Regulation and Transport Mechanisms of Eukaryotic Aquaporins

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Cover illustration: Tiles representing high resolution structure of Aqy1 from *P. pastoris*. Glycerol uptake in three different strains; *P. pastoris* X33 (wild type), *P. pastoris* GS115 *aqy1*Δ and *P. pastoris* GS115 *aqy1*Δ*agp1*Δ. Circular dichroism spectra of purified human AQP3 and human AQP7 as detergent protein complex and reconstituted into proteliposomes.

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“There is no such thing as failure.
There are only results”

- Tony Robins

List of publication

Paper I

Crystal structure of yeast aquaporin at 1.15Å reveals novel gating mechanism

Gerhard Fischer, Urszula Kosinska-Eriksson, Camilo Aponte-Santamaría, Madelene Palmgren, Cecilia Geijer, Kristina Hedfalk, Stefan Hohmann, Bert L. de Groot, Richard Neutze, Karin Lindkvist-Petersson

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Paper II

Yeast aquaglyceroporins use the transmembrane core to restrict glycerol transport.

Geijer C, Ahmadpour D, Palmgren M, Filipsson C, Klein DM, Tamás MJ, Hohmann S, Lindkvist-Petersson K

J Biol Chem. 2012 Jul 6;287(28):23562-70

Paper III

Differences in transport efficiency and specificity of aquaglyceroporins explain novel roles in human health and disease

Madelene Palmgren, Cecilia Geijer, Stefanie Eriksson, Samo Lasic, Peter Dahl, Karin Elbing, Daniel Topgaard, Karin Lindkvist-Petersson

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Paper IV

Overexpression and characterization of human aquaglyceroporins AQP3 & AQP7

Urszula Kosinska Eriksson, Madelene Palmgren, Karin Elbing,
Karin Lindkvist-Petersson

Manuscript

Abstract

Aquaporins are found in all kingdoms of life where they are involved in water homeostasis. They are small transmembrane water conducting channels that belong to the ancient protein family Major Intrinsic Proteins (MIP). Early on in the evolution, a gene duplication event took place that divided the aquaporin family into two subgroups; orthodox aquaporins, which are strict water facilitators, and aquaglyceroporins that except for water also transport small uncharged solutes.

The main questions that I have tried to address in this thesis are which regulatory mechanisms that are involved in aquaporin gating and to investigate transport differences in solute permeation. Specifically, we have investigated yeast and human aquaporins. To find answers to our questions, we have attempted to combine structural knowledge with functional analysis.

A high resolution structure of *P. pastoris* orthodox Aqy1 to 1.15Å generated new knowledge of regulatory mechanisms and functions of the long N-terminus that is common among fungi. We suggest that Aqy1 is gated by phosphorylation and by mechanosensation. An important functional role of Aqy1 in rapid freeze thaw cycles could be demonstrated. During this work, a single deletion strain was generated that now serves as the primary aquaporin expression platform in our laboratory.

Fps1 is a regulated glycerol facilitator that is important for yeast osmo-regulation. The regulatory mechanism is still not known but here we show that a suppressor mutation within the transmembrane region restrict glycerol by its transmembrane core. Thereby, we suggest that post translational modifications in the regulatory domains of N- and C-termini fine tunes glycerol flux through Fps1.

The aquaglyceroporins are classified as having a dual transport function, namely being capable of facilitating the movement of both water and glycerol over the plasma membrane. In this study, we can clearly show that there are major differences in the substrate specificity and efficiency between the different aquaglyceroporins and that small changes affect the transport efficiency and specificity of the channels.

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Abbreviations

MD	Molecular Dimension
ATP	Adenosine Tri Phosphate
ADP	Adenosine Di Phosphate
Pi	Phosphate
cRNA	Complementary Ribonucleic acid
H^+	Proton
cAMP	Cyclic Adenosine Monophosphate
ORF	Open Reading Frame
RU	Respons Unit
Å	Ångström
POPE	1-Palmitoyl-2-OleoylPhosphatidylEthanolamine
POPS	1-Palmitoyl-2-OleoylPhosphatidylSerine
POPC	1-Palmitoyl-2-OleoylPhosphatidylCholine

Introduction

Lipid bilayer

Biological membranes are barriers that either enclose or separate compartments within or around a cell. One of their basal functions is to maintain an optimal intracellular milieu meaning that the biochemical environment is different on each side of the membrane. In short, the lipid bilayer constitutes of a two leaflets of phospholipids where the negatively hydrophilic heads point towards the aqueous surrounding and the hydrophobic tails point towards each other. The arrangement of negatively charged head groups lining the hydrophobic interior of the bilayer generates a dissolved "fat layer" , which is highly impermeable to charged and polar solutes yet permeable to small uncharged molecules like water and oxygen.

All organisms and cells need to be in constant exchange with their environment, not only in order to assimilate substrates that are vital for cellular processes, but also to excrete waste products and toxic substances. This is achieved through the incorporation of trans-membrane proteins, generating entry and exit routes for molecules in and out of the cell. In the early 1970´s, S.J Singer purposed the fluid mosaic model in which he postulated that the amphipathic part of transmembrane proteins is embedded in the membrane and the charged or polar residues face the aqueous surrounding. He also described the lipid bilayer as a two dimensional viscous solution with its component diffusing in the plane (1).

Transport

Transport across biological membrane is either passive or active. Classification is based on energy costs and sub-classifications are based on how the solute transport is mediated across the membrane.

Water movement in biological system is called osmosis and is driven by osmotic pressure. When water moves (spontaneous) from a low osmotic pressure to high osmotic pressure it lowers its free energy states by dissolving solutes. The concept of changing osmotic pressure or solute concentrations is fundamental for living organisms and is called osmo-regulation. This is especially important to unicellular organisms like yeast since drastic changes in their surroundings will affect the net flow of water. Upon an increase in external osmotic pressure, referred to as hyper osmotic shock, water will flow out from yeast cells and cell volume will decrease. This will also generate an increase of intracellular components. To regain lost water, yeast cells increases intra cellular osmotic pressure by production of a compatible solute. Water will flow into the cells until equilibrium is reached. In contrast, during hypo-osmotic conditions (low osmotic pressure

in the environment) cells will lose excessive compatible solutes in order to prevent water influx.

Passive transport

Passive diffusion is the transport of solutes across the membrane that is “free of charge” and substances move freely in and out of the cell at any time. The driving force is the solutes own concentration gradient where molecules move from high concentration to low concentration. Simple diffusion is the transport of substances through the lipid bilayer. In general this applies to small uncharged solutes.

Facilitated diffusion is a protein-mediated simple diffusion that is carried out by two sets of proteins 1) pore-facilitated transport where channel proteins do not undergo any conformational changes and the substrate only interacts weakly with the pore (e.g. aquaporins) 2) permeases-mediated transport with binding sites for their substrate and alter conformation between two states (glucose transporters).

Active transport

Active transport is when solutes are transported across the membrane against its concentration gradient at the expense of energy. The energy is usually generated by the hydrolysis of ATP to ADP and Pi. Ion pumps are an example of direct coupled active transport since ATP hydrolysis is directly involved in the transport mechanism. Contrary to active transport, is indirect active transport the utilization of a generated gradient as the driving force of transporting a substrate by co-transport.

Aquaporins

Water was long considered to cross lipid bilayers by passive diffusion. Even though some scientists argued that the high water flux in certain tissues such as renal tubule and red blood cells could only be explained by the presence of water channels. In 1983, Bob Macey showed that water flux in red blood cells could be inhibited by addition of mercuric chloride in a reversible fashion by adding a reducing agent. He also suggested that transport is aligned in a single file, through a narrow pore and the prevention of H^+ permeation could occur if the single file is not continuous (2).

Experimental evidence for this hypothesis was lacking until the beginning of the 1990´s when Peter Agre and colleagues, while trying to identify Rh blood group antigens, came across a 28 kDa polypeptide, which they called CHIP 28 (CHannel like Intrinsic Protein of 28 kDa). Further investigation identified that this protein shared homology with other proteins in the ancient Major Intrinsic Protein family that have the potential to transport water (3). In a simple yet ground-breaking experiment, the team produced the first proof of the existence of water transporters. To test for water transporting properties, cRNA from CHIP28 was microinjected into highly water impermeable frog eggs, oocytes from *Xenopus laevis*. Oocytes were then put into distilled water and were observed to swell and eventually burst, in contrast to controls (microinjected with buffer), which remained unaffected (4). This protein is today known as human Aquaporin1 (AQP1). In 2003, Peter Agre was awarded the Nobel Prize in chemistry for the discovery of water channels.

Conserved protein family

Homologues of human AQP1 have been found in all kingdoms of life. They can all be grouped into the large Major Intrinsic Protein (MIP) family. The number of family members is constantly increasing with today's gene sequencing tools. In MIPModDB more than 1000 MIP sequences from 341 organisms have been collected (5). This vast increase in the number of MIPs has also generated a deviating classification and spelling among authors, particularly for the mammalian aquaporins (6). This is most likely due to the classification being based on sequence homology rather than function.

