mainly ring stages parasites were suspended in 5 volumes 5% D-sorbitol (Sigma) for 10 min. The parasitised erythrocytes were centrifuged at 602g for 5 min and the supernatant discarded. The pellet was then resuspended in culture medium at 5% haematocrit and returned to the incubator. This culture contained only the rings and consisted of parasites in the trophozoite stage the following day.

Parasites were cryostored in liquid nitrogen. To cryopreserve a culture, the parasitised erythrocytes in the ring stage with a parasitemia > 2% were centrifuged and the cells were resuspended in an equal volume of 28% glycerol in P.B.S. The suspension were frozen in 0.5ml aliquots and stored in a liquid nitrogen refrigerator. To retrieve a culture, parasites were thawed at 37°C in a water bath and transferred to a 10ml centrifuge tube. After addition of 1ml 3.5% NaCl, prewarmed to 37°C, the cells were centrifuged at 602g for 5 min. The supernatant was removed and replaced with 1 ml 3.5% NaCl. The suspension was again centrifuged and the supernatant removed. This washing step was repeated once. The resulting pellet was resuspended in 10ml culture medium supplemented with 100-200µl of washed blood and placed in culture.

7.2 Isolation and purification of *Plasmodium falciparum* trophozoites

7.2.1. Trophozoite isolation by sorbitol lysis

Trophozoites were isolated mainly as described by Hoppe *et al.* (1992). 10ml of culture was sedimented at 602g, for 5 min and resuspended in 3ml of an 8% (w/v) sorbitol-supplemented serum-free RPMI-1640 medium (Sigma). After incubation for 1 min the cells were sedimented by brief centrifugation, and rapidly suspended in 1ml of serum-free culture medium by vortexing in order to lyse the infected-erythrocytes and release the parasites. Immediately after lysis, the protease inhibitors aprotinin, leupeptin and phenylmethylsulfonylfluoride (PMSF, Boehringer Mannheim) were added to final concentration of 10μg/ml, 10μg/ml and 0.1mM, respectively. Isolated trophozoites were further purified by immuno-affinity chromatography.

7.2.2 Trophozoite isolation by saponin lysis

Infected erythrocytes were washed twice in 10 volumes P.B.S. The pellet was suspended in 10 volumes of PBS containing 0.05 % (w/v) saponin in order to lyse the erythrocytes. It was then incubated for 2-3 min at room temperature and centrifuged at 1,500g for 10 min. The resulting pellet was washed twice in P.B.S. and resuspended in 5 volumes P.B.S. The protease inhibitors aprotinin, leupeptin and phenylmethylsulfonylfluoride (Boehringer Mannheim) were added to final concentration of 10μg/ml, 10 μg/ml and 0.1mM, respectively. Isolated trophozoites were further purified by immuno-affinity chromatography.

7.2.3. Immuno-affinity purification of trophozoites

Unlysed erythrocytes and erythrocytes membranes contained in the trophozoite preparation were adsorbed onto the immuno-affinity column. Anti-human erythrocytes (polyvalent immunoglobulin (G,A,M) rabbit, Sigma) were diluted 1 : 1000 in P.B.S. 5g of polystyrene beads (Pierce) were incubated in 20ml of the above solution for 2 hours at room temperature with gentle agitation. The beads were washed in P.B.S. and further incubated for 1 hour with 20ml 1% bovine serum albumin. in P.B.S. The beads were washed again in P.B.S., incubated with the trophozoite preparation for 30 min, packed in a column (20ml propylene seringe) and eluted under gravity with 2 washes each in 10ml P.B.S. The purified trophozoite were collected and centrifuged at 1,500g for 10 min.

7.3. Preparation of *P. falciparum* plasma membranes

7.3.1. Parasite plasma membrane isolation by fixation to a dithio-NHS support

The cross-linking reaction was performed by incubating 20µl glass beads aminopropyl (10nM) (Sigma), 180µl trophozoites (0.1-0.5mg protein) and 10 µl DSP (dithiobis succinimidyl propionate) (2mM) (Pierce) for 30 min, 1 or 2 hours at 4°C. Cross-linked beads were blocked for 15 min in Tris-HCl 1M pH 7.5. The beads were washed first in P.B.S., then twice in Vesicle buffer I (0.25M sucrose, 1mM E.D.T.A., 10mM Tris-HCl pH 7.4). Vesicles were prepared by nitrogen cavitation according to the method of Lever *et al.* (1977). The trophozoites linked to the beads were resuspended in 500µl vesicle buffer I containing aprotinin, leupeptin and phenylmethylsulfonylfluoride (10µg/ml, 10µg/ml and 0.1mM, respectively) and equilibrated at 4 °C under nitrogen pressure at 800 psi for 30 min. The homogenate was then incubated for 5 min at 37 °C with Dnase I (50µg/ml). The vesicles were washed twice in 0.25M sucrose, 10mM Tris-HCl pH 7.4.

