Funktionelle Charakterisierung von Aquaporinen aus *Plasmodium falciparum*, *Toxoplasma gondii* und *Trypanosoma brucei*

Functional characterisation of aquaporins from *Plasmodium falciparum, Toxoplasma gondii* und *Trypanosoma brucei*

DISSERTATION

der Fakultät für Chemie und Pharmazie

der Eberhard-Karls-Universität Tübingen

zur Erlangung des Grades eines Doktors

der Naturwissenschaften

2006

vorgelegt von

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Tag der mündlichen Prüfung:	10. März 2006
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The experimental part of this work was done between October 2002 and October 2005 at the Institute for Pharmaceutical Chemistry, the University of Tübingen under the supervision of PD Dr. Eric Beitz and Prof. Dr. J.E. Scultz.

I would like to thank to PD Dr. Eric Beitz Prof. and Dr. J.E. Scultz for giving me the possibility to work on this interesting theme and for constructive discussions which resulted in my PhD thesis.

I would like to thank to Prof. Dr. O. Werz and Prof. Dr. L. Heide for taking a part on my finale exam.

Thank you, dear colleagues in the lab, at the Institute for Tropical Medicine and the Prof. Dr. M. Duszenko's working group.

Special thanks to my husband Sergej, for support and constructive discussions during my work, to my family and to my dear friends.

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List of abbreviations

BSA	Bovine serum albumin
dNTPs	Desoxynucleoside triphosphates
DTT	Dithiotreitol
EtOH	Ethanol
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethanesulphonic acid
IPTG	Isopropyl-β-D-thiogalactoside
LB-broth	Luria-Bertani bacterial growth medium
MCS	Multiple cloning side
Ni-NTA	Nickel-nitrilotriacetic acid-agarose
ORF	Open reading frame
PVDF	Polyvinylidene difluoride
TEMED	N, N, N', N'-Tetramethylethylene diamine
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

1 Introduction

1.1 Aquaporins

1.1.1 Discovery of aquaporins

The cell, the basic unit of life, is essentially defined by its plasma membrane. The cell plasma membrane is a major barrier for the free movement of solvents. Its main purpose is to divide the intracellular content from the extracellular fluid. During cell evolution, specific channels evolved to transport nutrients, metabolic products etc. in and out of the cell.

The major component of a cell is water. The hydrophobic plasma membrane presents a significant energy barrier for water movement into and out of the cells. Water can diffuse across lipid bilayer membranes, although the activation energy (E_a) required for this process to occur is much greater than for diffusion (10-20 vs. 5 kcal mol^{-1}; Heymann et al., 1999).

However, studies on water flow through mammalian red blood cell membranes demonstrated that it occurs with an E_a of <5 kcal/mol whereas diffusion of water through pure lipid membranes occurs with a higher activation energy (E_a >10 kcal/mol; Engel et al., 2001). Furthermore, the high water permeability of mammalian red blood cells to water is strongly inhibited by mercuric chloride ($HgCl_2$). In addition, experiments with amphibian skin have shown that some epithelia are extremely “leaky” to water.

All together, these findings led to early speculations that beside simple diffusion water may enter the cell in an assisted manner probably through proteins that conduct water.

About one decade ago, Peter Agre showed that specific water channels (aquaporins) are indeed present in erythrocytes. Originally, this channel protein was named CHIP28. It was the first member of the aquaporin (AQP) family and was subsequently renamed to AQP1 (Preston et al., 1992).

To date the aquaporin family comprises more than 450 members (Zardoya et al., 2005) from all kingdoms of life e.g. from bacteria, protozoa, plants and animals. Thirteen aquaporins have been identified in humans designated as AQP0-12.

The aquaporin family consists of two major subfamilies: water specific channels (orthodox aquaporins) and channels which are permeable for glycerol, water and other small molecules (aquaglyceroporins).

AQP1 is the prototype of the orthodox aquaporins and it is exclusively permeable for water molecules (Sui et al. 2001). The *E. coli* GlpF, a prototypical aquaglyceroporin, has high permeability to glycerol, but low permeability to water (Fu et al., 2000).

1.1.2 Structural organisation of aquaporins

Initial inspection of the primary sequence of aquaporin AQP1 suggested that the protein consists of six α-helical transmembrane domains with an inverted symmetry between the first three and last three domains. The two loops between helix 2-3 and helix 5-6 contain an amino acid triplet Asn-Pro-Ala (NPA motif) which is highly conserved across the members of the aquaporin family (Fig. 1.1. A).

It was thought, that the six bilayer spanning helixes surround a water pore with two conserved NPA motifs in the middle (Fig. 1.1. B; Preston et al., 1992; Jung et al., 1994; Borgnia et al., 1999). This topology was named "hour glass" model. This "hour glass" conformation was later confirmed using X-ray crystallography (Sui et al., 2001). The functional unit of aquaporins is a tetramer with an independent water pore in each subunit.

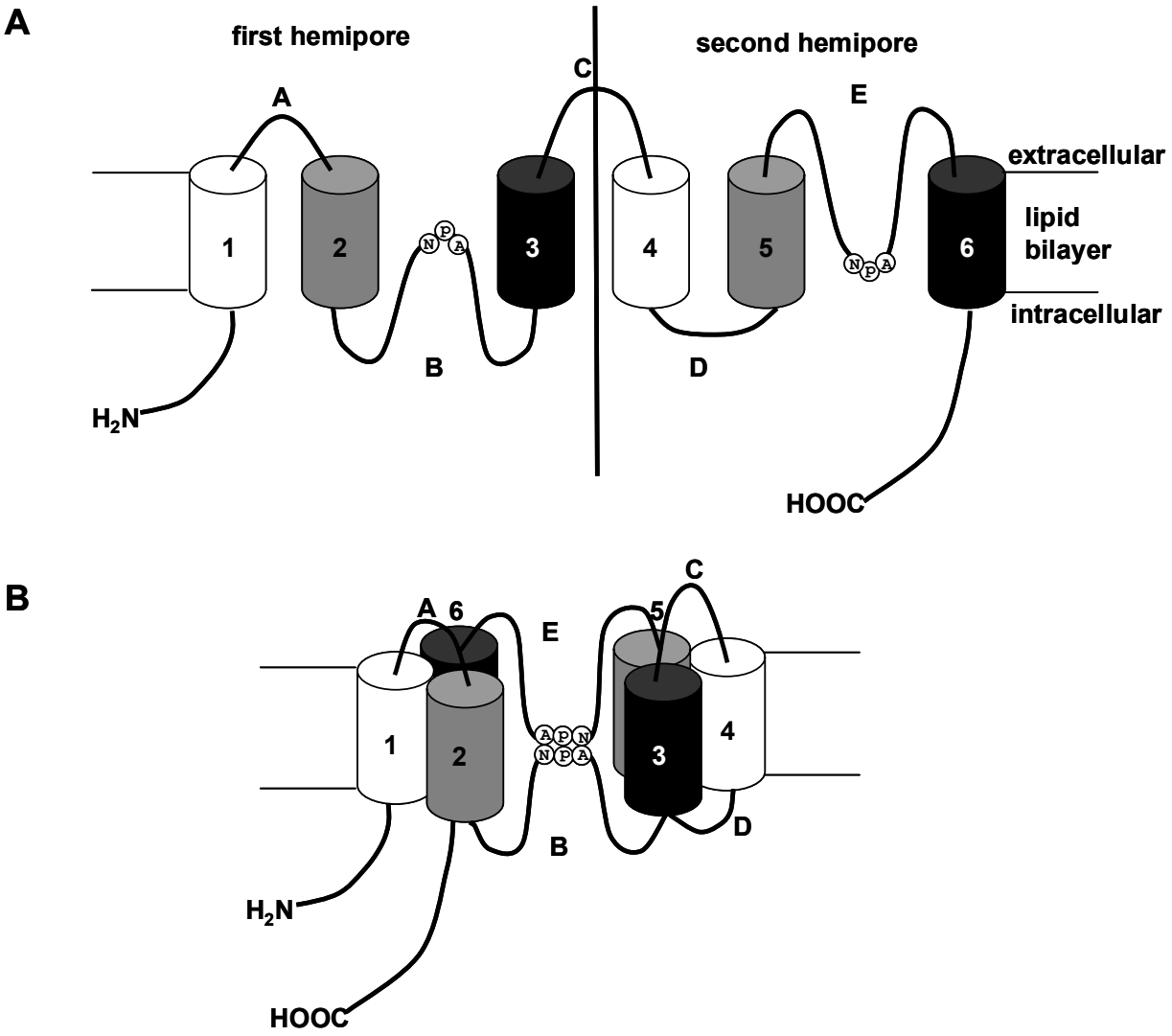

Fig. 1.1. Aquaporin-1 membrane topology and hourglass model. A: Aquaporin-1 contains six bilayer-spanning domains composed of two inversely symmetrical structures (first hemipore and second hemipore) and five loops (A, B, C, D, E) where loops A and C are extracellular, D is intracellular and B and E fold back into the membrane. N and C termini are intracellular. B: The NPA triads in loops B and E form a single aqueous pathway through the membrane.

Primary sequence analysis showed specific differences between aquaglyceroporins and orthodox aquaporins, such as the motive GLYY (Fig. 1.2., shaded in green) in the C-loop and an aspartate residue after the second NPA motif (NPARD) which are typical for aquaglyceroporins. In orthodox aquaporins the second NPA motif if followed by a serine (NPARS) (Fig. 1.2., shaded in yellow; Borgnia et al., 1999).

Another discrimination between aquaporins and aquaglyceroporins is the length of the extracellular C-loop which is about 15 amino acids longer in aquaglyceroporins (Engel et al., 2000; Beitz et al., 2004). Furthermore, the C-loop amino acid triad F[A/S]T is highly conserved in aquaglyceroporins (Beitz et al., 2004; Fig. 1.2. shaded in violet). In PfAQP this triad corresponds to a WET triplet with a highly conserved Thr. A marked difference between the WET and F[A/S]T motifs is the presence of a negatively charged Glu at the second position which might interact with a positively charged Arg in the pore constriction region.When the atomic structures of both, human orthodox aquaporin AQP1 and bacterial aquaglyceroporin GlpF, were reported, they revealed a surprising structural similarity (Beitz et al., 2004).

A few years ago, our group identified a single aquaglyceroporin in *Plasmodium falciparum* parasites (PfAQP; Hansen et al., 2002). The primary sequence suggested that the newly identified protein is an aquaglyceroporin with close a relationship to the *E. coli* GlpF. However, the characteristic NPA motifs are changed to NLA and NPS in the *P. falciparum* aquaporin (Fig. 1.2. shaded in red). Functional characterisation showed that this aquaporin has high water and glycerol permeability (Hansen et al., 2002).

Based on the GlpF structure PfAQP was modelled revealing that the amino acids of the pore constriction are identical to those of GlpF (Fig. 1.3.). Furthermore, the RD after NPS and

GLYY motifs which are characteristic for aquaglyceroporins, are present in PfAQP (Fig. 1.2.). However, the C-loop is shorter in PfAQP (13 aa) than in GlpF which is reminiscent of orthodox aquaporins.

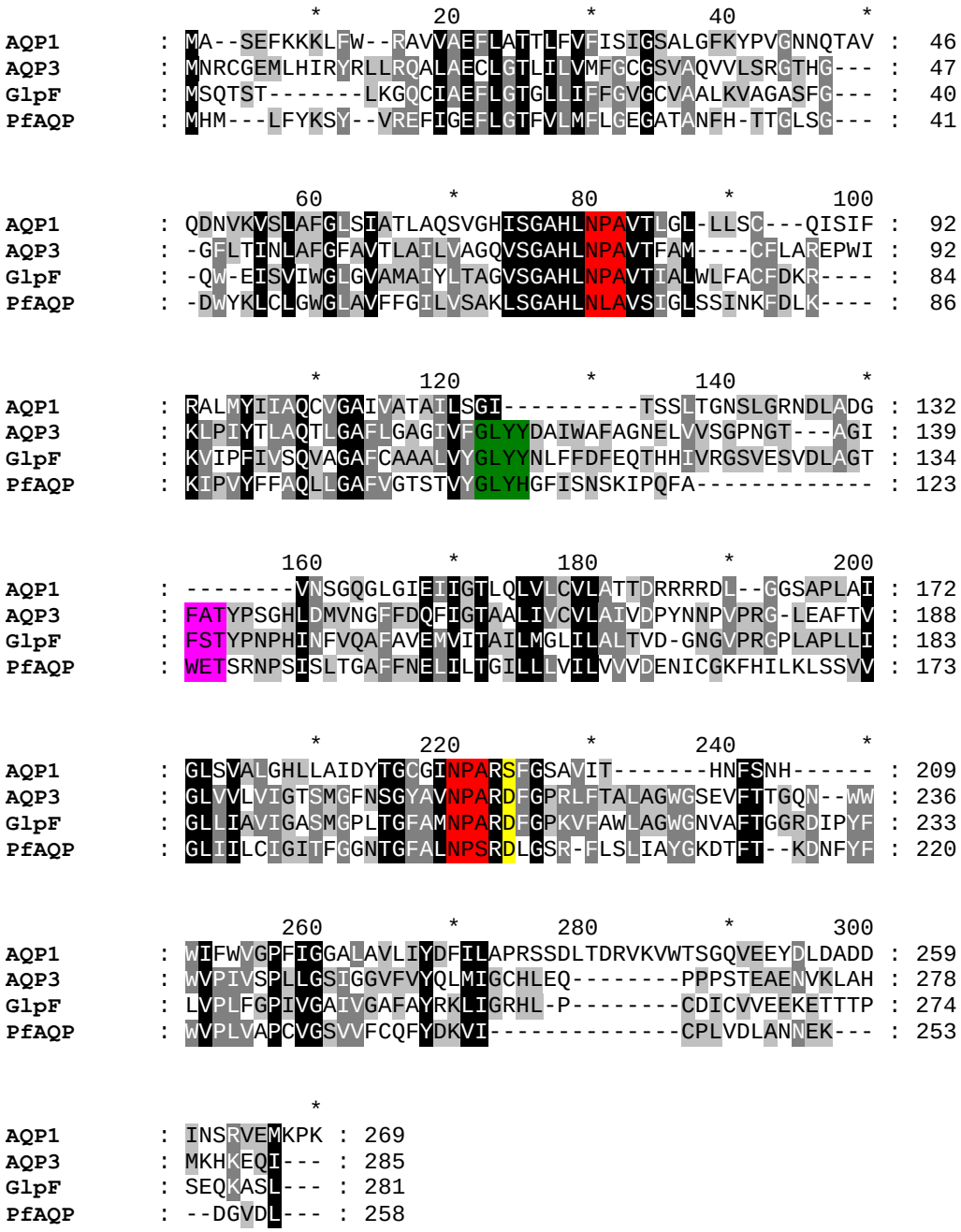

Fig. 1.2. Sequence alignment of human aquaporin-1 (AQP1), human aquaglyceroporin-3 (AQP3), *E. coli* aquaglyceroporin (GlpF) and *P. falciparum* aquaglyceroporin (PfAQP). The NPA motifs are coloured in red, F[A/S]T-WET triad is coloured in pink, GLYY is coloured in green and D or S after the second NPA motif is coloured in yellow. The alignment was generated using ClustalV.