The gene arrangement of CHIP28 and other homologues indicated that the DNA sequence is arranged in an inverted tandem repeat (3). Sequence analysis in more recent years have revealed that MIP:s arose from a gene duplication (7) but also that a gene duplication event split the protein family into the water facilitating aquaporins and the glycerol facilitating aquaglyceroporins (8,9). Since *Escherichia coli* possess a single water conducting channel (AQPZ) and a single glycerol transporting aquaglyceroporin (GlpF) in its genome, it has been suggested that the gene duplication arose early in the evolution (7,10).

Aquaporins are lacking in many microbes, however, they are more abundant in eukaryotic microbes than prokaryotes (11). This implies that aquaporins are not essential for basal cellular processes. Instead it was suggested that the presence of aquaporins in microbes are more of an ecological relevance, since aquaporins have been shown to have enhanced survival against rapid freezing during experimental conditions (11). In nature, microbes are exposed to rapid temperature changes or freeze thaw cycles, e.g. when rain hits the frozen ground or when microbes are liberated from warm-blooded animals by breathing or sneezing in freezing environment. Vice versa, microbes will thaw immediately for instance when inhaled in a frozen environment (12).

In plants, water-transporting proteins were first identified and demonstrated in 1993 (13). Plants have a much higher number of aquaporins in their genome than microbes and mammalians do; for instance, *Arabidopsis thaliana* was identified as having 35 AQPs (14), *Zea mays* has 31 AQPs (15) and rice has 33 AQP genes (16). Initially plant aquaporins were divided into four subfamilies; plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin-like intrinsic proteins (NODs), and small and basic intrinsic proteins (SIPs). But as many as 7 subgroups has been identified in the more primitive plants such as moss (17). The large number of aquaporins in plants are important for the movement of water in plants (18).

13 mammalian aquaporins have been found, AQP0-12. The names represent each aquaporins respective order of discovery. MIP from lens was known for a long time (though not its function) and after identifying AQP1 as a water channel, MIP was thereafter given the name AQP0. The most common classification is to place AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8 as aquaporins and AQP3, AQP7, AQP9 and AQP10 as aquaglyceroporins. The remaining two, AQP11 and AQP12, are gathered under the label superaquaporins. It should be noted that AQP6 and AQP8 are in some cases clustered with AQP11 and AQP12 as orthodox aquaporins (19). All aquaporins are distributed in a tissue specific manner and are highly abundant in water permeable tissues, as in the like kidney. They can be divided in three subgroups based on the solute that they transport; (1) classical aquaporins, (AQP1, 2, 4 and 5) that only transports water; (2) aquaglyceroporins (AQP3, 7, 9 and 10) that in addition to water also transport glycerol and small uncharged solutes; (3) unorthodox aquaporins, (AQP0, 6, 8, 11 and 12) that are not yet fully characterized (20).

From gene to structure

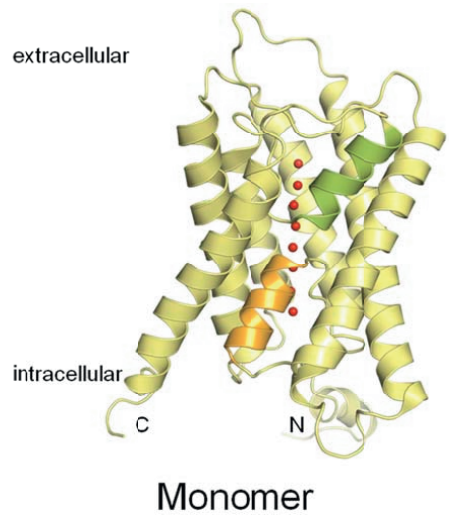
In the year of 1994, while structural information was still lacking, Peter Agre and co-workers used common molecular biology techniques together with predictions tools, to postulate a topology map for AQP1 describing of 6 transmembrane helices with both the N and C-termini on the cytosolic side (21). Loops B and E, each containing a highly conserved Asparagine-Proline-Alanine (NPA)-motif, were suggested to dip into the

membrane from opposite sides participating in the pore formation.(22) With a pencil and paper sketch, Agre and co-workers drew the hour glass model that was later to be widely adopted (23).

During the same period, a 2D projection map from electron microscopy (EM) on AQP1 revealed a tetrameric structure (24). The projection maps was improved in the following years using 3D cryo EM to confirm an α-helical bundle and the location of the water pore (25). Loops B and E were shown to protrude into the channel as half helices participating in the pore formation (26-28). It was not until 2000 that the first high-resolution structure was published. However, in contrast to previously known structures, it described an aquaglyceroporin, GlpF from *E. coli* (29). Between 2000 and 2010 several structures have been determined representing all five kingdoms of life. The animal kingdom is represented by the (human) AQP1 (25), AQP4 (30) and AQP5 (31), (bovine) AQP0 (32) and AQP1 (33), and (bovine) AQP0 (34), the plant kingdom by (spinach) SoPIP2;1 (35), the Fungi by (*P. pastoris*) Aqy1 (Paper I) (36), the Protists by (*E. coli*) AqpZ (37) and GlpF (29) and *M. marburgenis* AqpM (38) and last but not least the Monera by (*P. falciparum*) PfAQP (39).

The Aquaporin fold

All aquaporins so far have been shown to share a common fold of the transmembrane "core" unit (Fig. 1). The protein is situated as homo tetramers in the membrane displaying a four-fold axis where a given monomer interacts with two adjacent monomers. The typical monomeric aquaporin structure has six transmembrane tilted helices (H1-H6) in a right-handed bundle with both N- and C-termini facing the cytosol. Two loops dip into the membrane from opposite sides and fold back as half helices (HB and HE) making up a seventh pseudo helix. Each monomer has a pseudo two fold symmetry where H1-H3 rise from the first tandem repeat and H4-6 including HE represents the second repeat. Each monomer hosts one pore (25).



Figur 1. Structure of aquaporin monomer (Aqy1) PDB entry 2W2E. Six helices that spans the membrane (yellow) and loop B (orange) and loop E (green) form a seventh pseudo helix. Water molecules are displayed as red spheres.

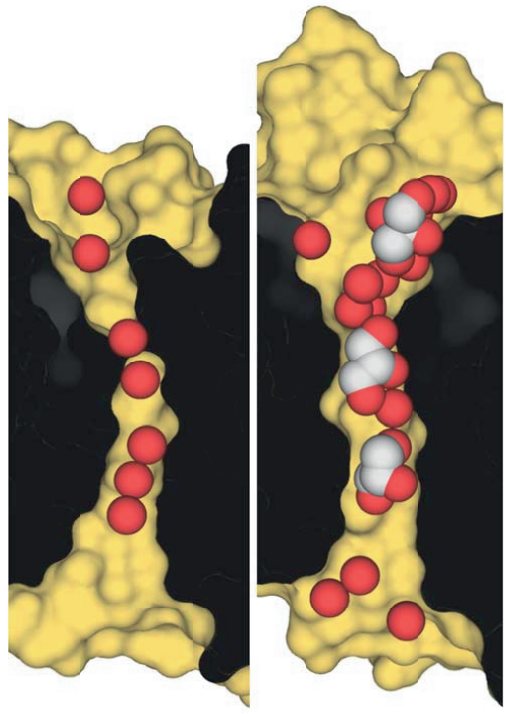
Constriction region

The narrowest part of the pore is located approximately one third down the pore (from the periplasmic side) and is called the aromatic/arginine (ar/R) -constriction region. In a water transporting aquaporin, this motif is built up by three hydrophilic aromatic amino acids (histidine, phenylalanine, cysteine in AQP1) where histidine is highly conserved. The fourth member of this site is represented by the positive arginine, which is conserved among all aquaporins. The amino acids are arranged in such a way that they create a small narrow passage, large enough for water to pass and hence responsible for size occlusion. In aquaglyceroporins (like in in GlpF), the corresponding amino acids in the aromatic region are a glycine, tryptophan and phenylalanine together with the conserved arginine. The amino acid "composition" generates a wider and more hydrophobic pore. The selectivity in GlpF compared to AQP1 in the constriction region has been suggested to be of a more steric relevance and the solute needs to adopt the right conformation (25,29,40-42).

NPA motif

In the center of the pore, where the two half helices meet, there are two highly conserved Asparigine–Proline-Alanine (NPA) motifs. This region is often referred to as the selectivity filter. The polar asparagines create a dipole and it thought to be partly responsible for the proton occlusion of the pore (41). High-resolution structures from AQP1 and GlpF have been used in real time MD simulations to elucidate how solutes move within the channel. The general picture is that large dipole moments are generated by the two asparagines within the NPA motif, forcing the water molecule to rotate 180°along the pore (40) (25,29,41).

The Grotthuss mechanism describes how protons move in a hydrogen network. Since aquaporins occlude protons, the proton wire must be disrupted. However, the mechanism behind proton occlusion is not experimentally proven. Information from high-resolution structures, in conjunction with real time MD simulation, a picture was created of water molecules moving through the channel in a single file passing two high electrostatic energy barriers at the aromatic region and the selectivity filter. In other words, the negative dipole moment of water will face the positive NPA motif when both entering and leaving this site with a total reorientation of 180° (40). But yet another contributing mechanism describing synchronized pair wise hopping of water molecules while passing the ar/R constriction region would also contribute to disrupt the Grotthuss mechanism (43).



Figur 2, Pore lining of a water transporting channel vs. glycerol facilitating channel. Left: orthodox aquaporin AqpZ (PDB entry 1RC2). Right: aquaglyceroporin GlpF (PDB entry1FX8).

