In Vitro Analysis and Modification of Aquaporin Pore Selectivity

Eric Beitz, Dana Becker, Julia von Bülow, Christina Conrad, Nadine Fricke, Amornrat Geadkaew, Dawid Krenc, Jie Song, Dorothea Wree, and Binghua Wu

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Abstract Aquaporins enable the passage of a diverse set of solutes besides water. Many novel aquaporin permeants, such as antimonite and arsenite, silicon, ammonia, and hydrogen peroxide, have been described very recently. By the same token, the number of available aquaporin sequences has rapidly increased. Yet, sequence analyses and structure models cannot reliably predict permeability properties. Even the contribution to pore selectivity of individual residues in the channel layout is not fully understood. Here, we describe and discuss established in vitro assays for water and solute permeability. Measurements of volume change due to flux along osmotic or chemical gradients yield quantitative biophysical data, whereas phenotypic growth assays can hint at the relevance of aquaporins in the physiological

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setting of a certain cell. We also summarize data on the modification of pore selectivity of the prototypical water-specific mammalian aquaporin-1. We show that replacing residues in the pore constriction region allows ammonia, urea, glycerol, and even protons to pass the aquaporin pore.

1 Introduction

The bursting of *Xenopus laevis* oocytes in a hypotonic bath was the Eureka moment of aquaporin (AQP) research. It functionally proved osmotic water permeability of the red cell and kidney CHIP28 protein, later termed AQPI (Preston et al. 1992). This now classical – though still commonly used – in vitro assay, together with reconstitution of the protein in liposomes, were indispensable tools for the determination of the Arrhenius activation energy of water permeation (Zeidel et al. 1992), estimation of the permeation rate, and analysis of aquaporin inhibition by mercurials (Preston et al. 1993). The obtained data immediately confirmed that aquaporins account for the so-far unexplained high water permeability of erythrocytes and certain epithelia (Solomon et al. 1983; Verkman 1989).

Later, permeability for small, uncharged solutes, such as glycerol and urea, was shown by the oocyte swelling assay and other in vitro experiments leading to the functional division of the aquaporin protein family into water-specific, orthodox aquaporins, and aquaglyceroporins (Ishibashi et al. 1994; Zardoya 2005). Coefficients of water (\(P_f\) or \(L_P\)) and solute (\(P_{solute}\)) permeability are now routinely measured to functionally categorize novel aquaporins. In vitro assays further revealed that the list of aquaporin permeants is not complete with water, urea, and glycerol (plus somewhat longer polyols), but also includes carbonyl compounds (Pavlovic-Djuranovic et al. 2006), antimonite/arsenite (Wysocki et al. 2001; Liu et al. 2002; Gourbal et al. 2004), silicon (Ma et al. 2006), ammonia (Holm et al. 2005; Zeuthen et al. 2006; Saparov et al. 2007), hydrogen peroxide (Bienert et al. 2007) and, in the case of AQP6, even anions such as nitrite and chloride (Yasui et al. 1999).

Current questions on the inner workings and intramolecular regulation mechanisms of aquaporins are addressed using in vitro analyses of specific aquaporin mutants. Where are filters located that allow aquaporins to select between different permeants? How do aquaporins exclude protons and other ions? How is osmotic and pH-dependent pore gating of certain mammalian, plant, and yeast aquaporins accomplished? Today, prediction of such selectivity and regulation mechanisms is a domain of molecular dynamics computer simulations and quantum mechanical calculations (de Groot and Grubmüller 2005; Chen et al. 2006). Yet, real-world in vitro measurements are needed to complement in silico data or to challenge theoretical models (Beitz et al. 2006).

Aquaporins are also becoming the focus of the pharmaceutical sciences. Their physiological and pathophysiological relevance and the consequent desire to therapeutically modulate cell water permeability (Beitz and Schultz 1999; Castle 2005; Jeyaseelan et al. 2006; Frigeri et al. 2007) started the search for small molecule aquaporin inhibitors (Brooks et al. 2000; Detmers et al. 2006; Huber et al. 2007;
Huber et al. 2008). This calls for industry-style high-throughput assay systems that allow for rapid screening of large compound libraries.