7.3.2. Magnetic streptavidin purification of parasite plasma membrane

The biotylination reaction was performed by incubating 250µl 1mg/ml NHS-SS-Biotin (sulfosuccinimidyl 2-[biotin-amido] ethyl-1,3-dithiopropionate) (Pierce) with 500µl trophozoite (2 mg protein) for 30 min at room temperature. The resulting biotinylated trophozoites were washed once in P.B.S., then twice in Vesicle buffer I (0.25M sucrose, 1mM E.D.T.A., 10mM Tris-HCl pH 7.4). Vesicles were prepared by nitrogen cavitation according to the method of Lever *et al.* (1977). The cells were resuspended in 500µl vesicle buffer I containing aprotinin, leupeptin and phenylmethylsulfonylfluoride (10µg/ml, 10µg/ml and 0.1mM, respectively) and equilibrated at 4 °C under nitrogen pressure at 800 psi for 30 min. The homogenate was then incubated for 5 min at 37 °C with Dnase I (50µg/ml). The biotinylated vesicles were incubated with 100µl of streptavidin immobilised on iron oxide (Sigma) previously washed with P.B.S., for 15 min at room temperature and then separated using a high gradient magnetic separation column (Magnetic Cell Sorting, Myltenyi Biotec) placed in a strong magnetic field. The magnetically labelled vesicles were retained in the column and washed with 1 ml 0.25M sucrose, 10mM Tris-HCl pH 7.4. The column was then removed and the magnetically retained vesicles were eluted with the same buffer. The separation of the magnetically labelled vesicles was also obtained by placing an eppendorf tube containing the solution in a strong magnetic field. The magnetic particles were bound to the side of the tube and washed with 1 ml in 0.25M sucrose, 10mM Tris-HCl pH 7.4.

7.4. Isolation of food vacuole

Vacuoles were isolated using a protocol adapted from those of Goldberg *et al.* (1990) and Saliba *et al.* (1998). 5ml of parasitized erythrocytes were washed twice in 50ml P.B.S. and resupended in 50ml P.B.S. containing 25mg saponin. After a short incubation (2-3 min), the trophozoites were centrifuged at 1,500g for 10 min. The pellet was washed twice with 1 ml ice-cold uptake buffer (2mM $MgSO_4$,

100mM KCl, 10mM NaCl, 25mM HEPES, pH 7.4) and resuspended in 10 volumes ice-cold water, pH 4.5. This suspension was triturated 5 times through a 27 ½ -G needle. The food vacuole preparation was centrifuged in a microfuge at 13,500g for 2 min and resuspended in 1ml uptake buffer. The suspension was then incubated for 5 min at 37°C with 10µl of 5mg/ml Dnase I, and centrifuged again. The resulting pellet was resuspended in 300µl ice-cold uptake buffer and triturated 3 times through a 27 ½ -G needle. It was then added to 1.2ml ice-cold 42% Percoll™ containing 0.25M sucrose and 1.5mM MgSO4 pH 7.4, and centrifuged at 13,500 rpm for 10 min at 4°C. The bottom of the gradient, containing the food vacuoles, was collected, suspended in 300µl uptake buffer to which was added 1.2ml of the Percoll™ solution and again centrifuged. The purified food vacuoles were collected, resuspended in 1ml uptake buffer and microfuged at 13,500g for 2 min to wash off the Percoll™.

7.5. Preparation of erythrocyte ghosts

Erythrocyte ghosts were prepared as described by Heidrich and Leutner (1974). The cells of 100 ml freshly drawn human blood were centrifuged at 120g for 10 min and then washed 3 times with a 5mM phosphate buffer, pH 8.0, containing 0.15M NaCl. The cells were hemolysed with 1,200 ml 5mM phosphate buffer, pH 8.0, by stirring at 0°C for 20 min. The resulting ghosts were then washed 5-6 times at 4°C with the same buffer by centrifugation at 20,000g for 10 min until they were white. Erythrocyte ghosts were stored at -80°C in 5mM sodium phosphate, pH 8.0.

7.6. Acetylcholine esterase activity

The acetylcholine esterase (AchE) activity was determined by a modification of the method of Ellman *et al.* (1961). Enzyme assays were carried out with 200 µl of freshly prepared 0.1M sodium phosphate pH 7.5, 0.5mM 2,2'-dinitro-5,5'-

dithiobenzoic acid, 0.6mM S-acetylthiocholine iodide to which was added 50µl of a suspension of erythrocyte ghost, trophozoites or parasite plasma membrane, in microtitre 96-well plates. 10µg of erythrocyte ghost protein, 3-5µg of trophozoite protein or 20µg of parasite plasma membrane protein were used to measure an initial rate. 50µl of P.B.S. or vesicle buffer was added to the control wells. Wells containing 200µl H_2O and 50µl of erythrocyte ghosts/trophozoites/vesicles suspensions were including to subtract any absorbance due to the cells. Changes in absorbance at 405nm were measured for at least 20 min, using a 7520 Microplate Reader Cambridge Technology, Inc.

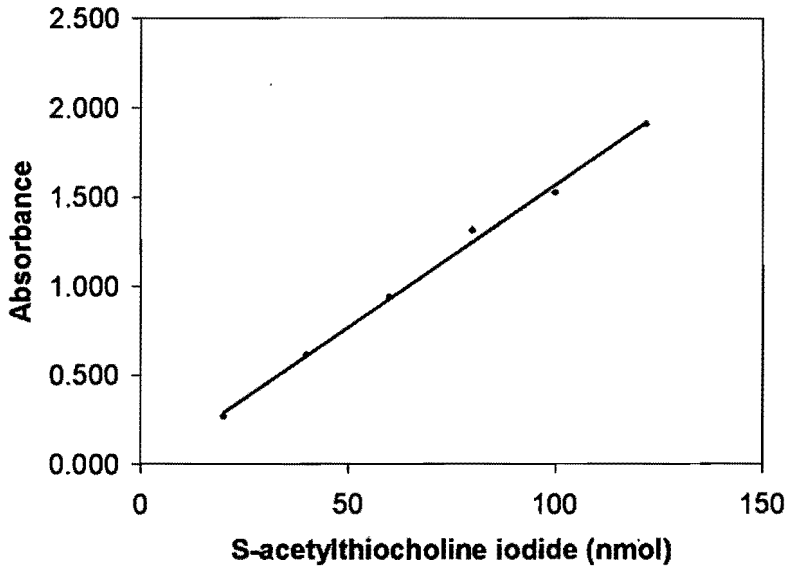

Figure 23: standard curve for the formation of 5-thio-2-nitro-benzoic acid by measurement of the absorbance at λ =405nm from 3 separate experiments (r^2 =0.9963).