Water vs. glycerol permeation

Since the overall three dimensional structure, constriction site and selectivity filter included, are similar for both aquaporins and aquaglyceroporins, only small changes within the aquaporin structure affects the preference of substrates, even giving rise to a gating phenomenon (44). Pore size of the strict water facilitators are generally narrower generating steric hindrance of larger molecules than water, while in the aquaglyceroporins the pore lining amino acids are generally more hydrophobic creating a "greasy slide"(Fig. 2) (29,45). In a substitutional study of the constriction site, where the pore diameter of a water transporting aquaporin was increased, the highly conserved amino acid in this region, glycerol and urea permeability was observed (46).

Regulation of aquaporins

Aquaporins are facilitators that allow passage of their permeates through their pore along the concentration gradient. Since they are involved in water homeostasis, it is important to be able to control the flux of water to maintain normal physiology in cells. This is highly relevant for unicellular microbes or plants where the surrounding conditions rapidly changes. A strategy to prevent water from leaking out is hence needed. Post-translational regulation of the transport through aquaporins is controlled in two ways, either by gating or by trafficking.

Many of the aquaporins have putative phosphorylation sites in their primary sequence, which opens up for the possibility of regulation by phosphorylation; this has also been confirmed experimentally. Spinach aquaporin SoPIP1;2, has been crystallized in both open and closed conformation, where it was suggested, based on structure and MD simulations, that a drop in pH or de-phosphorylation closes the structure (35). AQP0 in eye lens, has been showed to be positively regulated by a pH drop in the physiological range but also by Ca^{2+} levels (47). It is also suggested that rapid regulation in Aqy1 from *P. pastoris* occurs through phosphorylation together with mechanosensation (Paper I). This is important for survival during repetitive rapid freeze-thaw cycles (36). The aquaglyceroporin, Fps1, in *S. cerevisiae* has solved this by closing the pore upon osmotic chock to allow for the accumulation of intracellular glycerol and to prevent loss of waterto regain turgor (48).

Transport assays in aquaporins

General considerations of using yeast in transport assays

Yeast cell wall composition

Yeast is surrounded by a cell wall that is a rigid construction outside the plasma membrane. It fulfills four tasks; stabilization during osmotic conditions, physical protection, maintaining cell shape and functioning as a scaffold for proteins (49). The construction is built up by four polysaccharides that are organized into three layers. From the plasma membrane they appears as: the innermost chitin layer (N-acetylglucosamine), a middle load-bearing glucan-layer (consists of glucose that is cross-linked as either 1,6-β-glucan or 1,3-β-glucan), and the outer most protective mannan-layer (49,50). Cell wall components are susceptible to degradation by different agents targeting the different layers e.g. zymolyase hydrolyses 1,6-glucosides bonds. These compounds are used for cell wall degradation when making protoplasts, i.e. yeast cells without the cell wall that are very fragile and can easily burst (50).

Yeast and osmotic gradients

Aquaporins transport solutes by pore-mediated diffusion. This means that solutes will be transported down the concentration gradient (see Passive transport). By applying a higher concentration of the solute, in the media surrounding the cell, the solute will spontaneously be transported into the cell mediated by aquaporins. Transport rates are monitored directly (in the transported solute) or indirectly e.g. by the rate of swelling or shrinkage.

Deletion strains

In this thesis, the yeast species *Saccharomyces cerevisiae* and *Pichia pastoris* have been used in several assays. The benefits of working with yeast like *S. cerevisiae* are multiple; the availability and accessibility of different yeast strains, the number of deletion collections, and the variety of vectors and selection markers are a few examples (51). All information is gathered in the Saccharomyces Genome Database (SGD, www.yeastgenome.org). Molecular biology tools developed for *S. cerevisiae* have also been found to be applicable to other fungi like *P. pastoris.* It is also possible to use genes from *S. cerevisiae* in *P. pastoris,* so-called cross-complementation.

The ability to delete (remove the gene) or disrupt a gene (the coding sequence of the gene is manipulated in such way that gene transcript is interrupted), is a valuable tool in studying gene function through the loss of phenotype. In this thesis, we have generated two new deletion strains using both approaches (Paper I and Paper III). When a gene is deleted/disrupted it is also considered that gene replacement with an antibiotic resistance marker generates a selectable and stable strain (52). When the strain with the preferred

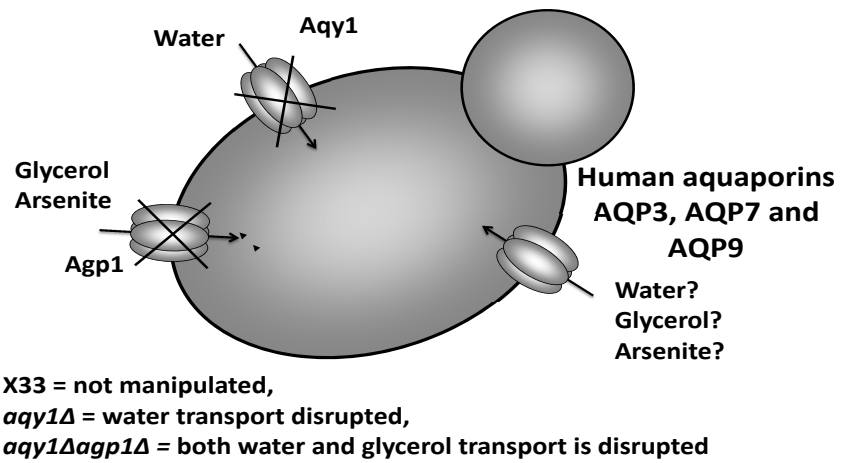
genetic background is generated, a recombinant gene is introduced into the cell to sustain in a plasmid or is incorporated in to the genome by homologue recombination. The application of deletion strains in Paper I have two functions; 1) reduce endogenous aquaporin expression that has been shown to be co-purified in high amounts, and 2) reducing background uptake in water transport assays. In Paper II, a second gene deletion, of the above-mentioned strain, was created to carry out transport assays (the strains are presented below).

P. pastoris GS115 aqy1::HIS4

P. pastoris GS115 is a strain auxotroph for histidine. Here, the HIS4-gene from *S. cerevisiae* (compatible with *P. pastoris*) was used to disrupt the *AQY1* allele (described in paper I). This is currently the primary strain of choice when producing recombinant aquaporins in our laboratory.

P. pastoris GS115 aqy1::HIS4 agp1::NATMX

A deletion cassette containing the resistance gene Nourseothricin, flanked by regions minimum 500bp length complementary to the upstream and downstream nucleotide sequence of the gene to be deleted, was used. After several rounds of optimizing every possible step in the transformation, we succeeded in deleting the complete gene ORF of an aquaglyceroporin homologue in the background to *P. pastoris* GS115 *aqy1::HIS4,* (generated the single deletion strain generated in Paper I) (Fig. 3). Phenotypic characterizations showed that the double deletion strain was able to grow on arsenite-containing plates, as opposed to the wild-type (X33 strain) and *P. pastoris* GS115 *aqy1::HIS4* strains. This strain has enabled comparative study of human aquaglyceroporins (Paper III).



Figur 3. Cartoon of yeast system used in transport assays. Both endogenous aquaporins *AQY1* and *AGP1* are deleted. The effect of deletions in transport assays is illustrated in PAPER I

Growth assay

In Paper II and Paper III we grew yeast cells on agar plates supplemented with various solutes. The basic idea is that the solute, e.g. arsenite, is supplemented into the solid growth media will be transported over the membrane by passive diffusion. Cells are spotted on the solid media in a 10x dilution series and differences in growth phenotype are observed. If growth, in comparison to control the strain (background strain), is affected the solute has been transported into the cultivated cells. If the cells take up e.g. arsenite, cells will exhibit reduced growth, as it is a toxic compound. Hence, this transport must then be through the expressed heterologous protein. Osmoregulation can also be tested in the same way by supplementing different osmoticum into the plates.

Transport assays

To measure transport through aquaporins in a system, three criteria need to be fulfilled; 1) a system with two compartments that is separated by a membrane 2) solute transport that correlates with a detectable signal and 3) a technique to measure the signal/substrate. Common systems used in aquaporin research are oocytes (frog eggs), yeast cells (unicellular organism), single cells (cultivated cells from certain tissue) and artificial vesicles (proteoliposomes).

Water transport using - Stopped Flow

The Stopped flow method (SFM) is used when studying fast reactions. The basic units of a SFM units are 1) two syringes (for reacting species) 2) a mixing chamber 3) an observation cell 4) a stopping syringe and 5) a recording system that can respond within a short period of time. The procedure is initiated by the two syringes that simultaneously push a fixed volume of reactants rapidly through the mixing chamber and through the observation cuvette. Reactants are then mechanical stopped by a third stopping syringe that prevents from further mixing of reactants and the detection is automatically initiated. The time that elapses between the initial mixing and to the arrival of the observation cell is equal to the dead volume of the reaction (53). The signal observed in the SFM is the change in intensity that is recorded in volts. The phenomenon observed is the scattering of cells/spheres. Hyper osmotic gradients, when applied, generate an outward movement of water; this results in a rougher surface and hence the scattering of more light. To evaluate transport rate recorded values are normalized and rate constants are calculated by using a single exponential curve-fit.