Aquaporin assay systems range from living cells, i.e., bacteria (Hubert et al. 2005; Mallo and Ashby 2006), yeast (Luyten et al. 1995; Jahn et al. 2004; Beitz et al. 2006; Pettersson et al. 2006), and mammalian cells (Solenov et al. 2004; Gao et al. 2005), via preparations of cell organelles (Laizé et al. 1995; Calamita et al. 2006), to artificial proteoliposomes (Zeidel et al. 1992; Borgnia and Agre 2001) and even planar lipid membranes (Saparov et al. 2001). Depending on the nature of the assay, information is gained about the biophysical properties or on the physiological relevance of an aquaporin if the survival of cells depends on its function. This chapter discusses quantitative and phenotypic aquaporin assays for various permeants as well as mutations of the prototypical water-specific AQP1 that modify pore selectivity.

2 Quantitative Assays of Water and Solute Flux

Quantification of water flux through aquaporins requires a two-compartment system in which an osmotic gradient can be rapidly established. The sudden disturbance in equilibrium will drive water across the dividing membrane to revert the system to equal osmolality. Resulting flow rates depend on the number of aquaporins as well as their permeability properties, and are monitored by the volume change over time. Permeability for osmotically active solutes can be similarly analyzed by creating a chemical gradient between compartments with equal osmolality. The solute flow will then generate an osmotic disturbance leading to secondary water flow and volume change. In either case, i.e., water or solute permeability assays, the magnitude and time scale of the volume change dictate the method to be used for monitoring (see Table 1).

2.1 Xenopus laevis Oocytes

Oocytes of the South African claw frog Xenopus laevis have been used in physiology research laboratories for decades. Due to their large diameter (≈1.2 mm; Table 1),

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Diameter (cm)</th>
<th>Surface (cm²)</th>
<th>Volume (cm³)</th>
<th>Surface/volume (cm⁻¹)</th>
<th>Swelling time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xenopus oocytes</td>
<td>1.2 × 10⁻¹</td>
<td>5.3 × 10⁻¹</td>
<td>9 × 10⁻⁴</td>
<td>5.9 × 10²</td>
<td>100</td>
</tr>
<tr>
<td>Yeast, red cells</td>
<td>10⁻³</td>
<td>3 × 10⁻⁶</td>
<td>5 × 10⁻¹⁰</td>
<td>6 × 10³</td>
<td>10</td>
</tr>
<tr>
<td>Bacteria</td>
<td>10⁻⁴</td>
<td>3 × 10⁻⁸</td>
<td>5 × 10⁻¹³</td>
<td>6 × 10⁴</td>
<td>1</td>
</tr>
<tr>
<td>Vesicles, liposomes</td>
<td>10⁻⁵</td>
<td>3 × 10⁻¹⁰</td>
<td>5 × 10⁻¹⁶</td>
<td>6 × 10⁵</td>
<td>0.1</td>
</tr>
</tbody>
</table>
easy handling and culturing, and the possibility to express eukaryotic membrane proteins by simply injecting in vitro generated cRNA, Xenopus oocytes have become a standard tool in transmembrane transport studies. Particularly advantageous for studying aquaporins is their low intrinsic water permeability. In nature, this property warrants survival of the oocytes in the hypotonic environment of a freshwater pond. In vitro, as an assay system, it ensures a low background water flux resulting in reasonable signal-to-noise ratios.

One to four days prior to the assay, cRNA (5 ng in 50 nl) encoding an aquaporin is injected, and the oocyte is incubated in isotonic medium for protein expression and insertion into the plasma membrane (Preston et al. 1992). Swelling is then initiated by abruptly placing the oocyte in diluted medium exposing it to an inward directed osmotic gradient, usually in the range of 100–150 mOsm. Under these conditions, the oocyte will swell up to 40% within 1–2 min and eventually burst (Fig. 1a). The volume increase is calculated from the increase of the area covered by the oocyte as documented by a video camera via a microscope (Fig. 1b). To test for solute permeability, sodium chloride from the incubation medium is isotonically replaced by the test solute to generate an inward directed chemical gradient (Hansen et al. 2002). Oocyte swelling due to solute influx and the secondary entry of water can reach