A standard curve for the formation of 5-thio-2-nitro-benzoic acid was set up by preparing dilutions of S-acetylthiocholine iodide ranging from 122 to 20nmol in 0.1M sodium phosphate, 0.5mM 2,2'-dinitro-5,5'-dithiobenzoic acid, pH 7.5, in a final volume of 200µl per well. 10µg of erythrocyte ghost protein was added to each well to initiate the reaction. After 20 min, absorbance values at 405nm were obtained as described above.

7.7. Parasite lactate dehydrogenase activity

Measurements of parasite lactate dehydrogenase (pLDH) activity were carried out essentially as described by Makler and Hinrichs (1993a). For these measurements, 5µg of trophozoites protein or membrane vesicle protein suspended in 20µl P.B.S. or vesicle buffer was added to 100µl Malstat™ reagent (Flow, inc.) and 25µl 0.24 mM phenazine ethosulfate (PES)/1.96mM nitro blue tetrazolium (NBT) in microtitre 96-well plates. 20µl of H_2O was added to the control wells. Wells containing 20µl trophozoites/ membrane vesicles and 125µl H_2O were included to subtract any absorbance due to the cells. Changes in absorbance at 620nm were measured for at least 20 min, using a 7520 Microplate Reader Cambridge Technology, Inc.

A standard curve for the formation of reduced 3-acetylpyridine adenine dinucleotide (APADH) was set up by preparing serial dilutions of a solution containing 0.5mM-7.8µM APADH (Sigma) and 0.18M L(+) lactic acid (sigma, 54.5mM Tris-HCl, 0.2% triton X-100, pH 9.0. Final volume of this solution in each well was 100µl, to which was added 25µl of 24mM phenazine ethosulfate (PES)/1.96mM nitro blue tetrazolium (NBT) and 20 µlH_2O. Absorbance values at 620nm were obtained as described above.

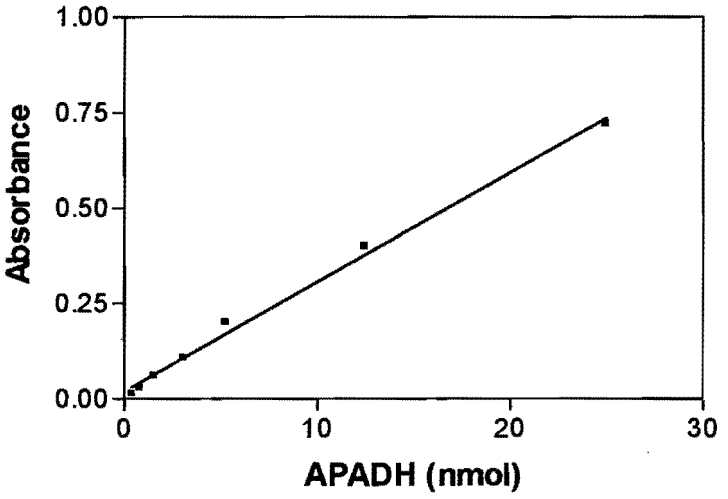

Figure 24: Standard curve for the formation of reduced 3-acetylpyridine adenine dinucleotide (APADH) by measurement of the absorbance at λ =620nm from 3 separate experiments (r^2 =0.994).

7.8. In vitro *Plasmodium falciparum* cytotoxicity assay

The parasite lactate dehydrogenase (pLDH) assay was used for the evaluation of parasite viability in the presence of drug according to the method developed by Makler *et al.* (1993b). This assay is based on the fact that pLDH activity is distinguishable from host lactate dehydrogenase activity using the 3-acetylpyridine adenine dinucleotide (APAD) analogue of nicotinamide adenine dinucleotide.

Chloroquine diphosphate (2μM) was serially diluted two-fold in culture medium, in microtitre 96-well plates. The suspensions were dispensed in quadruplicate at 100μl / well. To the control and blank well, 100μl of culture medium was added. Sensitivity assay were initiated by adjusting the initial parasitemia to 2% with 0-positive red blood cells (2% haematocrit) suspended in culture medium. 100μl of parasitised red blood cells was added to each well except the blank wells. To the blank wells, 200μl of unparasitised red blood cells (1% haematocrit) was added. Therefore, the final volume of each well was 200μl and the haematocrit of both the parasitised red blood cells and the unparasitised erythrocytes was 1%. The plates were incubated at 37°C for 48 hours in 93% nitrogen, 4% carbon dioxide, 3% oxygen.

At the end of the incubation period, the cultures were carefully resuspended and 10μl from each well of resuspended culture was transferred into another microtitre 96-wells plate that contained 100μl of Malstat™ reagent and 25μl of 0.24mM PES/1.96mM NBT solution. The reduction of the tetrazolium to the blue formazan salt is an indication of parasite survival. Absorbance values were measured at 620nm using a 7520 Microplate Reader Cambridge Technology, Inc.