Glycerol transport using ^{14}C Glycerol

Radiolabeled uptake experiments were performed using a hyper osmotic glycerol concentration gradient of 300 mM, to drive glycerol influx into the cells. Levels of radiolabeled uptake are measured by a liquid scintillator that detects radioactive decay as counts per minute (cpm). Here we have used a mix of glycerol with ^{14}C labeled backbone and unlabeled glycerol to intracellular label taken up by cells.

In the initial uptake experiment (using *P. pastoris* GS115 *aqy1Δ strain* single deletion strain), glycerol uptake in the background strain was very high. In the literature, active H^+/driven glycerol uptake is reported to be found to increase intracellular glycerol levels for osmoregulation in halotolerant *Pichia sorbitophila* (grows on 4M NaCl) (54). Glycerol can even be transported against the concentration gradient (54). Since the protononophore, cyanide *m*-chlorophenylhydrazone (CCCP) collapses the membrane potential and with that, the proton driven glycerol uptake (55). Thus, we tested CCCP pre-incubation for 10 minutes and glycerol levels decreased drastically. This implies that *P. pastoris* is capable of proton-driven glycerol uptake. Therefore, CCCP was used in pre-incubations of glycerol uptake measurements.

Arsenite uptake – ICP MS

Arsenic is a toxic heavy metal that occurs naturally in earth´s crust and is known to cause, among other things, neurological and respiratory diseases and several types of cancer (56). Arsenic is present foremost in the pentavalent form (H_3AsO_4) termed arsenate. In solution, arsenate exists as an oxyanion that mimics phosphate and therefore can enter cells through phosphate transporters; it is also known to inhibit enzyme activity. The trivalent arsenite is much more reactive and hence more toxic due to strong binding to thiolates and closely spaced cysteines in proteins, thereby inhibiting the function of the modified protein. In solution at physiological pH, it is most abundant in the protonated form $As(OH)_3$ (pKa of 9.2) that mimics glycerol and hence is taken up by the cell through the aquaglyceroporins (57,58). This has been shown for the aquaglyceroporin Fps1 in *S. cerevisiae* and in the mammalian AQP7 and AQP9 (59-61) (Paper III). Detection of arsenite levels is not performed in our laboratory but the method used for this purpose is called Inductively Coupled Plasma Source Mass Spectrometry (ICP-MS).

Yeast aquaporins

Pichia pastoris

P. pastoris was discovered in 1919 and its application has been in biotechnology (62). It was initially used during the 1970s to produce Single-Cell Proteins (SCP) with the application to produce high protein food products for mainly used in animal feed (63). In more recent years, *P. pastoris* has developed into an excellent host for producing eukaryotic recombinant proteins at high yields (64,65).

P. pastoris is the name for a certain set of related yeast strains (66). The prototrophic *P. pastoris X33* and the histidine auxotroph *P. pastoris GS115,* which are the most commonly used strains for protein over-production, originate from the same parental strain classified as *Komagataella phaffii.* The first genome to be sequenced and published was the *P. pastoris* GS115 in 2009, which can be found at www.pichiagenome.org. The release aimed to increase the number of genetic tools and engineered strains to enhance the usability of *P. pastoris* in biopharmaceutical production (67).

Strict water facilitating Aqy1

The first aquaporin in *P. pastoris* was discovered in 2009 by solving a collected dataset of what was thought to be human aquaporins 1 (Paper I). AQP1 had been expressed in *P. pastoris*, purified and used in crystallization setups. The intriguing part of the discovery was that the resolution of diffraction was very high (1.15 Å) so that each amino acid could be mapped into the electron density with high accuracy. But the expected amino acid sequence from AQP1 did not fit. However, could parts of the amino acid sequence (from the unknown protein) be extracted from the electron density map and used to identify homologues proteins. Since homologoues were found in other *Pichia* strains the crystallized protein was most likely an endogenous protein from *P. pastoris.* The *P. pastoris* genome was not public at the time but a sequence attained from Cregg and co-workers with high sequence homology to yeast orthodox aquaporin, Aqy1 (56 % identity) could nicely be built into the map! No other aquaporin homologues were found in the genome sequence during that time as stated in Paper I, even though it is very rare for yeast to lack aquaglyceroporin.

Since the endogenous aquaporin was co-purified and prone to crystallize, an *AQY1Δ* deletion strain was made. Aqy1 was generated (Paper I) and studied by structural and functional analysis (Paper I). Crystallization procedures could be repeated showing that the Aqy1 structure is a gated channel that is blocked by its N-terminal. A tyrosine residue (Tyr31) in the N-terminal sticks into the channel and blocks the pore (Paper I, fig. 2). In this work, Molecular Dynamics simulations and water transport assay, using the SFM

method were applied to elucidate the regulatory mechanism of Aqy1 and Aqy1 variants (The work is presented in Paper I).

Cells expressing wild type protein showed increased water permeability (compared to the background strain) and are therefore functionally expressed and localized to the plasma membrane. Tyr31 was also identified to be responsible of blocking the channel since water transport was increased in the Tyr31Ala variants. Moreover, the involvement of the N-terminus was demonstrated since a truncation of the terminus (ΔN36), showed a remarkable increased water transport.

A serine residue, (Ser107) located in helix 4, was identified to be a part of a phosphorylation consensus sequence and thereby a potential player in the regulation of the channel. This is a likely event since posttranslational modifications has shown to be a part of the regulatory mechanism in aquaporins. (68). Collaborating groups performing MD simulations observed a widening of the pore when mutating this residue to an aspartate, (mimicking a phosphorylated serine). Further MD simulations also identified mechanosensitive gating as a second regulatory mechanism. The basic idea is that pressure changes within the membrane are transmitted from helix 6, which is in contact with the membrane, to the cytoplasmic side of helix 4 and the channel would open. This is not a farfetched idea since in hyper osmolar media, rapid volume changes in unicellular organisms membrane must generate increased lateral pressure within the membrane and hence put increased pressure on membrane proteins. Under these circumstances cells would lose water rather quickly unless the water-transporting channels will close. Therefore mechanosensation would mediate a fast and direct response upon sudden changes in the environment.

Aquaglycerporin Agp1

A second member of the aquaporin family in *P. pastoris* was found in 2012, when transport assays of human aquaglyceroporins were performed using the *aqy1Δ* deletion strain of *P. pastoris*. Both glycerol and arsenite background uptake was remarkably high in all assays. At the time of this study, the *P. pastoris* genome had become public. The gene Pipas_chr4_0784 encoding “an unnamed protein product” was identified to have 32 % identity to aquaglyceroporin Fps1 from *S. cerevisiae* and ≥40% identity to human aquaglyceroporins. With the sequence data in mind, Pipas_chr4_0784 was most likely an aquaglyceroporin. By deleting Pipas_chr4_0784, the background uptake of glycerol and arsenite was fully abolished (Paper III). The new *aqy1Δ Pipas_chr4_0784Δ* double deletion strain also gained an arsenite tolerant phenotype. Nomenclature of genes identified in *P. pastoris* should follow *S. cerevisiae* nomenclature and if the protein is an orthologue to the gene/protein in *S. cerevisiae* it should adopt the same name. Pipas_chr4_0784 is not that similar to Fps1 since there is a large difference in the length of the N- and C- termini (several other Fps1 orthologs are found in other yeasts, e.g.

Ashbya gossypii, with long N- and C- termini). Therefore, the gene was given the new name, AquaGlyceroPorin1, Aqp1.

The functional role of Aqp1 has not been further investigated in this work.

Saccharomyces cerevisiae

Yeast *Saccharomyces cerevisiae* has been used for thousands of years in bread baking and wine/beer making due to it skills to ferment glucose to ethanol and carbon dioxide in the absence of oxygen (69). It is a well-studied organism that is used as a model system in many different research areas. *S. cerevisiae* is a unicellular eukaryotic microbe that has been found to possess four aquaporin genes in its genome. *AQY1* and *AQY2* encode for two orthodox aquaporins while *FPS1* and *YFL054C* encode for two aquaglyceroporins (70). Mutations in *AQY1* and *AQY2* in laboratory strains have generated strains of *S. cerevisiae* without functional orthodox aquaporins, which indicates that aquaporins in microbes are not vital. *YFL054C* is not as well studied as Fps1, however, passive diffusion of glycerol has been observed in the presence of ethanol (71).

Aquaglyceroporin Fps1

Fps1 was first discovered in 1991 as a suppressor mutant (fdp1 **s**uppressor) in *S. cerevisiae*. The protein was identified to have the six transmembrane domains and the highly conserved NPA sequence that is homologous to other MIP family members (72). Unlike the other MIP found at that time, the yeast protein has an N-terminal extension of approximately 250 amino acids, and a C-terminal extension of approximately 150 amino acids (72). This protein belongs to the aquaglyceroporin subgroup and has been shown to transport glycerol and metalloids like arsenite and antimonite, but also other compounds such as ethanol, urea and acetic acid. (48,59,73,74). Fps1 is a gated channel that upon hyper osmotic shock closes to help yeast cells regain turgor during osmotic stress (75). Fps1 has shown to be regulated by phosphorylation during arsenite stress (68). The N- and C –terminal domains have been proven to be involved in the regulation of Fps1, and years of work have pinpointed parts of these domains that are particularly important for the regulation of the protein transport activity. Consequently, these parts have been given the names, N- and C-terminal regulatory domains (NRD and CRD) (75-78). Regulatory mechanisms where the termini are directly involved in blocking the pore and also mechanosensitive gating have been suggested, but the exact mechanisms are not yet fully understood (76,78).