It was shown that the C-loop has an important role in water permeability in PfAQP (Beitz et al., 2004). Introduction of a mutation into the WET triad which is an analogue to FST in the GlpF C-loop, (glutamate to serine, PfAQP $E_{125}S$), resulted in a high reduction of water permeability (Fig. 1.3.). A possible explanation may be that Glu125 interacts with Arg196 and interruption of this interaction influences on water permeability.

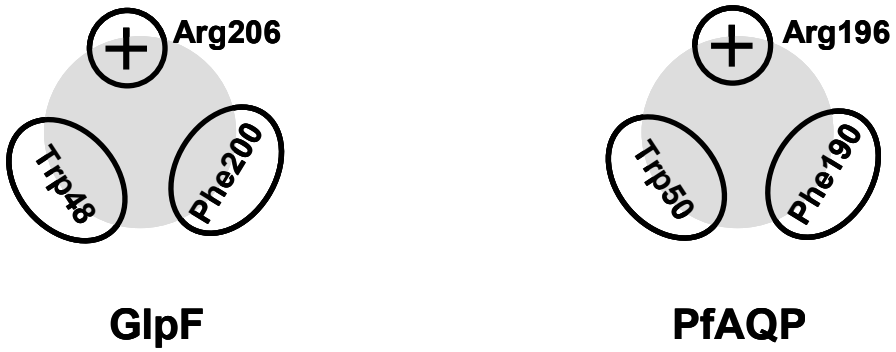

Fig. 1.3. Schematic presentation of the outer pore constriction in *E. coli* GlpF and PfAQP as viewed from the extracellular side.

1.1.3 Parasite aquaporins

Malaria, toxoplasmosis and Chagas' disease are infectious diseases caused by protozoa that affect more than 300-400 million people around the world and represent a major health problem in developing countries (Gelb et al., 2002). Because of the high infection rate and rapid development of drug resistant parasite strains, new strategies for drug development are needed.

Parasites from the phylum *Apicomplexa* and *Euglenozoa* (genus *Kinetoplastida*) are particular human pathogenic (Baldauf et al. 2000; Beitz et al., 2005).

The phylum *Apicomplexa* includes a large number of obligate intracellular parasites, among which are the human pathogens *Plasmodium* (malaria), *Toxoplasma* (AIDS related encephalitis), *Cryptosporidium* and *Cyclospora* (severe enteritis) as well as many parasites of veterinary importance (*Eimeria*, *Theileria*, *Sarcocystis* and *Babesia*).

The parasite life cycles can be relatively simple, involving only a single host (*Cryptosporidium*), whereas the others require interspecies transmission often between an insect and a mammal (Beitz et al., 2005, Roos et al., 2005). Some parasites are specialists, restricted to special species and tissues, e.g. *Plasmodium falciparum*, the others are more general and can infect almost any tissue of warm blooded animals, e.g. *Toxoplasma gondii*.

A characteristic organelle for all apicomplexan parasites is the apicoplast. It is postulated that apicomplexan parasite have acquired this organelle by the ingestion of an eucaryotic plastid containing alga (plastid of red alga and euglenoids; Dzierszinski et al., 1999).

Genome analysis showed that *P. falciparum* expresses only one aquaporin whereas *Kinetoplastida* parasites (*Trypanosoma cruzi*, *Trypanosoma brucei* and *Leishmania major*)

express from 3 to 5 aquaporins. Interestingly in the *Cryptosporidium parvum* genome no aquaporin gene was found at all (Beitz et al., 2005).

1.2 *Plasmodium falciparum*

It is estimated that there are around 300 million cases of malaria world-wide and up to 2.7 million deaths from malaria each year. Four species of malaria are infectious for humans: *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. *P. falciparum* is the most lethal species for humans.

Malaria parasites enter their vertebrate host via the bite of an infected *Anopheles* mosquito. Via the blood stream the parasites (sporozoites) first invade liver cells. Within two days, up to 32 new merozoites are released into the blood stream, where they are about to invade the red blood cells of their host.

The different stages of the asexual intra-erythrocytic phase of the parasite life cycle are schematically represented in Fig. 1.3. First, the malaria parasite invades an erythrocyte and during this process the parasite is being enclosed with a so-called parasitophorus vacuole membrane (PVM) (Fig. 1.3. a). Approximately, during the initial 15 hours after invasion (the so-called "ring" stage) the parasite presumably lies dormant (Fig. 1.3. b). There is a progressive increase in metabolic activity 15 h after invasion when parasites enter the trophozoite stage (Fig. 1.3. c).

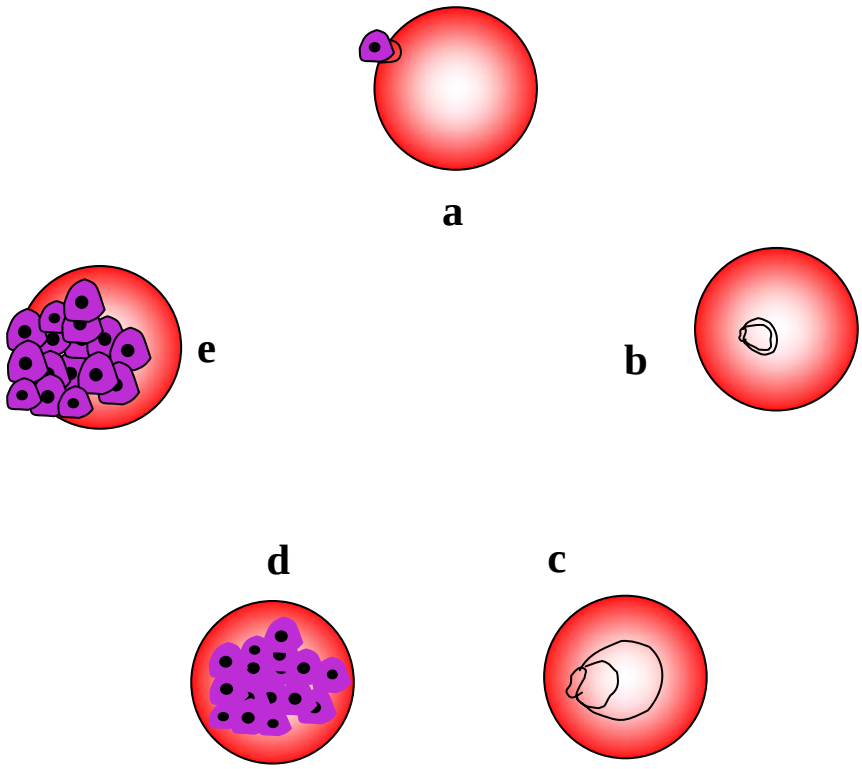

Fig. 1.4. Schematic representation of the different stages of the asexual erythrocyte phase of the life cycle of the malaria parasite *P. falciparum.* a: invasion a merozoite. b: represents "ring" stage. c: trophozoite stage. d: schizont stage. e: the host erythrocyte bursts and releases merozoites and a new cycle begins.

Protein, RNA and DNA synthesis is extensive in this stage of intra-erythrocytic development. Due to the fact that malaria parasites lack a functional citric acid cycle, their energy supply is fully dependent on glycolysis. During parasite growth glucose utilisation and lactate production is increased by up to 100 times the rate of uninfected erythrocytes (Roth et al., 1988).

Through the process of endocytosis, host proteins, especially haemoglobin, are internalised and further digested to small peptides and amino acids in the food vacuole and subsequently used for protein synthesis.

Protein degradation is accompanied by ammonia production. Due to the high level of haemoglobin degradation toxic ammonia has to be released or converted into a less toxic compound like urea. Inspection of the *P. falciparum* genome did not reveal sequences of any enzyme involved in the urea cycle except for arginase. From this information the question arose at which rate ammonia is produced during the erythrocytic stage of parasite development and how it is released.

About 36 h post invasion the parasite occupies approximately one third of the total volume of the host cell. Approximately 40 h after the trophozoite stage the parasite enters the "schizont" stage (Fig. 3 d).

At this stage, the parasite subdivides into 20-32 daughter merozoites. At "schizogony" the host cell ruptures and parasites are released (Fig. 3 e). This happens 48 h after invasion of the host erythrocyte.

During its life cycle the malaria parasite faces drastic osmotic changes during kidney passages. Further, there is the need for massive biosynthesis of glycerolipids during development in the blood stage because of fast growth and division (Hansen et al., 2002). PfAQP may facilitate rapid water movement during kidney passage and thus protect the parasite's membrane integrity. It may further provide access to the serum glycerol as a precursor for lipid biosynthesis during the parasite development in the intraerythrocytic stage.

Channels and transporters play a central role in parasite survival within the host organism. Hence, they are interesting as potential drug targets or may be used as uptake routes of cytotoxic drugs into the parasite cell.

The physiological function of PfAQP is unknown. One aim of this work is to characterise its permeability properties which may give hints on its physiological function.

1.2.1 GAPDH enzyme, possible roles in the cell

Glucose is the sole energy source of malaria parasites. Ten catalytic steps convert glucose to pyruvate to produce 2 mols ATP per mol of glucose. Conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate in the presence of NAD^+ and inorganic phosphate is catalyzed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The GAPDH enzyme is a homotetramer with a molecular mass of approximately 150 kDa (Fig. 1.5). Each subunit is composed of an N-terminal NAD^+ binding domain and a C-terminal catalytic domain (Cown-Jacob et al., 2003). The NAD^+ binding domain is conserved among various dehydrogenases (Rossman fold), but the structural changes in the adenosine binding pocket of *Leishmania mexicana* GAPDH can be used as a specific drug target (Bressi et al., 2001; Suresh et al., 2001).

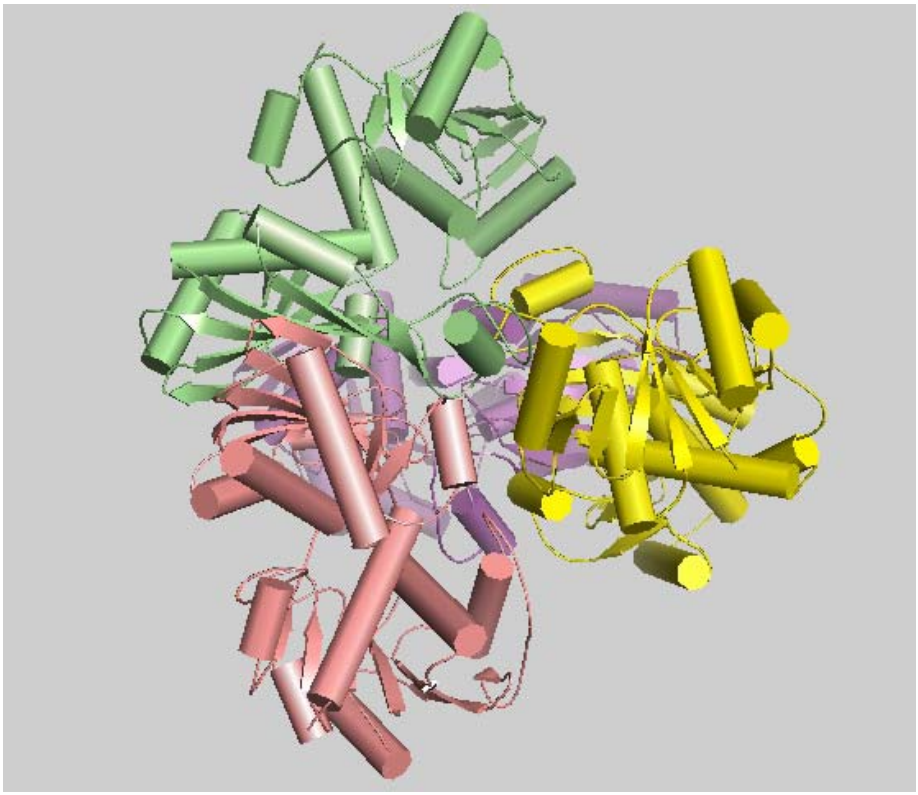

Fig. 1.5. Structure of the GAPDH homotramer. (This was prepared using PyMOL). GAPDH is a tetramer composed of four identical polypeptide chains which are coloured in pink, violet, green and yellow.
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Besides numerous other GAPDH structures from various species, the *P. falciparum* GAPDH structure has recently been resolved (Satchell et al., 2005).