Fig. 1 Osmotic aquaporin swelling assay systems using Xenopus laevis oocytes or proteoliposomes. (a) Image series of a swelling and bursting Xenopus oocyte under hypotonic conditions (140 mOsm gradient) taken every 20 s for 3 min. The white circle denotes the area of the oocyte at time 0 s. (b) Relative volume increase of the oocyte shown in (a) (black curve) compared to a non-expressing control oocyte (gray curve). (c) Increase of light scattering due to volume change of liposomes in a stopped-flow machine. Shown are curves for proteoliposomes carrying purified human AQP2 (black curve) and for pure liposomes (gray curve). The steeper black curve indicates more rapid water flux
approximately the same rate as in osmotic assays. The actual solute permeability coefficient of aquaglyceroporins, however, is usually at least two orders of magnitude lower ($P_{\text{solute}} < 1 \text{μms}^{-1}$) than the water permeability coefficient of orthodox aquaporins ($P_f \approx 50–200 \text{μms}^{-1}$). Water permeability of non-expressing oocytes is $10–20\text{μms}^{-1}$, whereas permeability for glycerol and other solutes is hardly detectable.

Besides employing swelling assays to follow solute uptake into cells, one can also use radioactively labeled compounds, if available, and determine the intracellular accumulation of the nuclides, after certain time points, in a scintillation counter (Beitz et al. 2006). This method provides a direct measure of the uptake rather than an estimate via indirect osmotic effects.

Interindividual differences between the oocytes, i.e., slight variances in size, shape, or aquaporin expression levels, call for several repetitions (5–10 or more), in order to minimize error. In principle, the oocyte system can also be used for the determination of the Arrhenius activation energy, which requires measurements at different temperatures. This is, however, rather tedious due to the multiple replications that are needed for significant statistics. Such biophysical parameters can be obtained more easily in a stopped-flow machine using vesicle preparations or artificial proteoliposomes carrying the aquaporin to be studied.

### 2.2 Secretory Yeast Vesicles and Proteoliposomes

With diameters of only 50–200 nm, vesicles and liposomes are located at the opposite end of the scale as compared with *Xenopus* oocytes (Table 1). Consequently, the compartment volumes are dramatically different, i.e., $\approx 9 \times 10^{-4} \text{cm}^3$ for the oocytes and $\approx 5 \times 10^{-16} \text{cm}^3$ for the liposomes. When calculating the surface-to-volume ratios, liposomes and oocytes differ by 3–4 orders of magnitude. This difference explains why the swelling or shrinkage of liposomes in an osmotic gradient is complete within 10–100 ms (Fig. 1c; Zeidel et al. 1992; Borgnia and Agre 2001) instead of several minutes, as in the case of oocytes (Fig. 1b; Preston et al. 1992). Clearly, a rapid data sampling system is required to monitor the process.

Practically, a stopped-flow machine is used, in which small volumes (50–200μl) of a vesicle suspension and an osmotically different solution are injected into an observation cuvette. Dead time should be around 1 ms. Osmotic water flow then changes the vesicle diameter that is monitored by quantification of the light scattering intensity. Short assay times and small sample sizes allow for multiple repetitions of the measurements. Temperature control, e.g., for the determination of the activation energy, is easily accomplished by bathing the machine parts that hold the test suspension/solution and the cuvette in a thermostat.

Test vesicles can be obtained from a mutant yeast strain expressing a temperature sensitive Sec6-protein (Laizé et al. 1995). Under non-permissive conditions, the mutation results in a cytosolic accumulation of secretory vesicles. When an aquaporin is heterologously expressed in this yeast strain, it will be present in these vesicles.
together with multiple other endogenous membrane proteins. This particulate frac-
tion can be collected by centrifugation and can be directly used for assaying. If a 
pure system is wished or required, one needs to engage in a more time-consuming 
procedure that involves purification of the aquaporin protein and functional recon-
stitution in proteoliposomes (Zeidel et al. 1992).