Fps1 in involved osmoregulation

As mentioned previously, yeast cells are highly exposed to drastic changes in their environment. Unicellular organisms have adapted systems to produce compatible solutes to be able to control intracellular osmolarity and hence regulate the water flux, in and out of the cell (79). *S. cerevisiae* use glycerol as a compatible solute and has the ability to

produce glycerol by itself. Two important enzymes, Gpd1 and Gpd2, are responsible for converting a glycolytic intermediate (dihydroxyacetone phosphate) to glycerol (80-83).

Upon a hyper osmotic shock, the cell immediately starts to lose water and as a consequence also turgor pressure and cell volume. This event triggers Fps1 inactivation / closure. Signaling pathways activate the transcription of *GPD1* and *GPD2* genes. An intracellular glycerol concentration increases by the enzymatic activity of Gpd1 and Gpd2. When intracellular osmotic pressure exceeds surrounding osmotic pressure, water will enter the cell and as soon as the cell regains volume and turgor, Fps1 releases excessive glycerol and the cell can continue to divide (75).

A hypo-osmotic shock is the reversed condition, where the cell is subjected to low osmolarity media and hence water flow into the cell. As a consequence, cell volume and turgor pressure increases. To lower internal osmotic pressure, the cell releases excessive glycerol through Fps1. Cells incapable of releasing excessive glycerol will burst. Cells lacking Fps1 are sensitive to hypo-osmotic shock, due to the inability to quickly release turgor pressure to prevent bursting (75).

Potential regulation mechanism of Fps1

As mentioned previously, Fps1 regulatory properties are not fully understood and to investigate details in terms of open and closed states, it is common to quantify intracellular levels of glycerol after hyper- and hypo- osmotic conditions. In Paper II, the regulatory properties of Fps1and a set of Fps1 variants were investigated. A single amino acid substitution, Fps1-N228A, located in the N-terminus (identified in previous work), generates a constitutively open Fps1 (unable to regulate glycerol flux through the pore). A second amino acid substitution, Fps1-N228A-G519S, located in the membrane-spanning helix 6, was identified to reverse the hyperactive state of Fps1. The Fps1-N228A-G519S variant behaved as a regulated Fps1 in both hyper- and hypo- osmotic conditions as well as in the glycerol transport assay. This suggests a potential regulatory mechanism where the tranmembrane core is affected by phosphorylation events in the regulatory domains to restrict glycerol flux through Fps1 (Paper II). What is interesting in this finding is that MD simulations of Aqy1, in Paper I, showed that Aqy1 was also identified to undergo rearrangement upon increased pressure from the membrane. The participating helices were 4, 5 and 6. The suppressor mutation in Fps1 is located in helix 6, and this glycine in particular is described in the literature to be part of a highly conserved GXXXG motif, which is frequently occurring in alpha helical membrane proteins such as aquaporins (84). When this glycine is substituted to a serine, as in the Fps1 suppressor mutant, a polar and larger amino acid is introduced and would likely induce local rearrangement within helix 4 and 6, which in turn generates a restricted channel. Therefore, it is likely that phosphorylations of the Fps1 regulatory domains induce changes in the pore that causes the closure.

Human aquaporins

There are 13 aquaporins found in humans, AQP0-AQP12, which are frequently divided in two subgroups; orthodox aquaporins, with the primary function to transport water and aquaglyceroporins that in addition to water also transports small uncharged solutes, such as glycerol and urea (for further reading on classification see section conserved protein family). Aquaporins are highly present in water permeable tissues.

To identify physiological roles of human aquaporins, it is common to study phenotypes using knockout mice. However, this is not always translational to humans. For instance, it has been shown that expression patterns of aquaglyceroporins in mice, rat and humans differ and hence conclusions drawn from studies in knockout mice can be slightly misleading for human physiology. It is likely that humans are more complex in terms of redundancy in pathways than rodents and therefore it is more difficult to pinpoint which aquaglyceroporins that are involved in the shuttle of metabolic compounds such as glycerol or toxic substances such as arsenite. In humans four aquaglyceroporins are found; AQP3, AQP7, AQP9 and AQP10. AQP 10 was the last aquaglyceroporin to be discovered and is located mainly to the intestine and has shown to be permeable to water, glycerol, erythriol and xylitol (85,86). In this work I have studied AQP3 AQP7 and AQP9.

Aquaporin 3

The first human aquaglyceroporins was cloned from rat kidney by three individual groups in 1994 (87-89). The identified gene codes for a 292 amino acid long polypeptide, that has a potential phosphorylation site located in the third extracellular loop (N141) (88). The protein showed to have approximately 20 % sequence homology to AQP1 and AQP0 but 40 % sequence similarity to bacterial aqualgyceroporin GlpF. AQP3 expression in rat was found in several tissues like kidney, brain, spleen, urinary bladder, intenstine and colon (87-89). AQP3 has later been identified to be abundant in human skin, (basal layer of keratinocytes) intestine, colon, stomach, kidney basolateral membrane, eye and also in adipocytes and liver (90-93).

AQP3 has been reported to facilitate transport of water, glycerol and urea and being inhibited by mecurials and phloretin (87-89) (Paper III). Independent studies have also shown that transport efficiency is negatively affected by low pH (94,95). In paper III, we elucidate the transport of water, glycerol and arsenite of AQP3 over-expressed in yeast.

Hydration of skin is vital to uphold its function (96). Mice lacking AQP3 demonstrated delayed wound healing, reduced skin elasticity and lowered glycerol and water content in epidermis (97). These phenotypes could be reversed by adding glycerol systemic or topical, suggesting that it is the glycerol transporting function of AQP3 is of importance

rather than water transport. The fitness of wild type mice improved as well, which confirms the importance of glycerol in skin. Glycerol is also the main ingredient in many moisturizing skin products (98). Experimental results indicate lower glycerol permeability from blood to basal keratinocytes in AQP3 null mice but also a lowered skin hydration, compared to wild type (99). Recent studies correlate AQP3 to tumorgenesis in keratinocytes, where impaired glycerol uptake leads to reduced levels of intracellular ATP, which is connected to cell proliferation (100). This finding makes AQP3 interesting as a determinant in tumorgenesis and a novel target for cancer therapy.

AQP3 has been located to the basal layer in parts of gastro intestinal tract and is thought to be involved in water and glycerol flux across this barrier, even though the function is not clear (92). Dextran sulphate sodium (DSS) is used to induce an inflammatory state in colon. DSS induced colitis in AQP3 KO mice, showed enhanced development of colitis and shorter lifespan (101). Interestingly, Beutler and co-workers identified that an amino acid substitution in AQP3 in position 43 (V43A, called Phoebus phenotype) played a role in DSS colitis. AQP3-V43A mice induced with colitis (by DSS), showed weight loss and bleeding (102). The mice lost 15 % of its initial weight and died on day 5 of the experiment, while DSS-treated wild type mice appeared indistinguishable from that of untreated mice (102). These studies suggest that AQP3 play an important role in gastrointestinal tract and it is most likely due to its glycerol transporting function. Since a single amino acid substitution (V43A) seems to progress colitis as in the KO mice it is most likely that AQP3 lose its glycerol transporting function. In Paper III using the yeast system, we could detect accumulation of glycerol in a pH dependent manner. To further investigate if the Phoebus phenotype was caused by a loss of glycerol transport via AQP3, we introduced this amino acid substitution. Glycerol transport in AQP3-V43A was significantly suppressed, however, a small uptake was still present (in comparison to the background). Since water transport assays did not show water transport in NMR measurements but some water transport in the SFM experiments (Paper III), we suggest that it is the loss of glycerol transport in the basal lateral membrane that generate the severe phenotype in Phoebus mice. Since AQP3 is the sole identified aquaglyceroporin in basal layer of epidermis and basal membrane in epithelial cells in the intestine, it is tempting to speculate that AQP3 may be an important provider of glycerol for a both of these barriers (98) (101). Moreover, it would be highly relevant to investigate if Phoebus mice display any other phenotypic traits that has been observed for other AQP3 null mice.

Aquaporin 7

AQP7 was first identified and cloned from rat testis in 1997 (103). This was a 269 amino acid long protein that in the oocyte system showed transport of water (not inhibited by mercurial), glycerol and urea. In the same year, a gene encoding for a 342 amino acid long polypeptide was found in human adipocytes (fat cells) that in oocytes assays was permeable to water, in a mercurial inhibited fashion, and to glycerol. AQP7 has also been

identified in gastro intestinal tract and kidney (104). In comparison to other human aquaporins, AQP7 has longer termini with a putative cyclic-AMP protein kinase phosphorylation consensus site in the N-terminus (105). This gene was initially named AQP9 and short thereafter referred to as adipose AQP, AQPap or AQPL, today it is referred to as AQP7 (106). In our study we use AQP7 protein with accession number O14520.