Historically, mammalian GAPDH was considered as an enzyme involved in glycolysis with its main role in energy metabolism. Expression levels of the GAPDH remain constant under most experimental conditions and it has been frequently used as an internal control for studying the regulation of expression of other genes.

Independent from the traditional role in glycolysis, GAPDH is a protein with multiple intracellular localisations and diverse activities (Sirover et al., 1999). These new activities include regulation of the cytoskeleton, membrane fusion and function, export of nuclear RNA, DNA reparation and, particularly intriguing, apoptosis. It is not clear whether PfGAPDH has additional functions in parasites besides in glycolysis.

1.3 *Toxoplasma gondii*

Toxoplasmosis is an infectious disease caused by the protozoan *T. gondii* which is, as *Plasmodium*, a member of the protozoan phylum *Apicomplexa*. Infections by *T. gondii* infect humans and animals world wide**.** Under certain conditions, toxoplasmosis can cause serious pathology; especially in individuals whose immune systems are compromised, e.g., AIDS patients.

While *Plasmodium* parasites multiply exclusively in the erythrocytes and liver cells, *Toxoplasma gondii* invades various cell types. The *T. gondii* genome is sequenced (www.tigr.org).

1.4 *Trypanosoma brucei*

Trypanosomatids like *Apicomplexan* parasites are frequent topics of research for two reasons: their pathogenicity and divergent biology. Among others, tripanomatids include African Trypanosomes (*T. brucei*), American trypanosomes (*T. cruzi*), and *Leishmania* spp. which cause chronic, often fatal diseases in humans.

The life cycle of *T. brucei* parasites includes two different stages: i.e., the long slender form in the blood, lymphatic fluids and in the cerebrospinal fluid of its mammalian host and the procyclic form in the mid gut of the tsetse fly.

In the mammalian bloodstream *T. brucei* has neither a functional Krebs cycle nor oxidative phosphorylation nor does it store any carbohydrates. Consequently, the energy metabolism of

T. brucei depends totally on glycolysis with glycerol as an alternative substrate (Bakker et al., 1997). It was reported that due to the differences between mammalian and trypanosomal glycolysis this pathway is a potential drug target for drug design against African sleeping sickness (Michels et al., 1998). The first seven glycolytic reactions of glycolysis in trypanosomes are placed in a peroxisome like organelle, the so-called glycosome (Parsons et al., 2004). The presence of a glycosome represents a connection to the plant kingdom similar to the apicoplast in *Apicomplexan* parasites. Accordingly, several plant-like proteins are present in the glycosome.

Under aerobic conditions, in the glycosome glucose is converted to 3-phosphoglycerate which is metabolized into pyruvate in the cytosol. The NADH produced in the glycosome is used to reduce dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (Gly-3-P). Further, Gly-3-P is reoxydised by molecular oxygen via glycerol-3-phosphate oxidase in the mitochondria and DHAP is returned into the glycosome. Under anaerobic conditions, equimolar amounts of pyruvate and glycerol are produced. Glycerol has to be rapidly exported out of the glycosome otherwise it has a toxic effect on the parasite. Suppression of glycolysis by inhibiting the release of glycerol or pyruvate is a potential therapeutic approach against sleeping sickness (Bakker et al.,1997).

The aim of this work was to characterise protozoan aquaporins (*P. falciparum, T. gondii* and *T. brucei*) in order to obtain information about the water and solute permeability profile, the possible pore size, the similarities and differences between these and other aquaporins. From such functional properties physiological functions may be derived.

2 Materials

2.1 Enzymes, kits and chemicals

Ambion, Austin (USA): mMessage mMachine Transcription Kit

Amersham Pharmacia Biotech, Freiburg: ECL Plus Western Blot Detection System, Hyperfilm ECL, Thermosequenase Fluorescent Labelled Primer Cycle Sequencing Kit with 7-deaza-dGTP

AppliChem, Darmstadt: HEPES, Acrylamide 4K-Solution 30 %

Appligene, Heidelberg: Taq DNA polymerase with 10× reaction buffer

Biogenes, Berlin: C-terminal hexadekapeptid (CPLVDLANNEKDGVDL) from PfAQP, rabbit anti-serum against this peptide

BIO-RAD, München: BIO-RAD Protein-Assay Dye Reagent Concentrate

Celllabs, Brookvale (Australia): Malaria Ag CELISA

Dianova, Hamburg: Goat anti-mouse antibodies horseradish peroxidase conjugated, goat anti-rabbit antibodies horseradish peroxidase conjugated

Macherey-Nagel, Düren: Nucleotrap

Merck, Darstadt: Imidazole, glycine, sodium acetate, glucose, sodium hydrogenphosphate, sodium chloride.

Millipore, Molsheim (France): QTUOO EX, QUANRUM EX

MWG-Biotech, Ebersberg: Oligonucleotides

New England Biolabs, Schwalbach/Taunus: Restriction endonuclease, BSA, T4-Polynucleotide kinase, 10× Kinase buffer.

Peqlab, Erlangen: Agarose, peqGOLD Protein Marker

Promega, Madison: Wizard MiniPreps Plasmid Purification Kit

Qiagen, Hilden: Ni^{2+}-NTA-Agarose, pQE Expression vector, purified mouse monoclonal RGS-His antibody (BSA free), QIAamp DNA Blood Mini kit

Roche (Boeringer), Mannheim: Restriction endonucleases, Klenow-polymerase, alkaline phosphatase, Rapid DNA Ligation Kit, dNTP´s, Complete Protease Inhibitor Cocktail Tablets

Sartorius, Göttingen: Cellulose acetate filter with pore size 0.2 µm

SERVA Electrophoresis, Heilderberg: Coomassie-Brilliant-blue G250, VISKING dialysis Tubing 8/32 and 27/32 with MW 12000-14000

Sigma, Deisenhofen: Glycerol, TRIS, EDTA, Xgal, IPTG, Ponceau S, Tween 20, TEMED, BSA, dihydroxyacetone, methylglyoxal, RPMI 1640 medium, Giemsa solution, sorbitol

Stratagene, Heidelberg: Plasmid pBluescript II SK (-), *E. Coli* XL1- Blue MRF^{-}

2.2 Equipment and materials

Amersham Pharmacia Biotech, Freiburg: Electrophoresis power-supply EPS 3500

Bender and Hobein, Ulm: Vortex Genie 2

Biometra, Göttingen: TRIO-Thermoblock thermocycler

BIO-RAD, München: Trans-Blot SD Semi Dry Transfer Cell

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Branson, Danbury (USA): Sonifier B-12

Eberhard-Karls-Universität, Tübingen: Chambers for gel electrophoresis

Eppendorf, Hamburg: Table centrifuges 5410 & 5414, cooling centrifuge 5402, BioPhotometer

Hybaid, London (GB): Mini Oven MK II

Kähler, Hamburg: *Xenopus laevis* females

Knauer, Berlin: Semi-Micro-Osmometer

Kodak, New Haven (USA): Biomax MR X-ray films

Kontron-Hermle, Gosheim: Centrikon h401 & ZK 401, Rotor SS34

Leica, Germany: Leica MZ6 microscope

Macherey-Nagel, Düren: Porablot PDVF-Blotting membrane (0.2 µm pore size)

Millipore, Eschborn: Water purification system MilliQ UF Plus

MWG-Biotech, Ebersberg: LI-COR DNA sequencer model 4000

Sartorius, Göttingen: Balance BP 2100 S, analytic balance handy

Savant, Farmingdale (USA): Vacuum centrifuge speed vac concentrator SVC100H

Schleicher & Schuell, Dassel: Whatmanpaper 3 MM, Protran BA83 Cellulosenitrate 0.2 µm Blottingmembrane

Shimadzu, Kyoto (Japan): UV-VIS spectrophotometer UV-1202

Stratagene, Austin (USA): UV Stratalinker 2400

WPI, Sarasota (USA): Nanoliter-Injector, binocular microscope

2.3 Buffers and solutions

2.3.1 Molecular biology

2.3.1.1 DNA electrophoresis

TAE-buffer
40 mM TRIS-acetate, pH 8.0
1 mM Na_2EDTA

TBE-buffer
100 mM TRIS
80 mM Boric acid
25 mM Na_2EDTA

BX-sample buffer
0.5 % Bromphenolblue
0.5 % Xylencyanol
5 % Glycerol

2.3.1.2 Reaction buffers

Klenow buffer (10×)
200 mM TRIS-HCl, pH 7.9
60 mM $MgCl_2$
10 mM DTT
1 mg/ml BSA

Dephosphrylation buffer
500 mM TRIS-HCl, pH 8.5
1 mM EDTA

2.3.1.3 Media for *E. coli* cultures

LB medium
10 g/l Bacto trypton
10 g/l NaCl
5 g/l Yeast extract

LB medium with antibiotics
100 µg/ml ampicillin and/or
50 µg/ml kanamycin

LB-Agar plates
15 g/l agar in LB medium

LB-Agar plates with ampicillin
100 µg ampicillin/ml LB medium
LB medium with agar

LB-Agar plates with kanamycin
50 µg kanamycin/ml LB medium
LB medium with agar

2.3.1.4 Transformation of *E. coli*

CM-buffer
100 mM $CaCl_2$
100 mM $MgCl_2$
Solution was sterilised by sterile filtration (pore size 0.2 µm)

2.3.1.5 DNA precipitation and purification

3 M Na-acetate buffer (stored at room temperature)
98 % EtOH (stored at -25°C)
70 % EtOH (stored at -25°C)

2.3.2 Protein chemistry

2.3.2.1 Protein purification

Lysis buffer
50 mM TRIS-HCl, pH 8.0
10 mM DTT

Washing buffer
50 mM TRIS-HCl, pH 8.0
300 mM NaCl
10 mM Imidazole

30 mM Imidazole
10 mM DTT

Elution buffer
50 mM TRIS-HCl, pH 8.0
300 mM NaCl
150 mM Imidazole
10 mM DTT
1 mM NAD^+

Hypotonic phosphate buffer
7.5 mM Na_2HPO_4 pH7. 5
with protease inhibitors cocktail

2.3.2.2 SDS-Polyacrylamide gel electrophoresis

Running gel buffer
1.5 M TRIS-HCl, pH 8.8
0.4% SDS

Stacking gel buffer
0.5 M TRIS-HCl, pH=6.8
0.4% SDS

Electrophoresis buffer
25 mM TRIS-HCl, pH 8.3
192 mM glycin
0.1% SDS

Protein sample buffer (4×)
130 mM TRIS-HCl, pH 6.8
10% SDS
10% β-mercaptoethanol
20% glycerol
0.06% bromphenolblue
2% LiDS

Coomassie staining solution
0.2% Coomassie Brilliant-Blue G 250
10% acetic acid
50% methanol

Destaining solution
10 % acetic acid
30 % EtOH

2.3.2.3 Western blot

Towbin buffer

25 mM	TRIS-HCl, pH 7.5
192 mM	Glycin
29 %	Methanol
0.0375 %	SDS

TBS-buffer

50 mM	TRIS-HCl
150 mM	NaCl

M-TBS

5%	milk powder in TBS buffer

TBS-T

0.1 %	Tween 20 in TBS

2.3.2.4 Medium for *Xenopus* oocytes

ND96

96 mM	NaCl
2 mM	KCl
1.8 mM	$CaCl_2$
1 mM	$MgCl_2$
5 mM	HEPES, pH 7.4

OR-2

82.5 mM	NaCl
2 mM	KCl
1 mM	$MgCl_2$
5 mM	HEPES, pH = 7.4

These solutions were sterilised by autoclaving for more than 20 min at 121°C and 1 bar.