Percentage parasite survival was calculated using the following formula:

$$\text{Percentage parasite survival} = \frac{\text{Mean of control wells}}{\text{Mean of drug containing wells}} \times 100$$

7.9. Chloroquine accumulation in parasitised erythrocytes

Parasitised erythrocytes, synchronised in the trophozoite stage, with a parasitemia between 3-5% and the haematocrit set at 1% in culture medium were exposed to [^{3}H]-chloroquine (11.9Ci/mmol, Amersham) in 1.5ml eppendorf vials. The control consisted of uninfected erythrocytes suspended at a final haematocrit of 1% in culture medium. Unlabelled chloroquine was included for concentrations greater or equal to 100nM. The vials were incubated at 37°C for 1 hour. The incubations were terminated by centrifuging at 13,000 rpm for 2 min in a microfuge and the PRBC were washed twice with ice-cold medium. The eppendorf tip containing the PRBC was cut off and placed in a scintillation vial containing 4 ml of liquid scintillation cocktail and 1 ml 3.5% (M/V) sodium hypochlorite, to bleach the red colour of the haemoglobin. Samples were shaken overnight and the radioactivity was counted in a Packard Tri-Carb 4640 liquid scintillation spectrophotometer.

7.10. Chloroquine accumulation in parasite plasma membranes vesicles

Membrane preparations were thawed rapidly at 37°C and 40μg of membrane vesicle protein were diluted in vesicle buffer (0.25M sucrose, 10mM Tris-HCl pH 7.4) containing 2mM $MgCl_2$. Chloroquine accumulation experiments were carried out in the presence or absence of 2mM ATP. All reactions were carried out in a final volume of 1ml. To initiate chloroquine accumulation, [^{3}H]-chloroquine (11.9Ci/mmol, Amersham) was added. Unlabelled chloroquine was included for concentrations greater or equal to 100nM. When additional drugs were used, they were preincubated for 15 min prior to the addition of [^{3}H]-chloroquine. Incubations were carried out at 37°C for the specified length of time. The incubations were terminated by centrifugation at 13,000 rpm for 2 min in a microfuge. The membranes were then washed twice with ice-cold vesicle buffer. The eppendorf tip containing the membranes was cut off and placed in a scintillation vial containing 5 ml of liquid scintillation cocktail. Samples were shaken overnight and the

radioactivity was counted in a Packard Tri-Carb 4640 liquid scintillation spectrophotometer.

7.11. ATPase activity

The ATPase activity was determined using a colorimetric assay adapted from those of Chifflet *et al.* (1988) and Doige *et al.* (1992). Enzyme assays were carried out with 50µg of freshly isolated *Plasmodium falciparum* plasma membranes suspended in 450µl of reaction buffer (50mM Tris-HCl, 0.15M NH_4Cl, 2mM $MgCl_2$, 0.02% NaN_3, pH 7.4). To initiate the reaction 50µl of ATP in reaction buffer was added, giving a final concentration of 2mM ATP. A control containing 50µg of parasite plasma membranes suspended in 500µl of reaction buffer was included to subtract any absorbance due to the membranes. Another control consisting of 500µl reaction buffer containing 2mM ATP was added to evaluate the ATP hydrolysis. When drugs were used, they were preincubated for 15 minutes at room temperature prior to the addition of ATP and the appropriate controls were included. After 45 min. at 37°C, the suspensions were centrifuged at 13,000 rpm for 3 minutes to pellet the membranes. Aliquots of 100µl of the resulting supernatant were transferred to the wells of a 96-well microtitre plate. The reaction was stopped by the addition of 100µl of fresly prepared solution A (6% SDS, 3% L-ascorbic acid, 0.5% ammonium molybdate in 0.25M sulfuric acid). After 15 minutes, the phophoammoniumolybdate complex formed was stabilised by the addition of 100µl of solution B (2% sodium citrate, 2% sodium arsenite, 2% acetic acid). After 15 minutes, the absorbance at 710nm was measured using a 7520 Microplate Reader Cambridge Technology, Inc.

A calibration curve for the inorganic phosphate determination was set up by preparing dilutions of sodium hydrogen phosphate ranging from 2 to 20nmol in reaction buffer in a final volume of 100µl. These dilutions were assayed as described above.

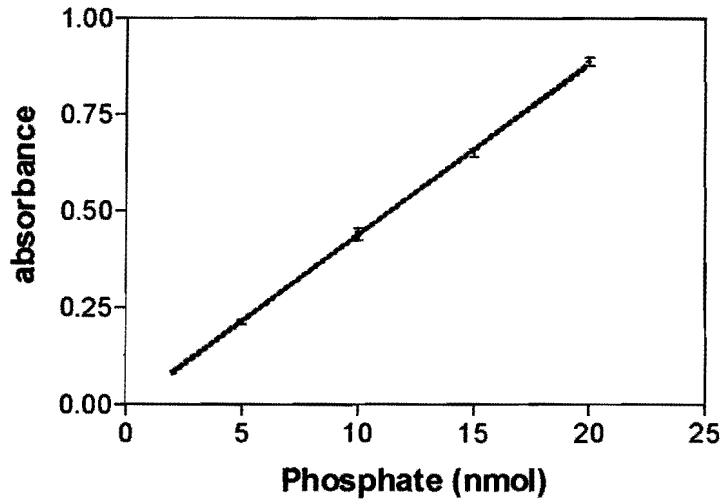

Figure 25: Standard curve for the inorganic phosphate by measurement of the absorbance at λ= 710nm from 3 separate experiments with errors bars representing the standard deviation (r^2=0.999).