AQP7, together with AQP9 has previously been identified to transport metalloids like arsenite (61). In this thesis, we show that AQP7 transport arsenite to higher extent, that AQP9 (Paper III).

In mice, AQP7 is the only aquaporin that have been identified in adipose tissue. KO mice for AQP7 was found to have low plasma glycerol levels after prolonged fasting suggesting AQP7 being responsible for glycerol flux in and out of adipocytes (107). Increased body mass fat, enlarged adipocytes and lowered glycerol release has also been observed (108). Feeding AQP7 KO mice a high fat diet, promotes obesity and insulin resistance (109). AQP7 is also expressed in kidney proximal tubule where it has the role of reabsorbing glycerol. KO of AQP7 showed hyperglyceroluria but no significant effect on water transport was detected (110).

Human natural variants of AQP7 such as R12C, V59L are both capable of transporting water and glycerol while in G624V variant the transport was abolished (111). Transport of AQP-G264V was investigated in Paper III also showing that glycerol transport was heavily suppressed, while arsenite transport was totally abolished. In studies in humans homozygous for AQP7-G264V (both alleles carrying the mutation), increased hyperglycerolurea was detected (112).

Aquaporin 9

Aquaporin 9 was first identified in 1998 in leukocytes (white blood cells) and has been found in various tissues like liver, lung, spleen testis erythrocytes and brain (103,113-115). The gene encodes a 295 amino acid long polypeptide. In the oocytes system, AQP9 was identified to transport water in a mercurial revisable fashion and urea. Glycerol permeation was not observed, but sequence homology to AQP3 and AQP7 placed AQP9 among aquaglyceroporins (113). In a reevaluation by the same group, AQP9 was identified to facilitate substrates including carbamides, polyols, purines, and pyrimidines in a phloretin- and mercurial-sensitive manner (116). The fact that AQP9 in other transport studies (using oocytes from *Xenopus Leavis)* showed enhanced permeability to water and glycerol, urea, sorbitol, uracil and arsenite, suggest that AQP9 is a rather promiscuous aquaporin (60,114,117,118).

The main expression site for AQP9 is the liver (119), and it was considered to be the sole aquaglyceroporin there until recently when both AQP3 and AQP7 were identified in

human liver (90). The two latter aquaglyceroporins has not been observed in mouse and rat liver. The role of AQP9 in liver, as a glycerol transporter, has been investigated. During fasting, mRNA levels of AQP9 increases and in contrast AQP9 expression is repressed by insulin (120). Expression of both AQP7 (adipose tissue) and AQP9 (in liver) are both increased in insulin resistant mice (120). In AQP9 KO mice, elevated glycerol levels has been observed and at the same time blood glucose levels was decreased compared with control mice (119). Since glycerol is a major substrate for gluconeogenesis, where glycerol is converted to glucose in the liver, it is believed that AQP9 play an important role in glycerol influx of hepatic cells.

The property of transporting such a variety of compounds makes AQP9 to have the broadest specificity among mammalian aquaporins studied so far. However, in our transport assay we observed that AQP9 transport arsenite almost to the same extent as AQP7 but glycerol transport is not equally good (Paper III). We could not able to detect water transport using SFM method or diffusion NMR (Paper III). Taken together, transport studies of AQP9 in literature are contradictory to our result and the possibility conclusions drawn that human AQP9 and rat AQP9 have different transport properties should be investigated (121).

The importance of the highly conserved arginine (in the Ar/R-constriction region) has been investigated by Liu and co-workers (61). They showed that by changing the highly conserved arginine in the constriction region to a similar residue like lysine in AQP9 (from rat), did not really affect either arsenite or glycerol transport (61). However, when substituting the same arginine to a smaller hydrophobic amino acid like alanine, the transport of both solutes was abolished. This suggests that a positive charged amino acid at this position is also important for the transport of glycerol and arsenite, as has previously been observed for orthodox aquaporins (46).

It would be most interesting to gain more detailed structural and functional insight of AQP9 since this aquaglyceroporin show broad specificity in transport assays and is suspected to occlude water, as shown in Paper III. So far there is no available three-dimensional structure of any mammalian aquaglyceroporin, only a projection map of AQP9 at 7 Å resolution, which confirms the typical aquaporin fold and a tetramer formation in the membrane. (122).

Overproduction of membrane proteins

Membrane proteins act as "communicators" between cells and their environment and therefore they are key players in living organisms and hence interesting as drug targets. Drugs need to be highly specific in order to target a single protein. In that aspect, a protein's three-dimensional structure would generate valuable information. However, structural insight into the membrane proteome is still lacking. Approximately 20-30 percent of the genome encodes for membrane proteins (123), but yet only 374 unique membrane protein structures have been generated using X-ray crystallography (approximately 20 of those are human proteins)(124). The biggest hurdle in contributing structural and functional characterization to the membrane protein field is the process of generating sufficient amounts of pure, solubilized and reconstituted proteins.

Today, only two three-dimensional structures have been solved using X-ray crystallography representing the subgroup of aquaglyceroporins (29,37). However, both are from microbial sources and structural insights of mammalian aquaglyceroporins remains to be solved. In Paper III (and in the previous chapter), we show that there are differences in solute specificity, selectivity and regulation among the three human aquaglyceroporins (AQP3, 7 and 9). The underlying mechanism would be highly relevant to study in more detail, to elucidate the role in human physiology as potential drug targets and also implications in medical treatment. But in order to get there, we must first understand the underlying mechanism of solute permeation among aquaglyceroporins. In order to perform crystallization setups and functional characterization, pure soluble protein is needed.

Overproduction in *Pichia pastoris*

P. pastoris has proven to be a successful system in generating recombinant eukaryotic membrane proteins. This is sufficient in the field of structural biology where milligrams pure protein is required for crystallization setup. Since it is a eukaryotic host, it has the machinery necessary to produce eukaryotic proteins like protein processing, protein folding and posttranslational modifications (125). *P. pastoris* fulfills many criteria of being a promising expression host for the aquaglyceroporins. First, it has been used to overproduce several eukaryotic aquaporins that has been successfully crystallized and whose 3D-structures have been determined using X-ray crystallography. Second, the protein yield of human aquaporin 1, expressed in *P. pastoris* was reported to be exceptionally high (65). Third, *P. pastoris* is an in-house system hence knowledge and equipment are easily accessible.

P. pastoris is a methylotrophic yeast that has the ability to utilize methanol as its sole carbon source (125). The metabolisation of methanol takes place in the peroxisome,

where the initial step of methanol conversion to formaldehyde is catalyzed by the enzyme alcohol oxidase (Aox) in the presence of oxygen. Aox has poor affinity for oxygen, and to compensate for that this enzyme is produced to high levels. Two AOX genes are present in the genome, AOX1 which is controlled by a strong promoter and AOX2 under the regulation of a weak promoter. Both genes are controlled at the transcriptional level and are tightly regulated. AOX genes are completely repressed in the presence of other carbon sources like glucose and glycerol (63,126,127). The fact that AOX1 is such a strong promoter is utilized to drive recombinant protein expression in a controlled manner by feeding methanol. The recombinant gene is integrated into the genome behind the AOX1 promoter (125).

Construct design

Sitting at the drawing table designing constructs for protein expression there are a few things to consider. It is beneficial to generate constructs with affinity-tags fused to either N or C-terminal since tags might affect the expression of the protein. The advantage of using a C-terminal fused tag is that a detection of the tag is a verification of a full length protein expression. The length of a poly-histidine tag is also to be considered. Increased length (from six to ten histidines) can reduce expression levels but increase affinity for Ni-NTA resin, either by increasing the affinity of the tag or that the tag will be more exposed to the resin (128). In later years, high-throughput strategies have used Green Fluorescent Protein (GFP) fusions for quantification and detection during the expression and purification process (129-131).

How to choose a well expressing clone

Three constructs was prepared for each gene (AQP3 AQP7 and AQP9) N-terminal fused His-tag including a C3 protease cleavage site, C-terminal fused His_6-tag and an untagged protein. All constructs have been transformed into three different *P. pastoris* strains, X33, GS115 *aqy1Δ::HIS and* GS115 *aqy1Δ::HIS4 aqp1::NATMX,* at three different occasions.

"Jackpot clone" is a term used by Invitrogen to describe the clone that has multiple insertions of a recombinant gene fragment at the site of recombination behind the strong AOX1 promoter (125). The frequency for this event to occur is rather low, approximately 1-10 % of all transformants (132). A common strategy to screen for potential "jackpot clones" is to let transformants grow on agar plates containing increasing zeocin concentration. This means that you will have to check at least hundreds of cells to be able to identify such clone. In Paper IV, we have further optimized this method. The idea is to apply the method in a 96 well plate format. We can also show how nicely the expression increase for clones that is selected at higher concentration of zeocin. If these are a true jackpot clones, with multiple recombinant gene insertions behind the AOX promoter, are not verified at the genetic level but it is likely since high protein expression was confirmed. However, it has been reported that increased gene dosage correlates with

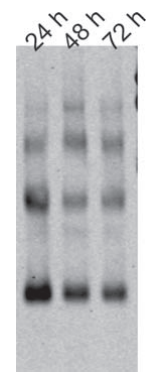
increased protein expression (133). Moreover, to screen for transformants that originates from different constructs is a lot of work and by using this 96 well based format saves time. We also observed that low number of cells in each spot reduce the number of false positives since the antibiotic concentration in the plate is constant. Taken together, the frequency of multiple insertions is considered to a rare event (1-10%) and during this work I have observed 0-5 mutants on high zeocin concentration (since this method was applied), the minimum number of cloned that should be screen are at least in a 96 well plate.