2.3.3 Media for *Plasmodium falciparum* cultures

All solutions for parasite cultures were made under sterile conditions, and sterilised by autoclaving or sterile filtration (filter pore size 0.2 µm)

2.3.3.1 Complete RPMI 1640 medium with glucose

RPMI 1640 medium (SIGMA 500 ml)
20 µg/ml gentamicin sulphate
25 mM HEPES
2 mM L-glutamine
10 ml human serum
0.1 µM hypoxanthine
50 ml albumax (Albumax II, Gibco)
store at 4°C

2.3.3.2 Complete RPMI 1640 medium without glucose

RPMI 1640 medium (Gibco 500 ml) without glucose:
20 µg/ml gentamicin sulfate
25 mM HEPES
2 mM L-glutamine
10 ml human serum
0.1 µM hypoxanthine
50 ml albumax II (without glucose)
store at 4°C

2.3.3.3 Albumax II (10× concentrate)

RPMI 1640 medium (with or without glucose)
20 µg/ml gentamicin sulfate
25 mM HEPES
2 mM L-glutamine
10 ml human serum
0.1 µM hypoxanthine
25 g albumax II
pH 7.0-7.4
store at -25°C

2.3.3.4 Solution for parasite synchronization

5%	sorbitol

2.3.3.5 Giemsa staining of thin blood films

98%	methanol
6.7 mM	phosphate buffer pH 7.1
	Giemsa stain

2.3.4 Buffers and solutions for enzyme assays

2.3.4.1 GAPDH enzyme assay

50 mM	TRIS-HCl buffer, pH 8.0
300 mM	NaCl
1 mM	NAD^+
50 mM	Na_2HPO_4
10 mM	DTT
5 µg/ml	GAPDH

2.3.4.2 Assay for ammonia determination

122 mM	TEA buffer, pH 8.0
0.56 mM	ADP in TEA buffer
6 mM	NADPH in TEA buffer
71 mM	α-ketoglutarate in TEA buffer
7.4 U/ml	enzyme in TEA buffer

2.4 Primer list

primers name	sequence	position	restriction place
PfAQPs	5'-actagatctcaatgcatatgtta-3'	-11-12	Bgl II
PfAQPF190Yas	5'ctggatcctaaatctcttgatgggttaagtg catatcca -3'	564 -602	BamH I
PfGAPDHs	5'- tagcatgcatggcagtaacaaaac ttgg -3'	1-20	Sph I
PfGAPDHEcoRVs	5'-aagatatcgaagtagttgcta ttaacgac-3'	65-86	EcoR V
PfGAPDHas	5'-ggaagcttagttgttagtaat gtgtac-3'	994-1014	HinD III
Tg1AQPs1	5'-tagaattcgagtgagactgcttctatg-3'	-23-3	EcoR I
Tg1AQPas	5'-ttaagcttccgtgccgccgttca ctctt-3'	1-28	HinD III
Tg1AQPs2	5'-tagaattccgagcatctcatcttcaat-3'	96 -111	EcoR I

3 Methods

3.1 Molecular biology methods

3.1.1 Polymerase chain reaction (PCR)

PCR was used to amplify fragments from plasmid or genomic DNA and for introduction of endonuclease restriction sites or point mutations. The DNA template (1-10 ng) was incubated with DNA polymerase (1-2 U), dNTPs (200 µM), and two oligonucleotide primers (each 200 nM), whose sequences flank the DNA sequence of interest in a total reaction volume of 50 µl. The annealing temperature was calculated as follows:

$$T_m = 60 + 0.41 * \%GC - 600 / n \text{ [for primers } \geq 25 \text{ bp]}$$

%GC represents percent of the GC content of the primer and n is the number of the bases of the primer

$$T_m = 2 * (AT) + 4 * (GC) - 4 \text{ [for primer } \leq 24 \text{ bp]}$$

AT and GC represent the number of A + T and G + C bases, respectively, in the primer sequence.

If the annealing temperature for the two primers of a PCR reaction was different, the lower annealing temperature was used.

The reaction was done in a thermocycler with heatable lids to avoid volume and concentration changes. The temperature program used for the PCR reaction is shown in Table 3.1.

Denaturation		95°C	5 min
20-40 cycles	Denaturation	95°C	50 s
	Annealing	Tm	50 s
	Extension	72°C	50 s
Fill up		72°C	10 min

Tabele 3.1 Program used for polymerase chain reaction

3.1.2 DNA agarose gel electrophoresis

DNA agarose gel electrophoresis was used to separate and isolate DNA fragments or to analyse the size of DNA fragments.

0.5-1 g of agarose was added to 50 ml of TEA buffer and melted in a microwave oven. DNA samples were mixed with BX buffer (1:10), loaded on the agarose gel (1-2%, TEA buffer) and electrophoresis was performed in TEA buffer at 80-100 V for 1 h at room temperature. The size of the DNA fragments was determined using λ-Marker (EcoR I/HinD III digested λ-phage DNA) and π-Marker (Msp I/Ssp I digested pBlueskript II SK(-)-vector). DNA fragment sizes of λ-Marker are 21226, 5184, 4973, 4277, 3530, 2027, 1904, 1584, 1330, 983, 831, 564, 125 bp and π-Marker DNA fragments are: 489, 404, 312, 270, 242, 215, 190, 157, 147, 110, 67, 57, 34, 26 bp. Detection of DNA was done by adding ethidium bromide (10 µg/100 ml) to

the gel solution. After electrophoresis, DNA was visualised in UV light (302 nm) and photographed.

3.1.3 Plasmid isolation

Plasmid isolation was done from 3-4 ml of bacterial cell (12-16 h at 37°C) using Wizard-Mini preps kits.

3.1.4 Photometric determination of DNA concentration

The absorption of nucleic acid solution was measured at 260 nm. The nucleic acid concentration was calculated using the following equation: c [µg/µl] = E260 * f * (dilution), f = 0.02 (for oligonucleotides, ss-DNA, RNA), f = 0.04 (for ds-DNA, plasmids).

3.1.5 DNA digestion with restriction enzymes

Double stranded DNA molecules were digested with 1-5 U of the restriction enzyme per µg of DNA at the optimal temperature (usually 37°C) in the buffer recommended by the supplier. If digestion was done with two or more enzymes, the most compatible buffer was used. In case that it was not possible to do double digestion, enzymes were used one by one with purification steps in-between. Purification steps were done with the Nucleotrap-Kit.

3.1.6 DNA extraction from agarose gels

DNA bands were cut from the agarose gel with a scalpel. Afterwards DNA was extracted with the Nucleotrap-Kit (Marcherey-Nagel) following the manufacturer's instructions.

3.1.7 Generation of DNA blunt ends

After PCR or restriction digestion, the Klenow fragment of DNA-polymerase I was used to make DNA ends blunt. For one Klenow reaction 7.2 µl DNA (1-2 µg), 0.8 µl Klenow enzyme [1U/µl], 1 µl 10× Klenow buffer and 1 µl dNTPs [1 mM] was used and incubated for 45 min at 37°C. After this reaction DNA was purified using the Nucleotrap-Kit.

3.1.8 5'-DNA-phosphorylation

Treatment of DNA with the Klenow fragment or PCR results in strands without 5'-phosphate groups. For ligation these fragments need to be phosphorylated. One reaction contains 1-2 µg DNA (or all Klenow reaction), 1 mM ATP, 7 U T4-Polynucleotid kinase and 10× kinase buffer. The reaction was incubated at 37°C for 45 min.

3.1.9 5'-DNA-dephosphorylation

To avoid recirculation of blunt-ended plasmid DNA after EcoR V digestion, the 5´-phosphates were removed with calf alkaline phosphatase. 1-2 µg DNA were incubated with 0.2 U alkaline phosphatase in phosphatase buffer at 37°C for 45 min.

3.1.10 Ligation of DNA fragments

DNA fragments were ligated with DNA ligase, using the Rapid DNA Ligation Kit according to the supplier's instructions. The optimal vector/insert ratio was assumed to be 1:3.

3.1.11 Purification of DNA by precipitation with ethanol

To precipitate DNA from solution 1/10 of the total volume 3 M Na-acetate (pH 4.5) and 2 volumes of 98.9% ice-cold ethanol were added and stored at -20°C for 1 h. Afterwards the sample was centrifuged for 15 min at 12000 × g and 4°C. The supernatant was discarded and the pellet was washed with 750 µl of 70% ethanol. After centrifugation for 10 min at 12000 × g and 4°C the supernatant was again discarded and the pellet was dried in a vacuum centrifuge for 5 min.

3.1.12 DNA Sequencing

DNA sequencing was done using the Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham) with 7-deaza-dGTP. The DNA template was cycled together with a 5´-fluorescent sequencing primer. For one sequencing reaction 1 µg plasmid DNA, 2 pmol of primer and 0.7 µl 3% DMSO were used (13 µl total volume). 3 µl of this DNA-primer were added to 2 µl of each G/A/T/C reaction mixture. Finally, one drop of low melting wax was added to each sample. The sequence reaction was done in a PCR block using the following temperature protocol (Table 3.2):

Denaturation		95°C	5 min
30 cycles	Denaturation	95°C	20 s
	Annealing	56°C	20 s
	Extension	70°C	20 s

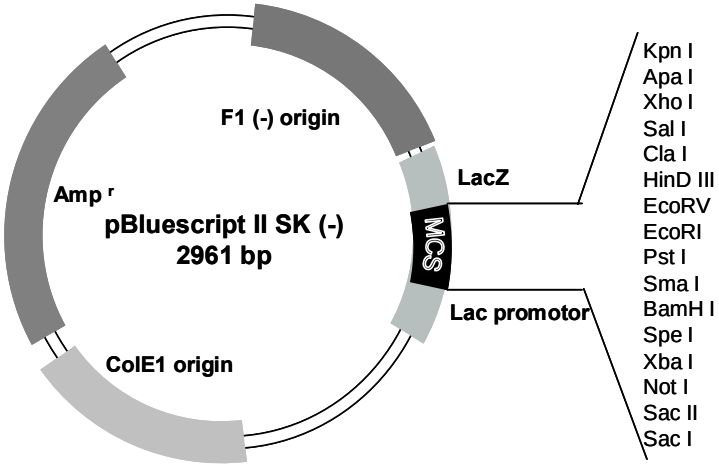
Table 3.2 Temperature program used for DNA sequencing

After adding 5 µl of formamide loading dye provided with the kit the samples were loaded on a 6% polyacrylamide gel. DNA electrophoresis and detection was done with a LI-COR DNA Sequencer model 4000 with TBE as a running buffer. BaselmagelIR V. 4.0 software (MWG-Biotech) was used for analysis.

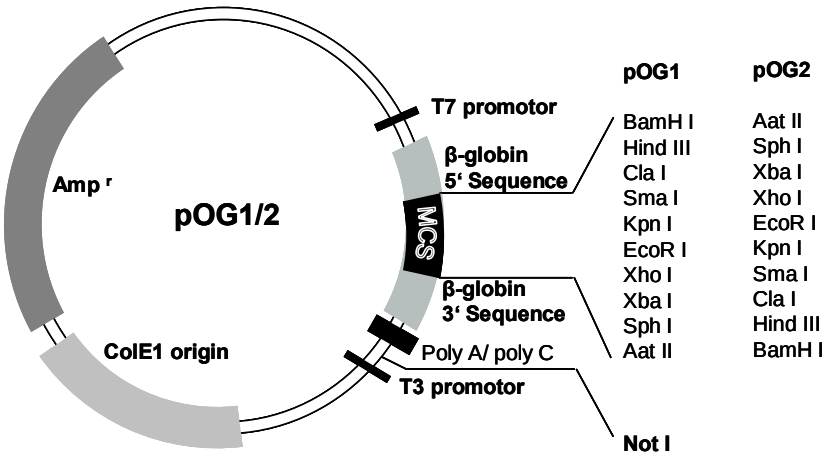
3.2 Cloning strategies

3.2.1 Vectors used

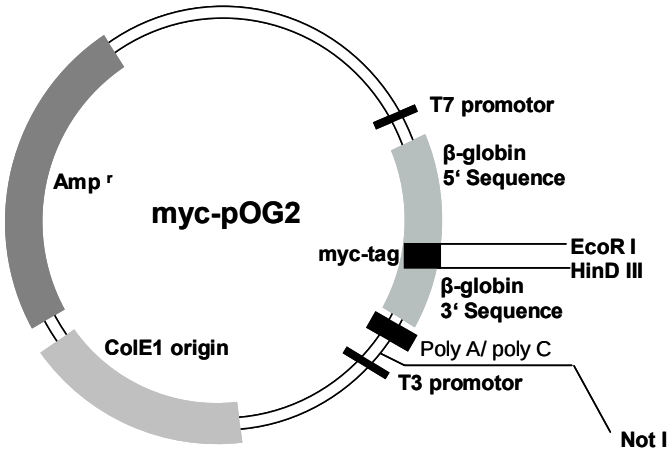
3.2.1.1 pBluescriptII SK (-) vector



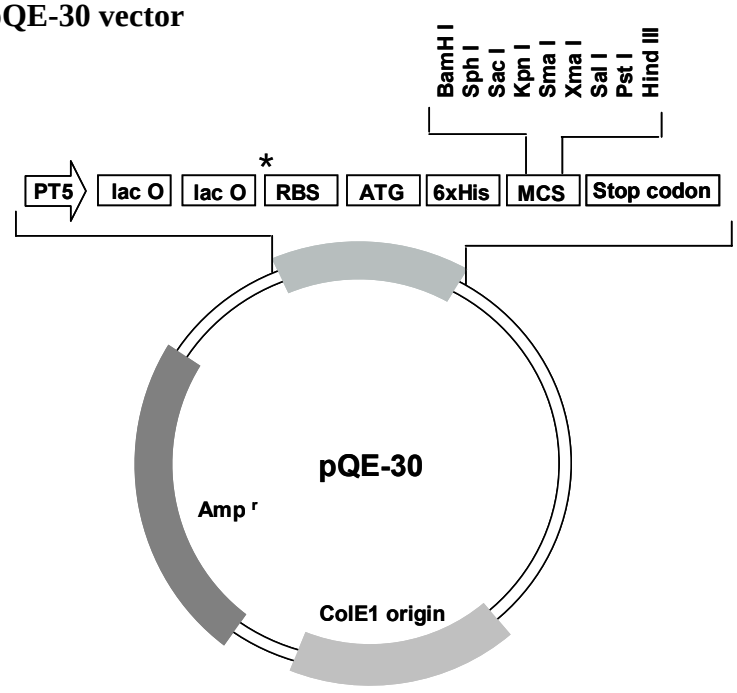
3.2.1.2 pOG1/2 vectors

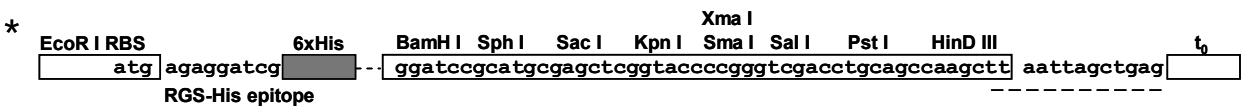


3.2.1.3 myc-pOG2 vectors



3.2.1.4 pQE-30 vector





3.2.2 Cloning strategy of the PfAQP $E_{125}S/F_{190}Y$ mutant

The PfAQP $E_{125}S$ mutant in the pOG1 vector was used as a template for a PCR reaction, with primer PfAQP $F_{190}Y$as to introduce a $F_{190}Y$ mutation. This resulted in a PfAQP $E_{125}S/F_{190}Y$ mutation. The length of the PCR product was 624 bp. After the purification by agarose gel electrophoresis, the PCR product was purified from the agarose gel, treated with Klenow enzyme and 5' phosphorylated. The purified blunt end PCR product was cloned into pBluescript II (SK-) via the EcoR V site (Fig. 3.1).