7.12. Preparation of antibodies to Pgh1

7.12.1. Preparation of competent cells

L-Broth medium was prepared by dissolving 5g Tryptone, 2.5g Yeast extract, 5g NaCl in 500ml distilled water. After sterilization by autoclaving, ampicillin was added to a final concentration of 0.1mg/ml. 500ml of L-Broth medium was inoculated with 3 ml of an overnight culture of *E. coli* and incubated at 37°C with shaking until the absorbance at 650nm of the culture reached 0.2-0.3. The cells were then centrifuged at 5,000 rpm in a JA-10 Beckman rotor for 10 min at 4°C and resuspended in 40ml of ice-cold 60mM $CaCl_2$, 10mM Pipes, pH 7.2 by gentle pipetting action. After 20 min incubation on ice, the suspension was centrifuged at 5,000 rpm in a JA-20 Beckman rotor for 5 min. The cells were resuspended in 4ml of 60mM $CaCl_2$, 10mM Pipes, pH 7.2, 15% glycerol. The resulting competent cells were frozen in liquid nitrogen and stored at -70°C.

7.12.2. Transformation of *Escherichia coli*

Two plasmid expression vectors pGex-3X constructs (Cowman *et al.*, 1991) encoding for the N-terminal ATP-binding site and the C-terminus portion of Pgh1 corresponding respectively to the amino acids residues 510-614 and 1252-1419 of Pgh1 (Foote *et al.*, 1989) as a Pgh1 fusion protein with glutathione-S-transferase (GST) were generously provided by Dr A. Cowman, Walter and Elisa Hall Institute of Medical Research in Melbourne, Australia. These plasmids were transformed either into *E. coli* strain JM-109 or XL-Blue. 10µl of plasmid DNA was incubated with 100 µl of competent cells for 1h30 on ice. This solution was transferred to a 42°C waterbath and heated for 2 min. 1 ml of prewarmed (37°C) L-Broth agar (5g tryptone, 2.5g yeast extract, 5g NaCl, 7.5g bacto-agar, dissolved in 500ml H_2O to which ampicillin was added after sterilization by autoclaving to a final concentration of 0.1mg/ml) was added and the cells were incubated at 37°C for 1 hour. Aliquots of this solution were spread on L-Broth agar plates containing 0.1mg/ml ampicilline and incubated overnight at 37°C.

7.12.3. Plasmid DNA purification

The DNA was purified using a small-scale plasmid DNA preparation system. 5 ml of an overnight culture of *E.coli* transformed with the recombinant pGex-3X was centrifuged for 10 min at 2,000 rpm and resuspended in 200µl of 25mM Tris-HCl, pH 8.0, 10mM EDTA, 50mM glucose. After 5-min incubation, 2µl of RNase I (10mg/ml, Boehringer Mannheim) was added. The membranes were lysed with 400µl of 5M NaOH, 10% SDS and incubated for 5 min on ice. The genomic DNA was precipitated with 300µl of 3M potassium acetate and incubated for 5 to 10 min on ice. After a 5-min centrifugation in a microfuge at 4°C, the supernatant containing the plasmid DNA was transferred into an eppendorf. The plasmid DNA was precipitated with 600µl isopropanol and incubated for 30 min at -20°C. The DNA was microfuged for 5 min at room temperature and washed twice with 70%

ethanol at room temperature. The resulting purified DNA was resuspended in 80µl TE-buffer (1mM EDTA, 10mM Tris-HCl, pH 8.0).

7.12.4. Enzymatic digestion of the plasmids

Enzymatic digestions were carried out with 10µl of plasmid DNA, 2µl of buffer H (SuRE/Cut buffer H, Boehringer Mannheim), 1µl of the specified restriction enzyme and 7µl of water. After 2 hours incubation at 37°C, the reaction was stopped by addition of 2µl of stop buffer (10mM EDTA, pH 7.5, 0.25% Bromophenol blue, 0.25% Xylene Cyanole and 30% Glycerol). Ten µl of the resulting digested DNA, the corresponding uncut plasmid and the λ-phage DNA marker (New England BioLab) were loaded onto an 1% agarose gel. Gels were electrophoresed at 40mA for 50 minutes and revealed with 1% ethidium bromide.

7.12.5. Sequencing of the Pfmdr1 gene fragments

Sequencing of the plasmid was performed by the method of Sanger (1977) using a Thermo Sequenase™ DNA polymerase (Amersham Pharmacia) and the Cys™ Thermo Sequenase Dye Terminator Kit (Amersham Pharmacia Biotech.) The plasmids were amplified on a Gene Amp PCR System 9700 (Perkin Elmer, Applied Biosystems). The sequencing primers consisted of a pGEX-3X forward primer: (23 mer) 4pmol/µl: 5'-GGG CTG GCA AGC CAC GTT TGG TG 3' and a reverse primer: (23 mer) 4pmol/µl: 5'CGG GGA GCT GCA TGT GTC AGA GG-3'. The sequencing was carried out on an ALFexpress DNA Automated Sequencer (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The electrophoresis was performed onto a 5% acrylamide gel for 13 hours at 55°C, using a standard gel cassette and 0.5mm spacers. Data were analysed by the ALFwin version 1.1 software (Amersham Pharmacia Biotech.).

7.12.6. Expression of the Pgh1-GST fusion protein

The expression of fusion proteins was performed basically as described by Smith and Johnson (1988). Overnight cultures of *E.coli* transformed with the plasmid of interest were diluted 1:10 in 900ml of L-Broth medium containing 0.1g/l ampicilline. After 1 hour of growth at 37°C, bacteria were induced with 0.1mM IPTG (isopropyl-β-D-thiogalactopyranoside, Promega). After a further 3-7 hours of growth, bacteria were pelleted by centrifugation for 10 min, at 5,000g in a GSA rotor (Beckman). At this point, bacteria pellets were frozen at -70°C before thawing and continuation of the protocol.