Expression of human aquaglyceroporins

Paper IV includes the report of the overproduction of AQP3 and AQP7. Overproduction of a membrane protein contains a lot of work regarding finding the most optimal conditions, which is not always published and will therefore not be available for others. Thereby, I will in next section complement Paper IV with some of the findings encountered during protein expression.

Cultivation parameters and cell disruption

P. pastoris has the ability to grow to high densities using bioreactors and generate approximately 500-600 g wet cells per bioreactor cultivation (1.5 L cultivation media at start). In comparison to cultivations in shaker flasks, which generate approximately 8-10 g wet cells / L cultivated media. In flasks, usually 6L is cultivated per time generating 45-50 g wet cells. Methanol induction time is optimally 24 hours for AQP3 (Fig. 4). Many parameters might affect the choice of cultivation procedure but yeast cell wall seems to be less rigid after cultivations for 24 hours and is much more easily to break up cells, (that is a necessary step to isolate membranes). After cultivation, cells are disrupted and total membrane is isolated. Here, shaker-flasks generates a noteworthy 2.5 times higher yield (recorded in weight) compared to cells cultivated in bioreactors. Cell disruption is an important step since unsuccessful cell disruption will generate lower yield of total isolated membrane in relation to the amount of cells that is disrupted from start. Analysis of pellet in bright field microscopy after initial preparative centrifugation reveals that sedimented material mostly contains undisrupted cells.

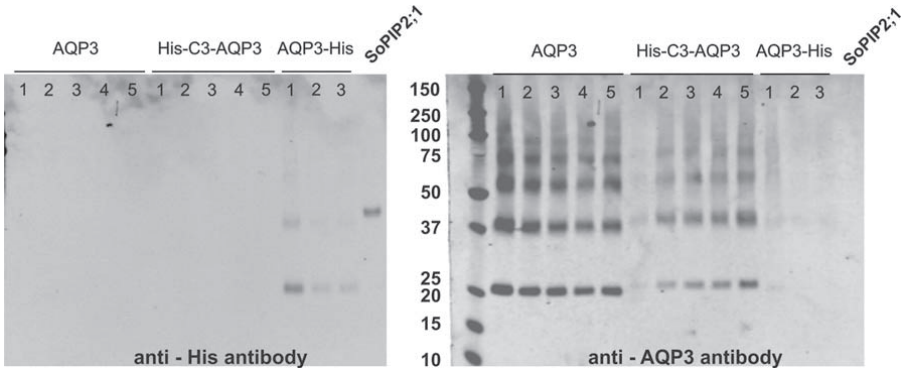


Figur 4. Western blot of time course expression study of AQP3.

Expression analysis using western blot-antibody issues

Clones that grow on high zeocin will be used in expression screens and their protein expression level is observed using western blot analysis. Here I would like to stress the importance of using two different antibodies, if possible. During the initial expression screen, in the beginning of this thesis, we could not observe expression of N-terminal tagged proteins. Then we assumed that the selected clone did not express the protein.

Even though it has been suggested that tags fused to the N-terminal in most cases generates higher expression level and is for instance the first choice at Structural Genomics Consortium (SGC) (134). Since I also work with untagged proteins, an antibody raised against the protein itself (here AQP3) was used in western blot analysis of an expression screen for untagged proteins. As usual, we could not observe any signal of the N-terminal His-tagged protein (left blot), however, when an antibody against the protein itself was used, observed expression pattern was totally opposite from what was expected (Fig. 5).



Figur 5: Western Blot of untagged, N-terminal tagged and C-terminal tagged AQP3 protein expressed in small scale expression screen. Equal amounts of total protein were loaded for each clone. Antibody directed towards AQP3 showed that N-terminally tagged protein express better than C-terminally tagged protein. Western Blot analysis is performed using fluorescent antibodies that are scanned at different wavelength. Left membrane is scanned at 800nm and left picture is the same membrane scanned at 680nm. His-tagged SoPIP2;1 mutant was included as control for both antibodies.

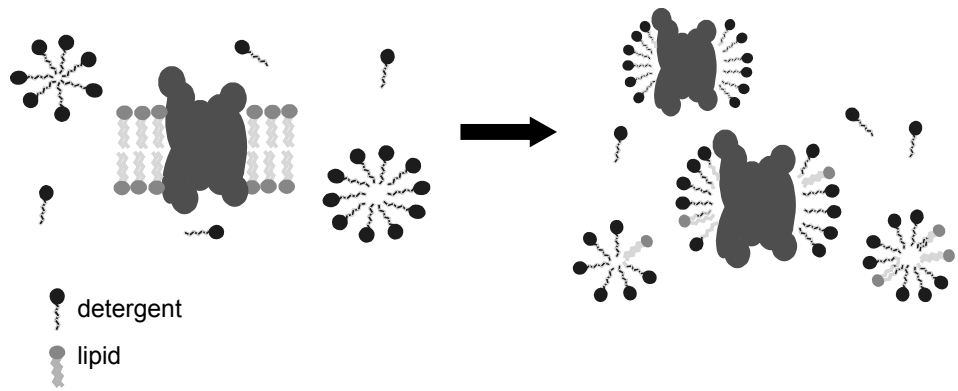
Increased expression in GS115 *aqy1Δ::HIS4 aqp1Δ::NATMX*

During the preparation of all strains used in the comparative functional analysis, which is presented in Paper IV, following observation was done. In the western blot analysis of identified clones (all isolated from high zeocin screening) I observed higher production levels than what was observed in the single deletion strain, which has been used for overproduction and purification in Paper IV. Due to the high expression, we cultivated them in bioreactors. However, AQP3-His had problems to grow already in the glycerol-containing batch phase (tested two times). In this phase, normal growing cell increase in density quite rapidly. The same clone grows perfectly fine in normal growth media, containing either glycerol or methanol expressing AQP3. We have also observe that AQP9 displayed much better expression in the double deletion strain and is able to grow without any problems in bioreactors.

Purification and characterization

Solubilization

The lipid bilayer shields the amphipathic surfaces of membrane proteins. To be able to extract and solubilize membrane proteins, detergents are used. Detergents are small amphiphilic molecules that cover the hydrophobic surfaces of membrane proteins with their hydrophobic alkyl chains. The formed protein detergent complex, (DPC) is often generalized as a micelle–like complex but suggest that it is a monolayer covering the hydrophobic surfaces (Fig. 6) (135,136). The important part is that the protein can remain stable and soluble in the DPC.



Figur 6 Solubilization of membrane proteins using detergents. Detergent dissolves both lipids and protein generating Lipid Detergent complex (LDC), Lipid Protein Detergent Complex (LPDC) and Detergent ProteinComlex (DPC). Solubilisation can also be considered as a purification step since all membrane proteins is not solubilized in the same detergent and hence it is important to screen for a detergent that can both solubilize and keep a given membrane protein stable in solution.

The choice of detergent is most often selected based on the application of extracted protein. If the aim is to crystallize the protein, it is highly recommended to choose a detergent with short alkyl chains to increase crystal forming contacts within the crystal. However, short-tailed detergents are often considered as harsh and hence denature the protein most likely since they cannot substitute the native membrane (137). In a review by Newstead, they evaluated parameters for crystallization that generated a resolved 3D structure. Glucopyranosides and maltopyranosides showed to be the most successful class of detergents that has been used in successful crystallization of α-helical protein (138). The difference is the head group as referred by the name. Initially I screened for a suitable detergent trying to have all types of detergent represented in terms of property e.g. uncharged, charged and amphiphilic. Nevertheless, glycosides and especially maltosides were consistently performing well in the screen. Presence of reducing agents like DTT improved solubilization for AQP3. Another approach that I initiated but never really continued to work with, was to use different ratios of detergent in one solubilization

condition. In paper IV, in figure 2, the final detergent screen is shown. Here we have decreased the number of detergents presented since they all were unsuccessful in solubilising AQP3 and AQP7.

Aquaporins generate a banding pattern on SDS page

Purification using Ni-NTA resin generated a sample with high purity. To evaluate the condition in terms of homogeneity and stability protein is subjected to size exclusion chromatography. The chromatogram displayed in Paper IV (Figure3) shows two peaks, the first peak, correspond to the void volume that is typical for protein that aggregates or form multimeric complexes, and the second peak corresponds to the purified correctly folded protein. Evaluation using SDS- PAGE, reveals a peculiar banding pattern that seems to be common among of aquaporins. Working with aquaporins for several years makes you blind to these phenomena and it is just accepted, but the reason for the banding pattern has been under speculation. SDS is the denaturing agent used in SDS-PAGE. The molecule is similar to other detergents, a 12 carbon long alkyl chain and head group. One conclusion drawn in a recent study was that SDS micelles are capable of preserving tetrameric formations of transmembrane domains (139) and especially if the protein has more or less intrinsic stability. In fact, we do see a more distinct multimeric banding pattern from AQP3 in comparison to AQP7 which rather generates a more distinct band corresponding to the monomer. AQP7 contain approximately 50 more residues than AQP3 and most are located outside the membrane in the N-and -C terminal parts. A potential reason for this difference is that the forces holding AQP7 tetramers together are weaker compared to AQP3 and thus SDS more easily breaks AQP7 apart. It is also noteworthy that Aqy1, which is studied in Paper I, migrates as a monomer (using Bis-Tris gels) but the S107D migrates as dimer using Tris-glycine gels.