Via EcoR I/BamH I restriction sites the PCR product was ligated into the pOG1 vector containing PfAQP mutant (PfAQP $M_{24}I$). By this procedure, the $M_{24}I$ mutant was replaced by the double mutant PfAQP $E_{125}S/F_{190}Y$.

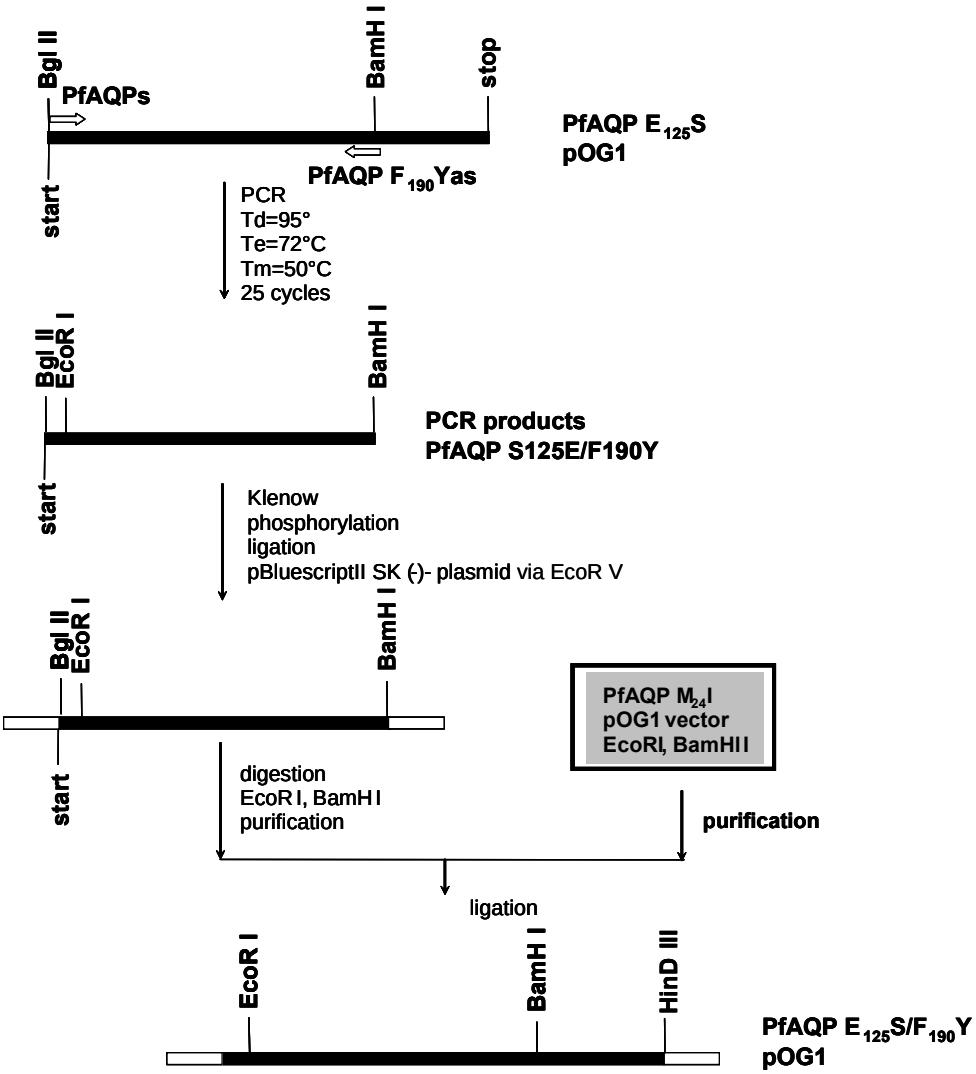


Fig. 3.1. Schematic presentation of cloning strategy of PfAQP $E_{125}S/F_{190}Y$ mutant

3.2.3 Cloning of the PfGAPDH open reading frame into the pQE-30 expression vector

To amplify the PfGAPDH ORF, *P. falciparum* (strain Binh1) genomic DNA was used (Fig. 3.2). The PfGAPDH ORF contains an intron. To avoid the intron two PCRs were carried out. The first product contained the full length ORF including the intron and the

one the part downstream of the intron. Both PCR products were cloned into pBluescript II SK(-). Afterwards, the PCR product containing the full ORF was cloned into pQE-30 via Sph I/HinD III and was digested afterwards with EcoR V and Hind III restriction enzymes. By this digestion only the first 86 bp PfGAPDH remained in the pQE-30 vector. Finally, the second PCR product (after the intron) was equally digested with EcoR V and Hind III restriction enzymes and ligated into the pQE-30 vector with the first 86 bp of PfGAPDH to complete the full length open reading frame.

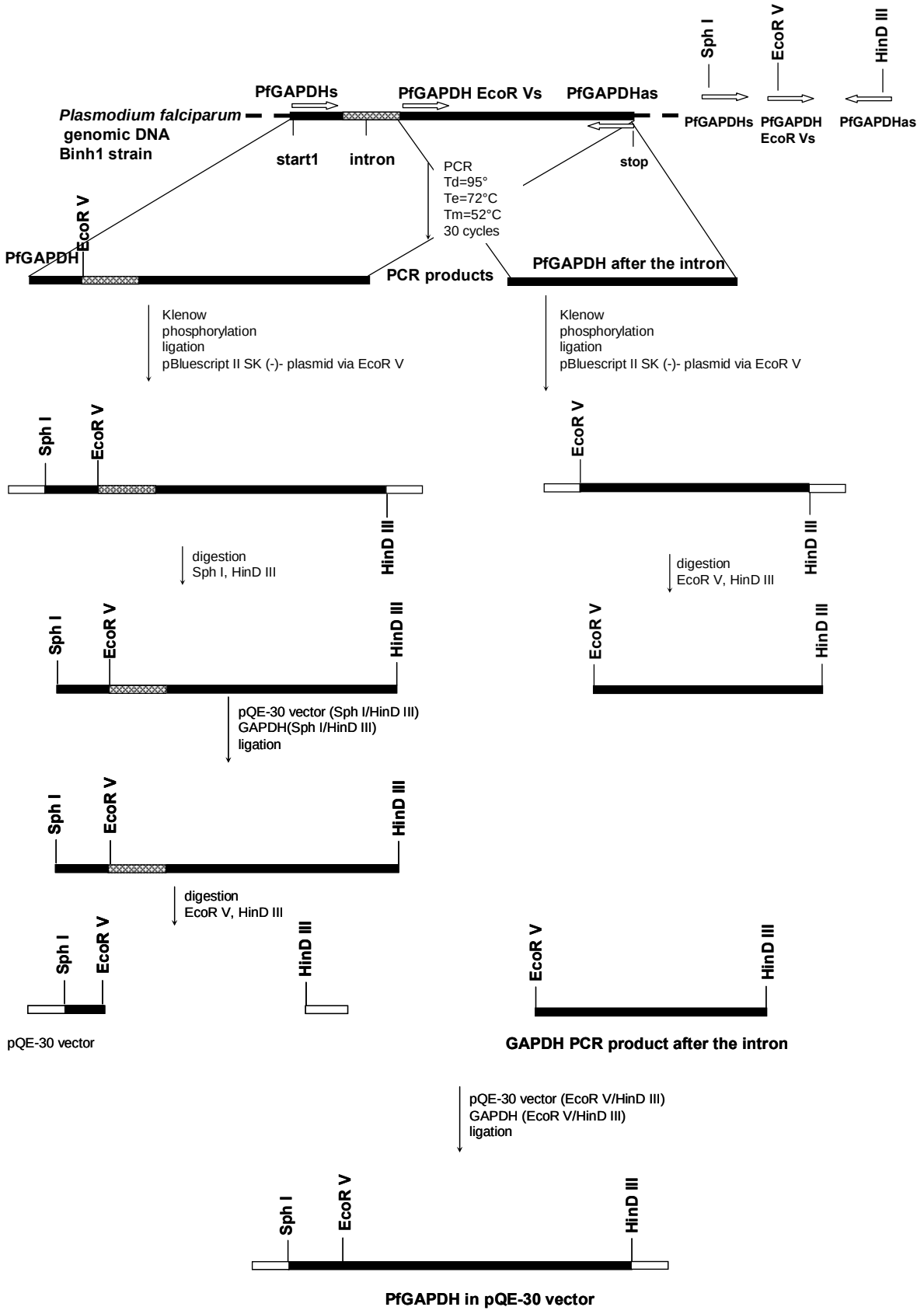


Fig. 3.2. Schematic presentation of cloning strategy of PfGAPDH

3.2.4 Cloning strategy of the *Toxoplasma gondii* aquaporin

The TgAQP open reading frame has two potential start methionines at positions M1 and M39. *T. gondii* genomic DNA was used for TgAQP M1 and TgAQP M39 PCR amplification. Condition for the PCR for both TgAQP M1 and TgAQP M39 were identical (Fig.3.3). The length of the PCR products was 821 bp and 733 bp, respectively. After electrophoresis, the separated fragments were purified, treated with Klenow fragment and finally 5' phosphorylated. The blunt ended fragments were then cloned in pBluescript II SK(-) vector via the EcoR V site. After this first cloning step, cloning into pOG2 vector was done. The pOG2 and myc-pOG2 vectors and TgAQP DNAs were digested with EcoR I/HinD III restriction enzymes and ligated.

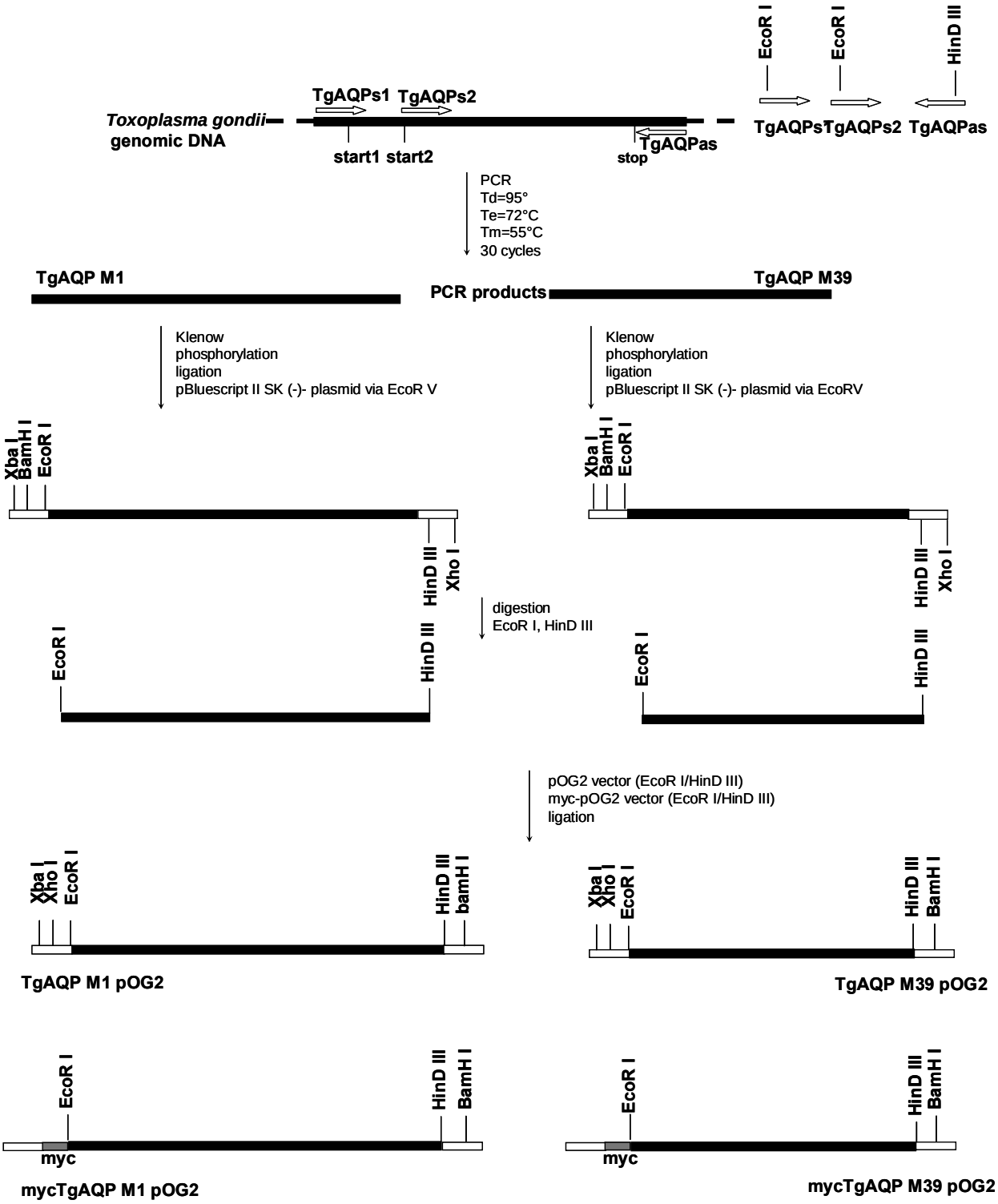


Fig. 3.3. Schematic presentation of cloning strategy of TgAQP M1 and TgAQP M39 open reading frames in pOG2 vector.