7.12.7. Purification of the Pgh1-GST fusion protein

The induced cell pellet was resuspended in 10ml ice-cold PBS containing 1mM phenylmethylsulfonylfluoride (PMSF, Boehringer Mannheim) and 20μg/ml Soybean Trypsin inhibitor (Sigma) and transferred to 14ml polypropylene tubes on ice. Cells were sonicated 4 times on ice for 20 seconds using an ultrasonic processor (Heat Systems Ultrasonic Inc., Model W-385) and Triton X-100 was added to a final concentration of 1%. After 15 min incubation on ice, the insoluble debris were pelleted by centrifugation at 10,000 rpm at 4°C for 5 min in a SS-34 rotor (Beckman). The supernatant was mixed with 2 ml of washed and swollen glutathione-agarose beads (Sigma) on a rotating platform at room temperature. After 20-min incubation, the beads were washed 5 times with ice-cold P.B.S. by repeated centrifugation. Fusion protein was eluted by competition with free glutathione using 2 X 2 min washes with 1 bead volume of 50 M Tris-HCl , pH 8.0 containing 5mM reduced glutathione (Sigma). The purified fusion proteins were freeze-dried and stored at -20°C.

7.12.8. immunization of rabbits

The fusion proteins were redissolved in water to a concentration of 1.2mg/ml and either 1ml of Freund's complete adjuvant (CFA, Sigma) or incomplete Freund's adjuvant was added to 200µl of the fusion protein suspension. New Zealand White rabbits were immunised by injection of 0.2mg of fusion protein together with Freund's complete adjuvant for the priming immunisation and incomplete Freund's adjuvant for the subsequent immunisations. Second and third immunisations were performed 30 days and 60 days respectively after the priming immunisation. 14 days after the last immunisation, 25ml of blood from each rabbit were harvested and incubated at 37°C for 1 hour. The red blood cells were removed by centrifugation and the resulting sera was stored at -70°C. 2 test bleeds of 5 ml each were performed respectively 10 days after the second immunisation and 7 days after the third immunization. As a control, 5ml of blood was taken before immunization of the rabbits and treated as described previously.

7.12.9. Preparation of antibodies to a N-terminal Pgh1 peptide

A peptide corresponding to the amino-acid 2-18 of Pgh1 to which was added a cysteine residue (GKEQKEKKDGNLSIKEEVC) was synthesised at the Eidgenössische Technishe Hochschule (ETH) Zürich, Switzerland. To activate the carrier protein, 200µl of a maleimidobenzyl-N-hydroxysuccinimide ester (MBS) solution (3.3mg of MBS in 330µl dimethylformamide) was added to 12mg of pure ovalbumine (Sigma) in 600µl PBS. After a 2 hours incubation, the unbound maleimide was removed by dialysis against PBS at 4°C. The coupling of 2mg of peptide dissolved in 20µl water to 2mg ovalbumin-benzoyl-maleimide in 100mM phosphate buffer pH 7.5 was carried out at 4°C for 20 hours. Aliquots of 200µg of the conjugate were stored at -70°C. These aliquots of conjugate were then injected to the rabbits as described in section 7.12.8.

7.12.10. Dot blots

Ovalbumin (0.14μg), activated ovalbumin (0.17μg) and a peptide (3μg) corresponding to the N-terminal 18 amino acids of Pgh1 were dissolved in P.B.S and pipetted onto nitrocellulose membrane (micro Separation, Inc.). The dried membranes were blocked for 2 hours in 5% milkpowder, 0.1% Tween-20 in P.B.S. and exposed to the rabbit serum against $Pgh1_{1-18}$ peptide diluted 1:2000 with in 5% milkpowder, 0.1% Tween-20 in P.B.S. for 2 hours. A negative control consisting of the rabbit pre-immune serum diluted 1:2000 was included. The membranes were rinced 3 times with 5% milkpowder, 0.1% Tween-20 in P.B.S. and exposed to a horseraddish peroxydase conjugated anti-rabbit (Sigma) at a 1: 4000 dilution for 1 hour. The membranes were again rinced with 5% milkpowder, 0.1% Tween-20 in P.B.S. for 15 minutes, followed by 3 washes with PBS/0.1% Tween-20 and finally rinced with PBS. Visualisation was performed with the Pierce SuperSignal CL-HRP chemiluminescent substrate.

7.13. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out essentially as described by Laemmli (1970). SDS-PAGE was performed on a 10% acrylamide slab gel (3mm thickness) and a 5% stacking gel. Samples and molecular weight standards (Biolabs, Prestained, protein marker broad range) were dissolved in ½ volume of 3X reducing SDS-sample buffer (Biolabs, 187.5mM Tris-HCl, pH 6.8, 6% (w/v) SDS, 30% glycerol, 0.03% phenol red, 0.125M dithiothreitol). The sample and standards were heated to 95-100°C for 5 min before loading onto the gel. Gels were electrophoresed at 100 mV until the dye reached the bottom of the gel in a mini-gel apparatus (Bio-Rad). Gels were either silver-stained or processed for western blot analysis.

7.14. Silver staining of gel

SDS-PAGE gels were silver-stained as described by Wray *et al.* (1981). Gels slabs were soaked in 50% methanol, 0.7% formaldehyde for at least 1 hour. They were then rinsed twice with H_2O and stained for 15 min in a freshly prepared 0.8 % silver nitrate solution. This solution was prepared by adding the solution A (0.8g $AgNO_3$ in 4ml H_2O) to the solution B (21ml 0.36% NaOH, 1.4ml 25% ammonia) while shaking and by increasing the volume to 100ml. Gels were again rinced in H_2O and developed in 0.02% formaldehyde, 0.005% citric acid until the desired contrast was obtained. The development was stopped by placing the gel in 50% methanol, 10% acetic acid for 15 min.