Besides vesicles, whole-cell water and solute permeability has also been ana-
lyzed in stopped-flow light scattering assays (Table 1), e.g., of erythrocytes (Mathai 
et al. 1996; Liu et al. 2007), yeast protoplasts (Pettersson et al. 2006), and even 
bacteria (Hubert et al. 2005) with aquaporin deletions or aquaporins carrying point 
mutations. This showed, for instance, that the erythrocytes of Colton null individu-
als that fully lack AQP1 have 87% reduced water permeability (Mathai et al. 1996) 
and glycerol influx into erythrocytes of AQP9 knockout mice is significantly slower 
by 85% than into wild-type cells (Liu et al. 2007).

2.3 Mammalian Cells

Mammalian aquaporins can also be analyzed in their typical cellular environment 
using primary cell cultures (Solenov et al. 2004) or expression systems (Gao et al. 
2005). Changes in fluorescence intensity after an osmotic challenge are then corre-
lated to cellular water permeability coefficients. The possibility to automate fluores-
cence measurements in stably transfected cells allows for high-throughput setups in 
in which large libraries of small compounds can be screened for aquaporin inhibitors. 
However, a breakthrough has not yet been achieved (see Part V of this volume).

3 Phenotypic Assays

The biophysical assays described above provide valuable quantitative data and ac-
curately describe permeability properties of aquaporins on a molecular level. They 
cannot answer, however, whether the found parameters are relevant in the physio-
logical setting of a cell. Here, a phenotypic readout, such as cell growth or even cell 
 survival, is needed to appreciate the role of an aquaporin under normal conditions 
and in certain stress situations.

3.1 Leishmania

The identification process of an aquaglyceroporin as a major pathway for trivalent 
antimony in human-pathogenic Leishmania parasites illustrates the quality of the 
phenotypic assay approach (Gourbal et al. 2004). Antimonials are still the first-
line treatment for leishmaniasis. Antimony is therapeutically applied as Sb(V), in
the form of sodium stibogluconate or meglumine antimonate. In the parasite’s cytosol, pentavalent antimony is reduced to Sb(III) to form the active compound. The aquaglyceroporin LmAQP1 was shown to be directly linked to antimonial resistance of *Leishmania major* parasites, probably by facilitating Sb(OH)$_3$ release (Gourbal et al. 2004). Evidence came from a naturally selected antimonial-resistant *Leishmania* strain in which the LmAQP1 gene was strongly overexpressed. The role and physiological relevance of LmAQP1 was analyzed in in vitro cultures of *Leishmania* in a reversed setup, i.e., parasites were treated directly with the reduced Sb(III) form. Indeed, loss of the LmAQP1 gene as a result of targeted gene disruption rendered the parasites ten times more resistant to externally applied Sb(III) by prohibiting the uptake, whereas overexpression of the gene led to sensitization of the parasites to external Sb(III). Here, the EC$_{50}$ was even shifted by two orders of magnitude to lower concentrations. This is clear evidence that LmAQP1 permeability for trivalent antimony is physiologically relevant in *Leishmania* parasites as a drug resistance pathway.

### 3.2 Yeast

The yeast system is particularly well suited for designing phenotypic growth or cell toxicity assays due to a long list of advantageous factors and available tools, e.g., the ease of generating gene disruptions, the possibility to express mammalian membrane proteins, usability of a wealth of nutritional selection markers, rapid growth, etc. Phenotypic yeast assays for glycerol, ammonia (or methylamine), arsenite/antimonite, and hydrogen peroxide permeability have been described.

Yeast cells rapidly and accurately adjust the internal glycerol concentration in order to compensate for osmotic imbalances between the cytosol and the environment. They express an aquaglyceroporin, Fps1, which has the ability to open and close the channel in an osmotically regulated fashion (Luyten et al. 1995). Under hyperosmotic conditions, the channel closes to retain the intracellular glycerol; additionally, glycerol is metabolically produced to reach adequate concentration levels. In turn, under hypotonic conditions, the Fps1 channel opens and releases glycerol into the medium, whereas glycerol biosynthesis ceases. Point mutations in the N- and C-terminus of Fps1 revealed that both termini mediate channel gating because the yeast lost its ability to adapt to osmotic changes, as seen by reduced growth (Karlgren et al. 2004).