3.3 Microbiological methods

3.3.1 Competent cells

5 ml of LB medium were inoculated with a single bacterial colony (XL1-blue MRF˙or BL21 (DE3) [pREP4]) and incubated over night at 37°C at 200 rpm. 1 ml of the culture was transferred into 50 ml fresh LB medium and incubated at 37°C (200 rpm) until an OD_{600} of 0.3-0.4 (approximately 2-3h) was reached. The culture was cooled on ice for 10 min and centrifuged at 2500 × g for 20 min at 4°C. The pellet was re-suspended in 10 ml ice cold $CaCl_2$ (0.1 M), incubated on ice for 20 min and centrifuged once more (2500 × g, 20 min, 4°C), the pellet was re-suspended in ice cold 0.1 M $CaCl_2$ supplemented with 20% glycerol and incubated on ice for 2 h. 100 µl aliquots were stored at -80°C.

3.3.2 Transformation of competent *E. coli* cells

The DNA ligation reaction (20 µl) was diluted with 10 µl 10 × CM buffer (100 mM $CaCl_2$, 100 mM $MgCl_2$) and 70 µl sterile water. This was added to 100 µl competent cells, mixed gently and then incubated on ice for 25 min. Cells were heat shocked at 42°C for 50 sec. Afterwards, cells were incubated on ice for 10 min and 500 µl of LB medium were added. This mixture was incubated for 45 min at 37°C (200 rpm). 200 µl of the mixture was spread on LB agar plates with appropriate antibiotics. Plates were incubated in an inverted position for 12-16 h at 37°C.

The transformation with the pBluescript II SK (-) vector could be monitored by a blue-white screen by spreading 40 µl of IPTG (0.1 M) and 40 µl of X-Gal (2 %) on the LB agar plate prior to cell plating. Bacteria carrying pBluescript with recombinant DNA formed white colonies, those carrying a plasmid without an insert formed blue colonies.

3.4 Protein expression in *Xenopus laevis* oocytes

3.4.1 Oocyte preparation

A *X. laevis* female frog was anesthetised in 500 ml Tricain solution (1g/l) and afterwards the oocytes were isolated by making a 1 cm long incision on the left or right ventral side. A part of the ovarian sack was removed and oocytes were placed in ND96 medium. Oocytes were washed in OR-2 medium and incubated in collagenase solution (0.5 mg/ml in OR-2) for 20-30 min (200 rpm). The oocytes were washed in ND96 medium and selected under the microscope (stage V and VI).

3.4.2 cRNA synthesis

The *in vitro* synthesis of cRNA was done with T7 RNA-polymerase using the mMESSAGEmMACHINE-Kit (Ambion). 1-5 µg of Not I linearised pOG1/2 plasmid in a 20

µl reaction mixture was incubated for 2 h at 37°C. After digestion of DNA with 1 µl, 2 U/µl, DNAse I for 15 min at 37°C cRNA was precipitated by addition of 25 µl 7.5 M LiCl for 60 min at -20°C. The precipitate was centrifuged, washed with 70% ethanol, dried in a vacuum centrifuge (5 min) and dissolved in 25 µl RNAse free water. The concentration of synthesised RNA was measured photometrically and diluted to a final concentration of 100 ng/µl.

3.4.3 Oocyte injection

Oocyte injection was performed under the microscope with a nano-liter injector. The oocytes were injected with 50 nl of distilled water or 50 nl of cRNA solution (100 ng/µl). Injected oocytes were incubated at 15°C for 3-4 days.

3.4.4 Standard oocyte swelling assay

To measure water permeability, oocytes were abruptly placed in 1:3 times diluted ND96 medium. To measure solute permeability 65 mM NaCl in ND96 was exchanged with 130 mM a non-ionic compound (e.g. glycerol, dihydroxyacetone, methylglyoxal). The isosmolality of ND96 medium was checked with a osmometer (Knauer) (± 10 mosm/kg). Oocyte swelling assays were performed under the microscope at room temperature and monitored with a camera connected to a computer, where all calculations were done. From the change in oocyte surface the change in relative volume (V/V_0) was calculated. Using the following formula, water P_f [µm s^{-1}] (1) and solute P_s [µm s^{-1}] (2) permeability are calculated.

$$P_f = V_0 \cdot d(V/V_0)/dt \,/\, [S \cdot V_w \cdot (osm_{in} - osm_{out})] \qquad (1)$$

$V_0 = 9 \cdot 10^{-4}$ cm^3 (initial oocyte volume), $d(V/V_0)/dt$ (relative volume increase in s^{-1}), S = 0.045 cm^2 (oocyte surface area), V_w molar water volume (18 cm^3 mol^{-1}) and osmotic gradient ($osm_{in} - osm_{out}$)

$$P_s = [osm_{total} \cdot V_0 \cdot d(V/V_0)/dt] \,/\, [S \cdot (sol_{out} - sol_{in})] \quad (2)$$

osm_{total} = 300 mOsm (total osmolarity of the system), $V_0 = 9 \cdot 10^{-4}$ cm^3 (initial oocyte volume), $d(V/V_0)/dt$ (relative volume increase in s^{-1}), S = 0.045 cm^2 (oocyte surface area), and ($sol_{out} - sol_{in}$) = 130 mOsm (osmotic solute gradient).

3.5 Protein chemistry methods

3.5.1 Protein expression in *Xenopus laevis* oocytes

P. falciparum aquaporin (PfAQP), *T. gondii* (TgAQP M1, TgAQP M39) aquaporins, *T. brucei* aquaporins (TbAQP1, TbAQP2, TbAQP3), rat AQP3 and rat AQP1 were expressed in *X. laevis* oocytes after injection of 5 ng of cRNA and incubation at 16°C for three days.

3.5.2 Membrane protein preparation from *Xenopus laevis* oocytes

For the preparation of membrane proteins 5 oocytes were lysed in 500 µl hypotonic phosphate solution and homogenised by pipeting up and down on ice. The homogenate was centrifuged for 5 min at 500 × g at 4°C. Afterwards the supernatant was collected. Further, the supernatant was centrifuged for 30 min on 17000 × g at 4°C, the yolk on the top of the supernatant was soaked away with paper and the rest of supernatant was removed by pipeting. The pellet was resuspended in protein sample buffer and solubilised at 37°C for 1 h and stored at -20°C for later use in Western blots.

3.5.3 *Plasmodium falciparum* glyceraldehyde-3-phosphate dehydrogenase (PfGAPDH) expression in *E. coli*

For expression of PfGAPDH a pre-culture was made with *E.coli* BL21 [rep4] cells containing the plasmid with the PfGAPDH gene. Therefore, 5 ml of LB medium supplemented with 50 µg/ml kanamycin and 100 µg/ml ampicillin were inoculated with *E. coli* BL21 [rep4] cells and incubated over night at 37°C (210 rpm). The overnight culture was inoculated into 200 ml of LB medium supplemented with 50 µg/ml kanamycin and 100 µg/ml ampicillin. Protein expression in *E.coli* BL21 [rep4] cells was induced at an OD_{600} of 0.6 with 1 mM isopropyl-β-D-thiogalactoside (IPTG). After 14 h of incubation at 37°C the cells were harvested at 3000 x g for 10 min. Cell pellets were then stored at -80°C.

3.5.4 Purification of PfGAPDH

The cell pellet from 1 l culture was resuspended in lysis buffer (300 mM NaCl, 50 mM Tris-HCl, pH 8.0) and lysed using a French press. The particulate fraction was removed at 27,000 x g and the soluble, recombinant PfGAPDH protein was bound to 300 µl Ni-NTA agarose (Qiagen) for two hours on ice. After washing with 2 ml 50 mM Tris-HCl buffer pH 8 supplemented with 300 mM NaCl, 10 mM DTT and 1 mM NAD^+, then with the same buffer (2 ml) supplemented with 10 mM and 30 mM imidazole, respectively, the PfGAPDH protein was eluted in 2 ml (300 mM NaCl, 150 mM imidazole, 10 mM DTT, 1 mM NAD^+, 50 mM Tris-HCl, pH 8.0) and dialyzed against assay buffer (300 mM NaCl, 1 mM NAD^+, 50 mM Na_2HPO_4, 10 mM DTT, 50 mM Tris-HCl pH 8.0). Purified PfGAPDH protein was stored at room temperature at a concentration of 3 mg/l.

3.5.5 Determination of protein concentration using the Bradford method

1-10 µg of protein were pipetted to 800 µl distilled water and mixed. 200 µl of Bradford (BioRad) Dye Reagent Concentrate (5×), were added and mixed, and the absorbance was measured at 595 nm. The protein concentration was calculated using a BSA calibration curve.

3.5.6 Preparation of membrane proteins from *Plasmodium falciparum* blood culture

The erythrocytes were collected from 1 ml of blood culture (hematocrit 5%). 50 µl of the erythrocyte pellet were lysed in 4 volumes of phosphate buffer. The mixture was homogenised on ice by gently pipetting up and down. The mixture was centrifuged at 4°C for 30 min at 17000 × g. The supernatant was discarded, the pellet was washed twice with the same buffer and afterwards the pellet was suspended in 100 µl of sample buffer and incubated for 1 h at 37°C.

3.5.7 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE electrophoresis was done using the Hoefer apparatus. In table 3.3 the recipe of polyacrylamide gels are shown:

Stacking gel	4%	Running gel	12.5%
Stacking gel buffer	0.5 ml	Running gel buffer	1.5 ml
Water	1.2 ml	Water	2 ml
AA/Bis (37.5%):(1%)	0.3 ml	AA/Bis 37.5(%):1(%)	2.5 ml
TEMED	5 µl	TEMED	5 µl
APS 10%	20 µl	APS 10%	40 µl

Table. 3.3. The volumes of ingredients used for SDS-polyacrylamide gel

Protein samples were mixed with sample buffer and heated at 95°C for 5 min (PfGAPDH) or at 37°C for 1h (AQPs) before loading on a 12.5% gel. Electrophoresis was done at a constant current: 20 mA, 200 V for 45-60 min. Gels were stained with Coomassie Blue for 30 min with gentle agitation and afterwards destained or used unstained for electrotransfer for Western blotting.

To estimate protein size peqGold Marker was used Table 3.4:

peqGold Marker	MW (kDa)
Lyzozyme	14.4
β-lactoglobulin	18.4
RE Bsp981	25
Lactate dehydrogenase	35
Ovalbumin	45
BSA	66
β-lactoglobulin	116

Table3.4 The list of proteins and their sizes used for SDS-PAGE electrophoresis

3.5.8 Western blot

After SDS-PAGE, Western blot was done using the Semi-Dry-Electrotransfer and a PVDF membrane which was activated in methanol for 5 min. One 3 mm Whatman paper was soaked in Blot buffer, placed on the anode and gently pressed to remove air-bubbles. Then, the activated PVDF membrane was placed on the paper. On top of the PVDF membrane the SDS-PAGE gel was placed. One soaked 3 mm Whatman paper was placed on top. Again,

air-bubbles were removed. At the end, the cathode was placed on the top and transfer was carried out with a current of 2-3 mA/cm^2 of gel (20 V) for 2-3 h. After the transfer the gel was stained with Coomassie Blue for 30 min. The PVDF membrane was stained with Ponceau S for 5 min. When the proteins were visible the membrane was washed with deionized water and photographed. Afterwards, the membrane was incubated for 1 h in blocking solution (M-TBS buffer). Then, the membrane was incubated for 1 h with the primary antibody diluted in M-TBS buffer. After washing (1 x 15 min, 2 x 5 min) the membrane was incubated with the secondary antibody diluted in M-TBS buffer for 1 h. Washing was repeated. For chemoluminiscence a mixture of 2 ml of solution A and 50 µl of solution B of the ECL-Plus Western Blotting Detection Kit was prepared. The mixture was uniformly distributed over the surface of the membrane and incubated for 5 min. Afterwards, a film exposure with Hyperfilm-ECL was taken. Exposure periods varied from 5 sec to 5 min.