7.15. Western blotting

Samples were electrophoretically transferred to 0.45µm pure nitrocellulose membrane (Bio-Rad) using the Bio-Rad Mini Trans Blot system in a transfer buffer consisting of 0.025M Tris-HCl, pH 8.3, 0.192M glycine, 0.1% SDS and 20% methanol at 100mV for 1hour. Nitrocellulose blots were blocked for at least 3 hours using 10% milk powder in P.B.S./Tween 20 (0.1%), rinced with P.B.S./Tween 20 (0.1%), and exposed to the appropriate antibody for 1 hour. Affinity-purified antibodies to Pgh1 were used at a dilution of 1:1000 while antibodies against human immunoglobulins (Sigma) were used at a 1:5000 dilution. The nitrocellulose was rinced with P.B.S./Tween 20 (0.1%) and exposed to an alkaline phosphatase-conjugated anti-rabbit IgG (Sigma) at a 1:10000 dilution, for 30 min. The nitrocellulose was again rinced with P.B.S./Tween 20 (0.1%) and then with a wash solution (100mM NaCl, 100mM Tris-HCl, 5mM $MgCl_2$, pH 9.5). Bound antibodies were detected colorimetrically using the bromochloroindolyl phosphate/nitro-blue tetrazolium (Sigma) substrate system.

In some experiments, bound antibodies were detected by autoradiography with peroxydase-labeled anti-rabbit antibodies and the Boehringer Mannhein detection chemiluminescence detection kit.

7.16. Electron microscopy of parasite plasma membranes vesicles, trophozoites and parasitised erythrocytes

Samples were fixed with glutaraldehyde (0.8%)/formaldehyde (8%) in PBS for 1 hour at room temperature and washed in PBS, followed by addition of 50mM NH_4Cl for 30 minutes and 2 washes in PBS. Samples were dehydrated in 70% ethanol and embedded in LR White resin (Polyscience Inc.) which was then polymerised at 60°C for 28 hours. Then 1μm-thick sections were cut using a reichert Ultracut S ultramicrotome. Sections were mounted on copper grids and stained with 2% uranyl acetate and lead acetate. Samples were viewed on a JEM 200CX transmission electron microscope.

7.17. Immunoelectron microscopy

Sections mounted on copper grids prepared as described previously (section 7.15) were incubated in PBS/BSA (0.1%) for 30 minutes, and then with rabbit anti-$Pgh1_{1-18}$ antibodies diluted 1:50 in PBS/BSA (0.1%) for 2 hours at room temperature. After washing in PBS/BSA (0.1%), thin-sections were exposed to a goat anti-rabbit antibody labelled with 20nm-gold particles for 1 hour at room temperature. Sections were then washed in PBS/BSA (0.1%) and stained with 2% uranyl acetate. Samples were viewed on a JEM 200CX transmission electron microscope.

7.18. Protein determination

Protein determinations were performed by the method of Lowry *et al.* (1951) using a protocol developed by Peterson (1977). Samples were brought to a final volume of 1.0ml with water and 1ml of 2.5% sodium carbonate/0.025% copper sulfate/0.05% potassium tartrate/2.5% SDS/0.2M sodium hydroxyde was added. After 10 minutes at room temperature, 0.5ml of a 1:5 solution of Folin-Ciocalteu phenol reagent (Merck) was added. Samples were mixed immediately and aliquots of 200µl were transferred into the wells of a 96-well microtitre plate. After 30 minutes, absorbance values at 710nm were measured using a 7520 Microplate Reader Cambridge Technology, Inc.

Increased concentrations of Bovine serum albumin was used to set up standard curves.

7.19. Data analysis

Statistical significance (p-values) was established using the two-tailed Student's t-test using the computer program GraphPad Prism® (GraphPad software). The level of significance was set at $p \leq 0.05$. Data for dose-response curves were fitted using GraphPad Prism® (GraphPad software) and the concentration of chloroquine that inhibits 50% of parasite growth (IC_{50}) was determined from the curve using this program.

Annexe

Preparation of antibodies to a N-terminal Pgh1 peptide

Antibodies against Pgh1 peptide were prepared in our laboratory (M. Lindt). A peptide corresponding to the N-terminal 18 amino acids of pgh1 was synthesised and a cysteine residue was added at the C-terminus to permit coupling to a carrier protein via a maleimide function. Since the pgh1 peptide represents the N-terminus of the protein, the peptide was conjugated to a carrier via the C-terminus so that the N-terminus of the peptide was visible to the immune system. Antibodies directed against this peptide-conjugate should recognise the N-terminus of the pgh1 protein. The conjugation reagent used was maleimidobenzoyl-N-hydroxysuccinimide ester (MBS). This reagent reacts with amino groups on protein via the N-hydroxysuccinimide ester to yield protein-benzoyl-maleimide. The peptide was then conjugated by simply co-incubating the thiolated peptide with the protein-benzoyl-maleimide and injected to the rabbits as described in methods section 7.12.9. Dot blot experiments showed that the peptide as well as the carrier protein was recognised by the immune sera. Western blot experiments showed that the trophozoites contain a protein that is recognised by the anti-conjugate serum. The molecular weight of the protein is approximately 75kDa. Surprisingly, several bands were recognised in the sample of type B^+ blood, the dominant band being at 55kDa. Since the rabbit was immunised with an ovalbumin-peptide conjugate the cross-reactivity with human blood might arise out of homology between ovalbumin and serum proteins.).