Circular dichroism and reconstitution of protein into liposomes

Circular dichroism (CD) spectroscopy is a method that is a useful tool to be able to estimate secondary structures of proteins. The method has been around since the 1960s where it was shown that secondary structures reflect different types of spectra. Particularly, in the far ultraviolet (UV) region, 190-250 nm, α-helices generate two strong minima at 208 nm and 222 nm and a maximum at 217 nm (140). Aquaporins are alpha helical proteins and in solution (as detergent protein complexes (DPC)) generates nice spectra with two clear minima's (86,141). By using CD spectroscopy we could verify that both AQP3 and AQP7 contained alpha-helical structures and that most likely a properly folded protein in solution (Paper IV).

After a protein (applies to both soluble and membrane proteins) has been purified the best verification of a native folded protein is to perform an activity assay. If the proteins still are functionally active it is likely that the protein has its functionality and native fold. Aquaporins are pores, transporting a solute from one side of a membrane to the other side,

therefore there is no assay to test for activity in solution. A good way to test for transport of purified aquaporins is to incorporate the protein back into artificial spheres. This is done by using different sets of lipid extract that form artificial double-layered vesicles, also called liposomes. The purified protein will then be reconstituted into the liposome that is now called proteoliposomes. Lipids used for liposomes in aquaporin research has been both from *E.coli* lipid total extracts or from mixing different types of lipid species e.g. POPE, POPS and POPC (86),(141). A crucial step is the incorporation of membrane protein in to liposomes. Slow dilution or removal of detergent "force" proteins into liposomes since it is more favorable for the hydrophobic regions to be shielded in the liposome lipid double layer with decreasing detergent concentration. By evaluating proteoliposomes with CD, we concluded that AQP7 had been successfully reconstituted into the liposome. However, this was not observed for AQP3.

Summary of papers

Paper I

Crystal structure of yeast aquaporin at 1.15Å reveals novel gating mechanism

(2009) *PLoS Biol* 7, e1000130

Summary

In Paper I, the first and to date the only yeast aquaporin structure available are presented to 1.15 Å. This is the highest resolution reported so far for a membrane protein. Yeast aquaporins has the trait of long termini that has been suggested to participate in gating. The work presented in this paper show of an excellent combination of structural insights that generate clues of biological functions that can be tested in functional analysis.

Structural studies show that the protein is gated by its N-terminal by a tyrosine sticking into the pore and blocking water permeation through the channel. Two potential mechanisms were proposed to open the gate 1) phosphorylation of a serine close to the pore opening and to the tyrosine that block the channel and 2) by mechanosenstaion. The first mechanism was further strengthened by substitution studies by introducing an amino acid that mimics a phosphorylated serine at the predicted phosphorylated site. This generated increased water flux in experimental procedure. MD simulation predicted a pore opening of both mechanisms.

We could also show that Aqy1 increase survival rates drastically in *P. pastoris*. This fact underlines the relevance of possessing aquaporins in unicellular organisms in their natural environment.

P. pastoris GS115 *aqy1::HIS4* deletion strain that was generated in this work serves as the premier strain to express recombinant aquaporins today in our lab.

Paper II

Yeast aquaglyceroporins use the transmembrane core to restrict glycerol transport.

J Biol Chem. 2012 Jul 6;287(28):23562-70

Summary

Microbial aquaglyceroporins are a transmembrane protein that facilitates solutes across the membrane. Fps1 in *S. cerevisiae* (ScFps1) facilitates glycerol in a regulated fashion and regulatory domains have been identified in both N- and C-termini. Extensive amount of work have been invested trying to find out regulatory mechanism of this protein, but still it remains elusive. Here, we have contributed with a novel discovery of regulatory property in the transmembrane core.

An identified Fps1-homologue in *Ashbya gossypii* and growth assays indicated that AgFps1 behaves as a hyperactive channel in *S. cerevisiae.*

In complementation analysis, where Fps1 "membrane core" fused with either N- or C-termini (or both) from AgFps1, no osmosensitivity was observed. On the other hand, AgFps1 chimeras fused with N- or C- termini (or both) from ScFps1 gained sensitivity towards hyper osmotic growth conditions. This suggests that the core of Fps1 overrules the role of the termini, in regulating flux through the channel.

An amino acid substitution (Fps1-N228A) has been identified to generate an unregulated Fps1 that is constantly opened. This mutant is also sensitive for hyper osmotic conditions. In a screen for suppressor mutants, a second amino acid substitution situated in the transmembrane core, was identified. This substitution could reverse thehyperactivity of Fps1-N228A to function as a wild type Fps1. Thereby, we suggest that the role of the transmembrane core of Fps1 is to regulate glycerol flux through the pore. A potential mechanism would be that the regulatory domains (NRD and CRD) fine tunes the transport of glycerol by interplaying with the transmembrane parts.

Paper III

Differences in transport efficiency and specificity of aquaglyceroporins explain novel roles in human health and disease

Submitted to *J Biol Chem.*; under review

Summary

The role of aquaporins in human physiology has been studied, but the role of aquaglyceroporins is still lacking. First of all, we need to gain knowledge of the physiological role by understanding the biology of their substrate specificity for glycerol and arsenite. Several characterization assays have been performed for each aquaglyceroporin separately, most commonly using oocytes. The aim with this study was to 1) develop a system that can be used for further functional assays and that could express recombinant protein to high yields 2) compare and elucidate potential differences in transport specificity and selectivity among three human aquaglyceroporins 3) investigate the effect of amino acid substitutions that has implications in human physiology

In this study, we included AQP3, AQP7 and AQP9. Since we want to have high expression levels of our proteins, we aimed to perform all experiments in *P. pastoris*. This yeast specie is rarely used as model organism hence methodology and genetically modified strain are not available as for *S. cerevisiae.*

In both arsenite and glycerol uptake assays, no accumulation of neither substrates were observed. Since the most common approach to characterize a gene product is by loss of function, we have characterized a new aquaglyceroporin, and thereby the name AquaGlyceroPorin1 (Agp1). With this new strain in our hands, we have enabled the possibilities to perform transport studies of aquaglyceroporins to elucidate transport mechanism.

Comparative studies of human aquaglyceroporins, where several substrates has been included, are not to be found in literature. To our knowledge, we have for the first time performed such study in our new yeast strain. We were able to test glycerol, arsenite and water transport. Transport assays showed that AQP7 had higher capacity to transport glycerol and arsenite than AQP3 and AQP9. But AQP9 was almost equally good as AQP7 to transport arsenite. However, growth on arsenite containing plates clearly showed that growth of cells expressing AQP7 was poorer than cells expressing AQP9. AQP3 transported glycerol almost as good as AQP9 and the transport was shown to be gated in a pH dependent manner). At physiological pH, AQP3 did not transport arsenite. Water transport could only be observed in AQP7 using NMR diffusion, however, AQP3

indicated to have some water transport capability in SFM experiments. For all aquaglyceroporins, poor water transport was observed, as compared to orthodox aquaporins.

We also investigated transport in a set of varinats of AQP3, AQP7 and AQP9. Since individuals homozygous for the amino acid substitution AQP7-G264V show disturbed glycerol plasma level and hyperglycerolurea and amino acid substitution in AQP3-V43A (in mice) called Phoebus phenotype generate weight loss and bleeding upon induced colitis, we wanted to test whether we can state that the substitution in either aquaglyceroporins affect glycerol flux and hence responsible for the phenotypic outcome. Note that the glycine substitution found in AQP7 is the same glycine as the suppressor mutation found in Fps1 (Paper II).

We observed that arsenite transport was totally depleted while glycerol levels were heavily suppressed but not abolished. This opens up for the fact that solute permeation of different substrate through the pore is not the same.

Paper IV

Overexpression and characterization of human aquaglyceroporins AQP3 & AQP7

Manuscript

Summary

The task of generating sufficient yield of pure membrane proteins that can be used for further functional and crystallization setups are a difficult task. Several bottle necks that are encountered in the process are reflected in the literature. Many high throughput projects try to elucidate and shed light on key event that lead to a faster progress of gaining structural and functional information of membrane proteins.

To combine structural and functional assays we need pure protein. In this manuscript, we have summarized the path from optimizing clone selection to reconstitution of pure protein into proteoliposomes.

First, without any good expression level there will not be a good yield in the final step. To increase the odds, we made optimizations in the clone selection step using zeocin screening in a 96 well base format. A clone generating exceptional high protein expression is a rare event. Clone that is selected in this expression screen showed to express protein to higher levels than clones on lower zeocin concentrations.

Not all membrane proteins can be solubilised in any detergent therefore it is of high importance to identify a detergent that is capable of pulling the protein out from the membrane and into the solution, forming detergent protein complexes.

We have successfully extracted and purified two human aquaglyceroporins, AQP3 and AQP7.

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I´m running out of time so I have to wright this in Swedish, so try google translate…

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