3.6 Culture of blood stage *Plasmodium falciparum* and purification of genomic DNA

P. falciparum parasites (strains Binh1 and 3D7) were cultivated at 5% hematocrit (O^+ bloodtype) and 0.5-5% parasitemia in RPMI 1640 medium supplemented with 25 mM HEPES, 20 µg/ml gentamicin sulphate, 2 mM glutamine, 200 mM hypoxanthine, and 0.5% Albumax II at 37°C in a nitrogen atmosphere (90% N_2, 5% O_2 and 5% CO_2) (Trager et al., 1976).

3.6.1 Synchronisation of erythrocytic stage of *Plasmodium falciparum* parasites

For synchronisation with sorbitol parasites should be in the ring stage (not longer than 10-12 h). Everything was done under sterile conditions.

The parasite culture (10 ml) was centrifuged at 600 × g at room temperature for 5 min. The supernatant was discarded and then 10 ml of 5% sorbitol were added to the red cell pellet.The suspension was mixed gently with a pipette and left for 5 min at room temperature. Afterwards, the cells were collected (600 × g, 5 min), washed twice with complete RPMI 1640 medium. After 6 h the procedure was repeated (Lambros et al., 1979).

3.6.2 Giemsa staining of thin blood films from parasite cultures

The staining of malaria parasites was done by a standard procedure described in "Methods in Malaria Research" (Inger Ljungstöm et al., 2004). Shortly, thin blood films on slides were fixed in methanol for 30 s. After fixation, the slides were dried and stained in 10% Giemsa solution in phosphate buffer (pH 7.1) for 20 min. After staining, the slides were washed and analysed using a microscope at 100× magnification.

3.6.3 *Plasmodium falciparum* culture in medium supplemented with glycerol

Here, RPMI 1640 medium with and without glucose supplemented with 11 mM glycerol was used for the culture. Starting parasitemia was 0.2%, parasites in the ring stage were synchronized with sorbitol. The total culture volume was 5 ml, hematocrit 5%. Every day a 1 ml sample was taken, the cells were collected (600 × g, 5 min) and frozen at -20°C. Medium was changed every day after taking the samples. Samples cultivated in complete RPMI 1640 medium were used as controls.

3.7 Monitoring of *Plasmodium* proliferation by ELISA

A commercial ELISA based on the quantitation of the *P. falciparum* histidine rich protein 2 (HRP2) was used (Malaria Ag CELISA, Cellabs; Noedl et al., 2002). Briefly, 96-well plates were pre-dosed with 10 µl of dihydroxyacetone, methylglyoxal, glycerol, glucose, chloroquine, fosmidomycin and ammonium chloride dilutions in complete RPMI 1640 medium. Synchronized parasite cultures (80% rings) were diluted to 0.05% of parasitemia at a hematocrit of 1.5%. Then, 90 µl of the diluted cultures were added to each well of the pre-dosed plates. After 72 h of incubation at 37°C in 90 % nitrogen atmosphere the plates were harvested and twice freeze-thawed. The samples were transferred to ELISA plates, and analyzed according to the Cellabs protocol using an ELISA plate reader at 450 nm.

3.8 GAPDH enzyme assay

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyzes the following reaction:

glyceraldehyde-3-phosphate + NAD^+ + P_i $\rightleftharpoons$ 1,3-bisphosphoglycerate + NADH + H^+

The initial conversion rate of NAD^+ is measured as an increase in absorption at 340 nm. One unit is defined as the amount of enzyme that converts one micromole NAD^+ per minute at 25°C.

The assay was done in Tris-HCl buffer (50 mM, pH 8.0), supplemented with 300 mM NaCl, 50 mM Na_2HPO_4, 1 mM NAD^+ and recombinantly expressed PfGAPDH (5 µg/ml) or commercially available rabbit GAPDH (5 µg/ml). The reaction was started at room temperature by the addition of 360 µM DL-glyceraldehyde-3-phosphate.

3.9 Quantification of ammonia in the culture medium

Glutamate dehydrogenase (GLDH) catalyzes the conversion of α-ketoglutarat to L-glutamate in the presence of NH_4^+ and NADPH.

NH_4^+ + α-ketoglutarat + NADPH $\rightleftharpoons$ L-glutamate + NAD^+ + H_2O

The reaction was followed at 340 nm until the end point was read.

The assay was done in 122 mM TEA buffer pH 8.0 supplemented with 300 µl sample, 0.56 mM ADP, 71 mM α-ketoglutarat, 6 mM NADPH. After 5 min the reaction was started by addition of GLDH to a final concentration of 7.4 U/ml.

From the differences in absorption before and after the reaction the ammonium concentration was calculated based on a calibration curve with known ammonium concentrations.

4 Results

4.1 Modulation of water and glycerol permeability of the *Plasmodium falciparum* aquaglyceroporin by introducing a mutation in the pore constriction region

It has been suggested that the electrostatic environment at the extracellular pore entry of the *P. falciparum* aquaglyceroporin may regulate water permeability (Beitz et al., 2004). An $E_{125}S$ mutation introduced in the conserved WET triad in the extracellular C-loop of PfAQP affected water permeability while glycerol permeability was changed only minimally. It was proposed that by removing the charge at position 125 and thus probably disrupting an interaction of Glu125 with Arg196, the polarity of the constriction region would change. The pore layout of PfAQP $E_{125}S$ closely resembles that of GlpF, i.e. *E. coli* aquaglyceroporin with high glycerol and low water permeability.

During the course of this work I addressed the question whether the water permeability of PfAQP $E_{125}S$ could be improved again by introducing an additional mutation at the pore constriction region. I sought to mimic the AQP3 pore layout which contains a Tyr instead of the Phe present in GlpF (Phe200) and PfAQP (Phe190). AQP3 is an aquaglyceroporin with good glycerol and intermediate water permeability. Mutating Phe190 of PfAQP to tyrosine would introduce an additional hydroxyl group and increase polarity in pore constriction region (Fig. 4.1.).

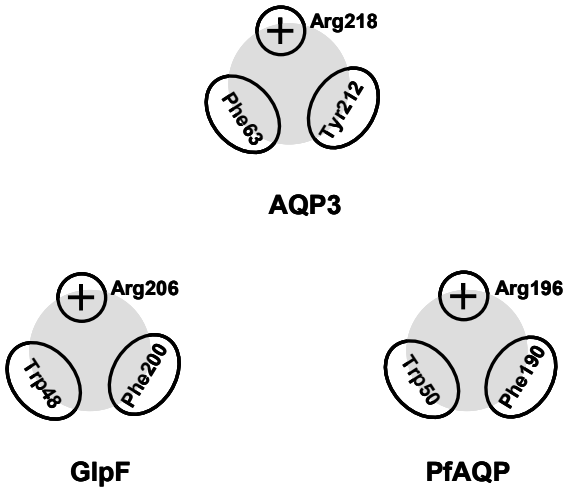


Fig. 4.1. Schematic presentation of the PfAQP, AQP3 and GlpF pore constriction region.

4.1.1 Expression and characterisation of PfAQP $E_{125}S/F_{190}Y$

To test this hypothesis I generated the double mutant PfAQP $E_{125}S/F_{190}Y$. This variant as well as PfAQP wild type were expressed in oocytes and characterised in standard oocyte swelling assays.

PfAQP $E_{125}S/F_{190}Y$ cRNA was injected into *X.* oocytes and protein expression was analysed by Western blot using a specific PfAQP antibody (Fig. 4.2.).

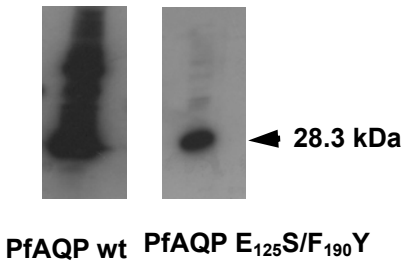


Fig. 4.2. Western blot analysis of PfAQP wt and PfAQP $E_{125}S/F_{190}Y$ expressed in *X. laevis* oocytes with anti-PfAQP antibody (on the SDS-PAGE gel 1.2 oocyte per line was loaded).

Osmotic swelling assays showed that the PfAQP $E_{125}S/F_{190}Y$ variant was water permeable. However, compared to wild type PfAQP (316 µm s^{-1}) its water permeability (28 µm s^{-1}) was 11 times smaller (Fig.4.3. A).

PfAQP $E_{125}S/F_{190}Y$ (0.55 µm s^{-1}) also showed glycerol permeability. Still, glycerol permeability was 3 times smaller than that of PfAQP wt (1.63 µm s^{-1}; Fig. 4.3. B).

The expression levels of PfAQP wt and PfAQP $E_{125}S/F_{190}Y$ were not equal (Fig. 4.2.). To avoid the influence of expression level on oocytes permeability results, the ratios between water and glycerol in wild-type and mutant were calculated. The water/glycerol ratio for PfAQP wt is 194:1, and for the PfAQP $E_{125}S/F_{190}Y$ it is 51:1 (Fig. 4.3. C). Comparing these data to those of the published PfAQP $E_{125}S$ mutation, where water permeability was reduced to background level, with the double mutant water permeability was gained.

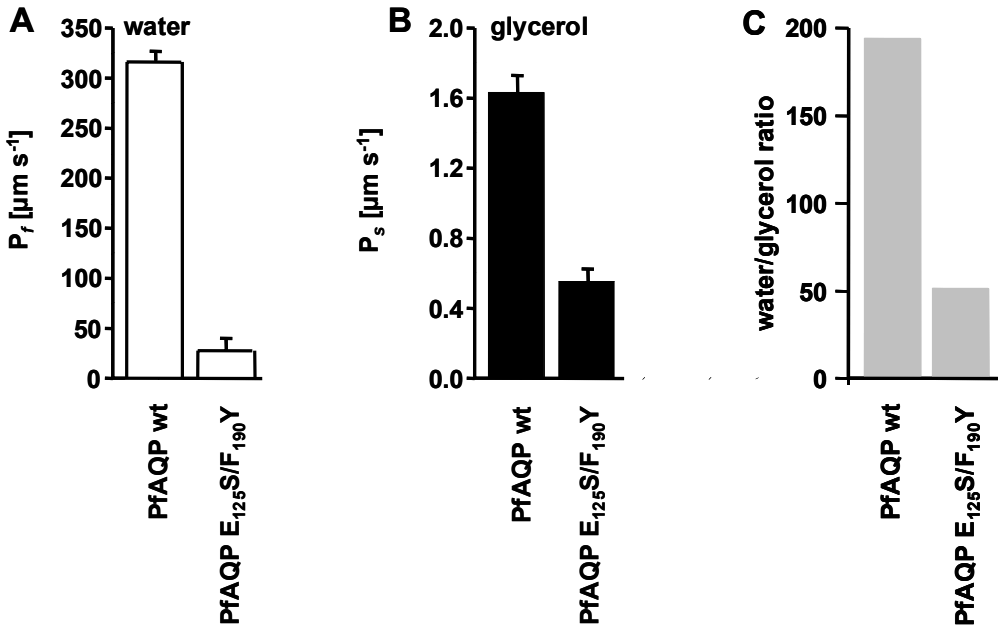


Fig. 4.3. Water and glycerol permeability of PfAQP wt and PfAQP $E_{125}S/F_{190}Y$ mutant.
A: water permeability P_f [µm s^{-1}] of PfAQP wt and PfAQP $E_{125}S/F_{190}Y$. B: glycerol permeability P_s [µm s^{-1}]. Control values for water (37 µm s^{-1}) and glycerol (0 µm s^{-1}) were subtracted from the sample values. C: water/glycerol ratio for PfAQP wt and PfAQP $E_{125}S/F_{190}Y$. All values are mean ± S.E.M. (n=5-6).

4.1.2 pH dependency of water permeability in PfAQP

Since a glutamate residue seems to be responsible for the high water permeability of PfAQP I wanted to test for a pH effect on water and glycerol permeability of the PfAQP. Shifts to low pH may change the protonation status of Glu125 and may alter permeability properties. The pKa value of the carboxyl group in glutamic acid is 4.4. As it is known pKa values depend on temperature, ionic strength and microenvironment. Amino acid pKa values in proteins might be different than in solution because of the influence of other amino acids and the environment.

To test water permeability a pH-range of pH 3-7 was used. For glycerol permeability I used a broader range of pH values from pH 3 to pH 9. The basic condition at pH 9 can led to amino acid deprotonation and may influence the possible interaction between Glu125 and Arg196. Swelling assays at the different pH values were performed with oocytes expressing PfAQP. The data show that water permeability P_f (Fig. 4.4. A) was not dependent on the pH in the range tested. Glycerol permeability was not significantly changed at different pH values, too ($2p>0.05$; Fig. 4.4. B).