This phenotype can be exploited for functional assays of aquaglyceroporins. Yeast growth is unaffected in hypertonic medium even in the absence of Fps1, i.e., a Δfps1 knockout strain, because the cells need to retain internal glycerol and would have closed the channel anyway (Fig. 2a, *upper two rows*). If a functional, unregulated aquaglyceroporin, e.g., the *Plasmodium falciparum* aquaglyceroporin PfAQP (Hansen et al. 2002), is heterologously expressed under these conditions, the cells will leak glycerol, cannot adapt to the osmotic conditions, and will stop growing (Fig. 2a, *bottom row*). The phenotype becomes even clearer in yeast, where enzymes
Fig. 2 Yeast-based phenotypic aquaporin assays for various solutes. (a) Glycerol release from yeast cells. The cells were grown in 1 M glycerol liquid medium and then plated on solid medium containing 1 M of impermeable sorbitol instead of glycerol, to establish a large outward gradient for glycerol. Cells that express regulated Fps1 grow as well as cells without the endogenous aquaglyceroporin, because the Fps1 pore will close under hypertonic conditions (upper two rows). Cells that express the unregulated aquaglyceroporin from Plasmodium falciparum (PfAQP) show reduced growth due to glycerol leakage and consequent osmotic stress. (b) Permeability assays for ammonia (Δmep1–3 strain; upper panel) and methylamine (Δfps1 strain; lower panel) at pH 5.5–7.5. Uptake of ammonia is facilitated pH-independently by expression of an ammonium transporter (AmtB, positive control) or pH-dependently by PfAQP with highest rates at neutral pH, i.e., at higher concentrations of uncharged ammonia. Diffusion of ammonia across the cell membrane is depicted by high background growth (middle row). Background is close to zero when cell growth depends upon the release of toxic methylamine (lower panel, middle row). Aquaporins with methylamine permeability (Fps1 and PfAQP) sustain cell growth in the acidic range according to the larger outward gradient of the uncharged form. (c) Permeability for arsenite, As(OH)_3. Aquaporins that facilitate the influx of toxic arsenite into the cells lead to reduced growth (PfAQP) compared to cells lacking the endogenous Fps1.

Phenotypic yeast aquaporin assays are the more useful when the permeant, e.g., ammonia, is not osmotically active, because here osmotic swelling assays using Xenopus oocytes or liposomes would not work. Yeast, however, requires uptake of ammonia as a nitrogen source for cell proliferation and can therefore be used as an assay system (Jahn et al. 2004). In yeast, three ammonium transporters, Mep1–3, facilitate ammonium uptake very efficiently and pH-independently. A respective triple knockout can only grow when alternative pathways exist, such as a heterologously expressed ammonia-permeable aquaporin, e.g., human AQP8, plant
TIP2;1 (Jahn et al. 2004) or PfAQP (Fig. 2b, upper panel; Zeuthen et al. 2006). The growth rate will be pH-dependent decreasing toward acidic pH, because aquaporins only conduct the uncharged ammonia form and not the protonated ammonium ion (pK_a 9.25). The readout in this assay is not always convincing due to high background growth in the control cells (Fig. 2b, upper panel), hence a variant based on the cytotoxic ammonia derivative methylamine was established (Beitz et al. 2006).

Methylamine is a chemical analog of ammonia, with high toxicity for yeast cells. Similar to ammonia, it exists in two forms, i.e., the uncharged methylamine form and the protonated, charged methylammonium. The latter prevails in the physiological pH-range due to a pK_a of 10.64 of the compound. The more acidic the pH, the farther is the chemical equilibrium shifted to charged methylammonium. Yeast cells take up methylammonium via the aforementioned Mep 1–3 ammonium transporters, resulting in an intracellular accumulation and inhibition of proliferation if the endogenous aquaglyceroporin is knocked out (Δfps1 strain; Fig. 2b, lower panel). However, functional expression of a methylamine-conducting aquaglyceroporin, such as the endogenous yeast Fps1, Escherichia coli GlpF, or PfAQP, rescues the cells when grown in an acidic medium (Fig. 2b, lower panel; Wu et al. 2007).