Recognition of the N-terminal 18 amino acid peptide of Pgh1 by rabbit serum against $Pgh1_{1-18}$ peptide

To examine whether the serum recognises the peptide, dot experiments were performed. Ovalbumin (0.14μg), activated ovalbumin (0.17μg) and $Pgh1_{1-18}$ peptide (3μg) were pipetted onto nitrocellulose membranes and incubated with rabbit serum against $Pgh1_{1-18}$ peptide. A negative control consisting of the pre-immune rabbit serum was included. Visualisation was performed with goat anti-rabbit antibodies conjugated to horseradish peroxidase using a chemiluminescent system. The immune serum recognised the carrier protein and the peptide whereas the negative serum did not recognised any of the antigens (Plate 19).

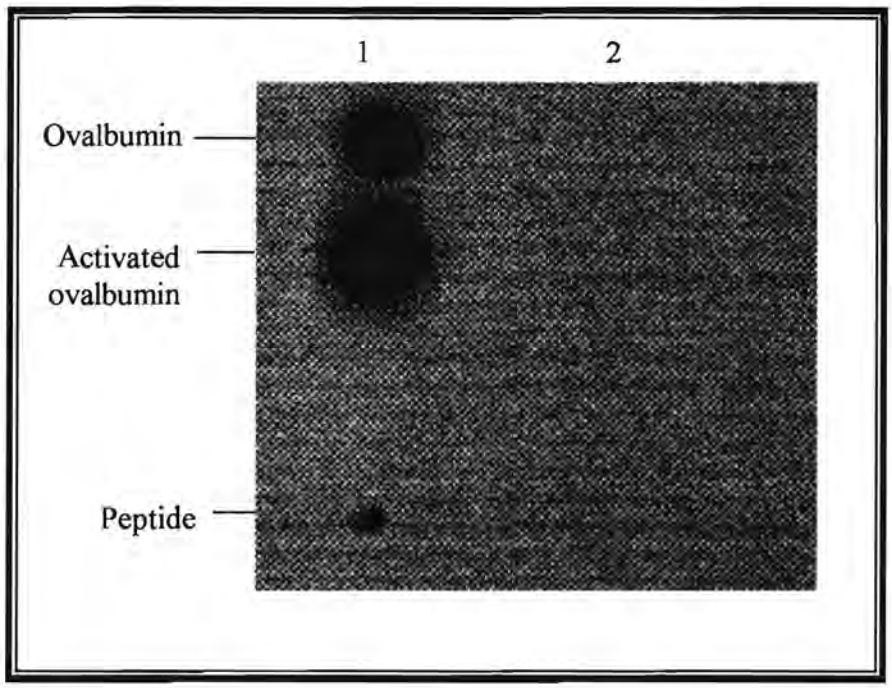

Plate 19: Dot blot profiles of ovalbumin (0.14μg), activated ovalbumin (0.17μg) and $Pgh1_{1-18}$ peptide (3μg) revealed with rabbit serum against $Pgh1_{1-18}$ peptide (lane1) and pre-immune rabbit serum (lane 2). Recognised bands were detected using chemiluminescence.

Recognition of Pgh1 in trophozoites by rabbit serum against $Pgh1_{1-18}$ peptide

Western blot of trophozoites revealed with rabbit serum against $Pgh1_{1-18}$ peptide were performed to determine whether these antibodies recognised Pgh1. Equal amount (20μg) of purified D10 trophozoite protein and full blood protein (serum and erythrocytes) of type B^+ were electrophoresed onto a 12.5% SDS-PAGE gel, transferred to nitrocellulose and probed with rabbit serum against $Pgh1_{1-18}$ peptide. Visualisation was performed with goat-anti-rabbit antibodies conjugated to alkaline phosphatase.

Western blot experiments showed that the D10 trophozoites contain a protein of approximate molecular weight of 75 Kda that is recognised by the anti-conjugate serum. Several bands are recognised in the lane containing the type B^+ blood, the dominant band corresponding to a molecular weight of 55 Kda (Plate 20).

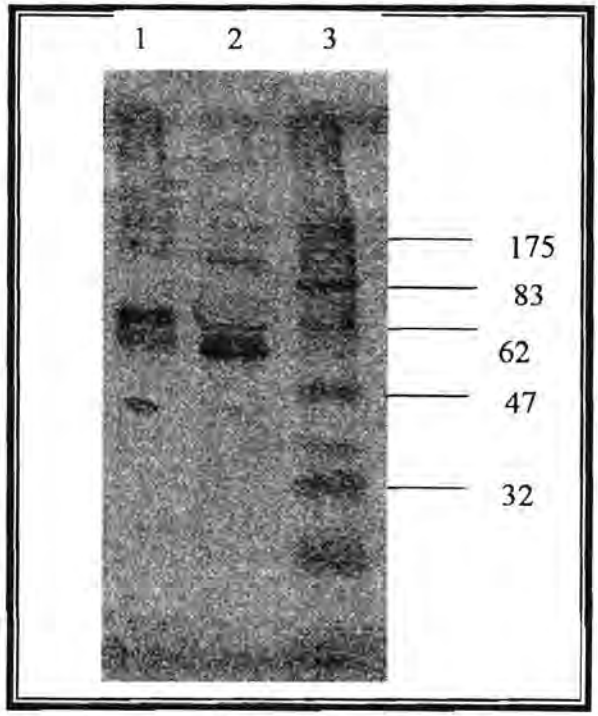

Plate 20: Equal amount (20μg) of purified D10 trophozoite protein (lane 1) and full blood protein (serum and erythrocytes) of type B^+ (lane 2) were electrophoresed, transferred to nitrocellulose and probed with rabbit serum against $Pgh1_{1-18}$ peptide. Recognised band were detected colorimetrically with goat-anti-rabbit antibodies conjugated to alkaline phosphatase. Lane 3 contains protein markers with approximate molecular weights expressed in kDa.

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