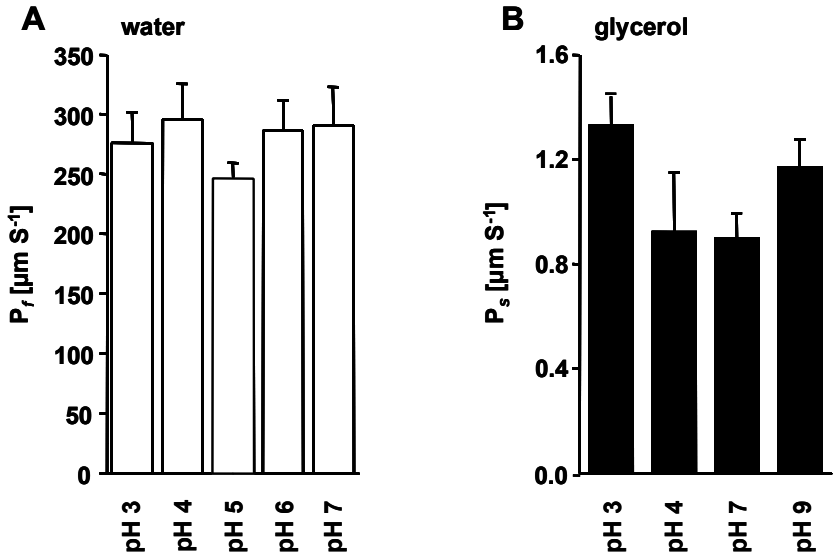


Fig. 4.4. Water and glycerol permeability of PfAQP at different pH values. A: P_f values for water permeability at different pH values. B: P_s values for glycerol permeability different pH values. All values are mean ± S.E.M. (n=5-6). (control values for water (9, 6, 6,10, 21µm s^{-1}) and glycerol (-0.018, -0.01, 0.024, 0.141 µm s^{-1}) at different pH values were subtracted from sample values. Two control values for glycerol have negative values because of oocyte shrinking).

4.2 Glycerol induction of PfAQP expression in *Plasmodium falciparum* blood culture

The physiological functions of PfAQP are unknown. It has been reported that parasites utilize serum glycerol to synthesise glycerolipids (Vial et al., 1975). Hansen *et al*. proposed that glycerol may be used as a backup energy source in *P. falciparum* in hypoglycaemia states when glucose uptake is restricted (Hansen et al., 2002).

After phosphorylation and oxidation glycerol could enter glycolysis. Further, glycerolipid synthesis using glycerol from the host should be favoured by the parasite over generating

glycerol from glucose due to the resulting shift in the NAD^+/ NADH ratio towards the oxidized form.

There are examples that glycerol content in the serum regulates aquaporin expression in humans (Carbrey et al., 2003). Expression of AQP9 in the hepatocyte plasma membrane is induced up to 20 fold when glycerol levels rise. AQP9 is permeable to glycerol and urea and to a wide range of different solutes including polyols, carbamides, purines, pyrimidines, nucleosides and monocarboxylates (Tsukaguchi et al., 1998).

After *in vitro* characterisation of PfAQP I moved to an *in vivo* parasite system and tested for glycerol induction of PfAQP expression in *P. falciparum* blood culture.

Parasite growth and expression levels of two cultures were analysed, one without glycerol and one with 11 mM glycerol supplementation (Fig. 4.5. A). Starting parasitemia was 0.2% with the parasites in the ring stage. After two days, parasitemia increased 4-5 fold and after five days 25-30 fold. Addition of glycerol led to an 8 fold increase of the PfAQP protein expression (Fig. 4.5. B). However, parasites did not survive in medium where glucose was replaced by glycerol. Apparently, plasmodia cannot use glycerol as the only energy source (Fig. 4.6).

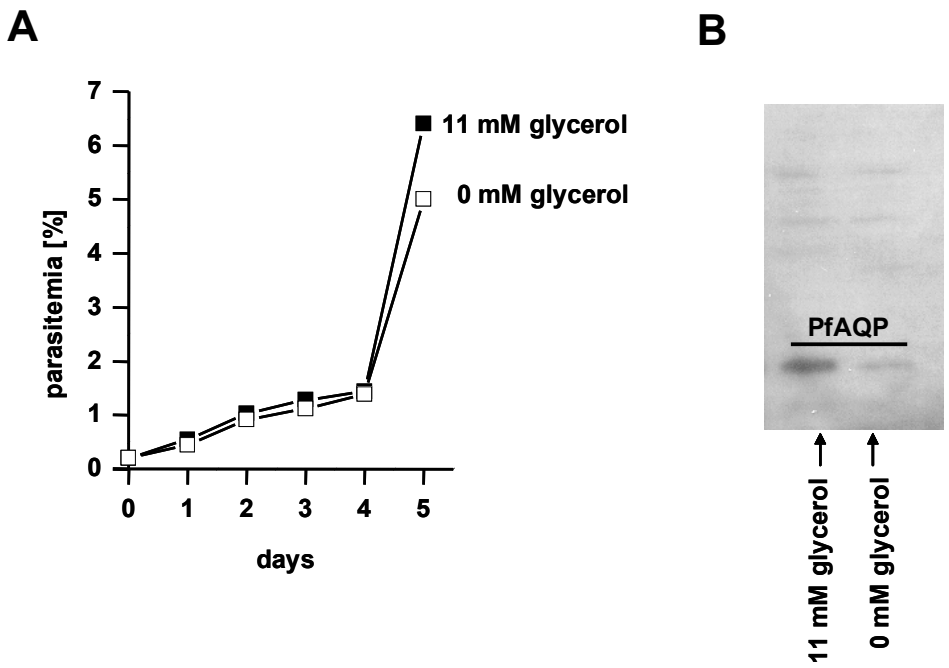


Fig. 4.5. Influence of glycerol on parasite proliferation and PfAQP expression. A. Growth curves of *P. falciparum* parasites in medium without (open squares) and with 11 mM (filled squares) glycerol addition. B. Western blot with samples taken after three days of parasite culture with and without glycerol (control).

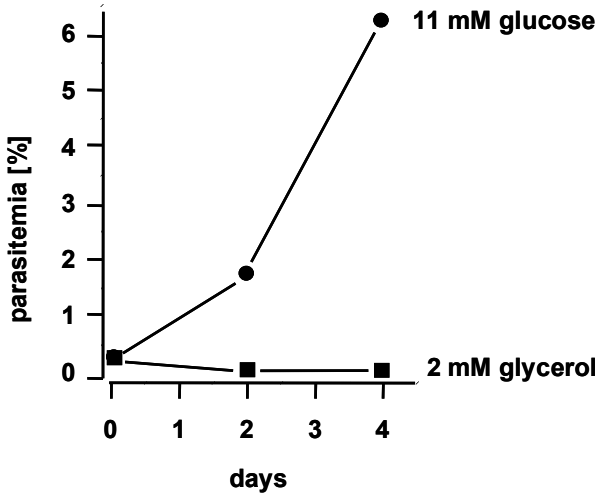


Fig. 4.6. Growth curves of *P. falciparum* parasites in medium supplemented with glucose (filled circles) or glycerol (filled squares) as an energy source.

4.2.1 Ammonia permeability of PfAQP

One of the central features in malaria metabolism is digestion of the host cell cytosolic proteins (predominantly haemoglobin) to be used as a source of amino acids for protein biosynthesis (Francis et al., 1997).

Protein biosynthesis and degradation is accompanied by the production of cytotoxic ammonia. Further, *P. falciparum* deaminates and oxidises glutamine to gain reduced NADH. One biochemical way of detoxification is to convert ammonia to urea. This detoxification process is accomplished in the urea cycle by most terrestrial vertebrates. In the plasmodia genomes, none of the enzymes of the urea cycle, except arginase, have been detected.

Alternatively, ammonia could be exported from a cell by ammonium transporters but these do not seem to be present in the *Plasmodium* genome. Ammonia diffusion through the membrane could occur, but the question is, would it be sufficient?

It was reported that a yeast strain lacking ammonium transporters could not grow if the only nitrogen source is ammonium chloride (5 mM). When the ammonium ion concentration is increased to 20 mM then yeast cells grow (Marini et al., 1997). Some aquaporins like plant aquaporins and human AQP8 were characterised as ammonia channels (Janh et al., 2004; Holm et al., 2005).

PfAQP as an aquaglyceroporin is also a good candidate for an ammonia channel. A yeast strain lacking all ammonia transporters and the endogen aquaporin Fps1 was used as an assay system to test ammonia permeability and indeed, it turned out that PfAQP facilitates ammonia permeation (Wu, personal comunication).

4.2.2 Ammonia production in *Plasmodium falciparum* parasites

To test ammonia production in parasites I used standard culturing conditions (Trager et al., 1976) with highly synchronised parasites (more than 85% were in ring stage). Starting parasitemia was 5%, total culture volume was 20 ml. Samples (1 ml) were taken at 0, 16, 24, 38, 48 and 64 h and ammonia was quantified. After each time point, bottles were gassed and again incubated at 37°C until the next sample was taken.

Non-infected erythrocytes were used as a control. Ammonia production in non-infected erythrocytes was absent, but in culture with parasites ammonia production was detected after 16 h. (Fig. 4.7).

Between 16 and 24 h there is a sudden increase in ammonia production in parasites. This time interval corresponds to the trophozoites developmental stage of *P. falciparum*. After 16 h parasites were in the trophozoite stage.

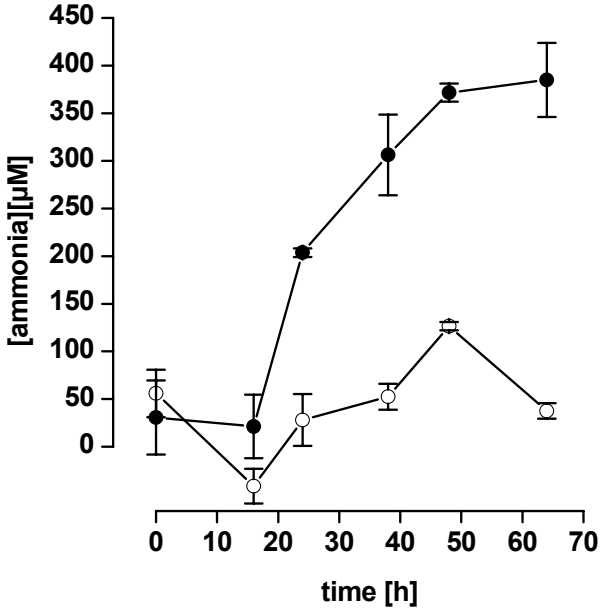


Fig. 4.7. Ammonia production in *P. falciparum* clone 3D7 culture (filled circles) and in non-infected erythrocytes (open circles). Ammonia levels in RPMI 1640 medium were subtracted from ammonia levels in parasite and erythrocytes samples. (n=3 ± S.E.M.)

The parasitemia between 16 and 24 h was 5%, hematocrit 5%. Ammonia production at this time point was 175 µmol/l corresponding to $2.75*10^{10}$ infected red blood cells (IRBC). The amount of ammonia produced by one parasite per hour was 0.8 fmol/parasite. Accordingly, one litre of IRBC produce 8.8 mmoles of ammonia per liter of triphozoite infected cells per hour whereas lactate production is 220 mmoles (one litre of packed erythrocytes contains $1.1*10^{13}$ cells). Thus, there is 20 times less ammonia production than lactate production in plasmodia (Ginsburg et al., 2002).

4.2.3 Effect of ammonia on growth of *Plasmodium falciparum* parasites

An ELISA was used to test growth of *P. falciparum* 3D7 in medium supplemented with various concentrations of NH_4Cl. Ammonium chloride was toxic for plasmodia 3D7 clone with an IC_{50} of 2.8 mM (Fig. 4.8.).

The parasite volume is 20 fl in the trophozoite stage. If ammonia is not transported out from the cell in 4.2 min the concentration of ammonia in the parasite cell might reach 2.8 mM. Thus, parasites live on the edge of death.

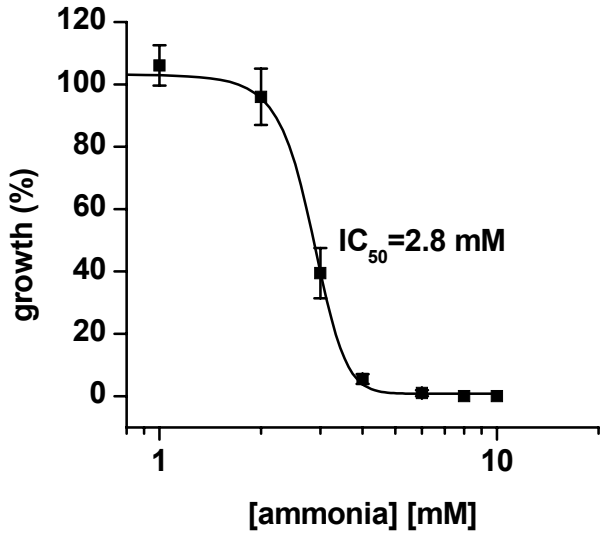


Fig. 4.8. Effect of ammonium chloride on growth of *P. falciparum* 3D7 clone. The IC_{50} for ammonium is 2.8 mM (n=2 ± S.E.M.).

4.3 Dihydroxyacetone and methylglyoxal permeability of PfAQP and AQP3

Except for water and glycerol, PfAQP is permeable for other uncharged solutes, such as polyols of up to five carbon-hydroxyls in length (Hansen et al., 2002).

Carbonyl containing compounds have not been tested before. Dihydroxyacetone (DHA) is a three carbon sugar with one keto group at the C-2 atom and methylglyoxal (MG) is a three-carbon compound with a keto and an aldehyde group.