For instance, a gradient from cytosolic pH 7.2 to pH 5.5 in the external medium establishes an outward gradient for uncharged methylamine that renders PfAQP expressing yeast ≈100 times more resistant (Zeuthen et al. 2006).

Yeast knockout strains have also been used for arsenite and antimonite uptake assays similar to the Leishmania system (Fig. 2c; Wysocki et al. 2001) and for phenotypic hydrogen peroxide permeability assays (Bienert et al. 2007). The yeast cells in the latter assays lack a transcription factor that renders them more sensitive to oxidative stress than wild-type cells. Uptake of hydrogen peroxide via aquaporins can then be analyzed by monitoring cell growth where high permeability for hydrogen peroxide is indicated by suppressed growth (Bienert et al. 2007).

This selection of aquaporin in vitro assays shows their wide application range. It should be mentioned that several other techniques have been employed to characterize more special permeability properties, such as for carbon dioxide leading to pH-shifts in the presence of carbonic anhydrase activity (Uehlein et al. 2003) and for the wide field of ion permeability using electrophysiology approaches (Yasui et al. 1999; Saparov et al. 2001; Holm et al. 2005; Beitz et al. 2006).

### 4 Modification of Aquaporin Pore Selectivity

With such diverse and complementary tools at hand, we set out to analyze, in vitro, which pore residues define selectivity for water, solutes, and protons in the prototypical, water-specific AQP1 (Beitz et al. 2006). For these studies, it was particularly helpful that the general layout of the aquaporin channels was well established due to the availability of several atomic structures of water-specific aquaporins, e.g., AQP1 (Sui et al. 2001), and aquaglyceroporins, e.g., E. coli GlpF (Fu et al. 2000). For more details than presented in this section, see the two preceding chapters.
4.1 General Aquaporin Pore Layout

All aquaporins form tetramers with an independent water pore in each monomer (Gonen and Walz 2006). Each monomer spans the membrane six times, and is further characterized by two short helices that only dip into the membrane and meet in the center of the channel. Two almost invariable Asn-Pro-Ala (NPA) motifs stabilize the stacking of the short helices and form a constriction, i.e., the so-called NPA region. The two asparagines are the capping amino acids at the positive ends of the short helices and act as hydrogen donors to the oxygen atoms of passing permeants. It is discussed that in this region (a) water is re-oriented such that hydrogen bonds between neighboring molecules in the water chain are disrupted, preventing the formation of a proton wire throughout the pore, and (b) a major energy barrier for proton conductance is formed due to a strong positive electrostatic field (de Groot and Grubmüller 2005).

The major part of the aquaporin pore is lined by hydrophobic residues that contribute a ladder of main-chain carbonyl oxygens to the inner pore surface. These oxygen atoms serve as hydrogen bond acceptors and probably compensate for the energy cost of hydrogen bond breakage when a molecule is isolated from the bulk solution at the pore mouth.

The narrowest pore constriction is located close to the extracellular entrance (Fig. 3a, b). In water-specific aquaporins, it is 2.8 Å in diameter, perfectly matching a water molecule, whereas aquaglyceroporins accommodate larger molecules, such as glycerol and urea, due to a wider constriction of at least 3.4 Å. This constriction is referred to as the aromatic/arginine (ar/R) constriction and is formed by four residues, i.e., Phe56, His180, Cys189, and Arg195 in human AQP1 (Fig. 3b), and Trp48, Gly191, Phe200, and Arg205 in E. coli GlpF (Fig. 3a). A histidine is typical for water specific aquaporins, which, together with the highly conserved arginine, provides strong hydrophilicity. In GlpF, and essentially in all other aquaglyceroporins, the ar/R region is more hydrophobic due to the lack of the

![Fig. 3](image-url) Layout of the aromatic/arginine (ar/R) region of the E. coli aquaglyceroporin GlpF (a), the human water-specific AQP1 (b) and generated mutants of AQP1 (c). The circumference of a glycerol molecule is indicated by the oval; the black bars in (c) mark the length of the alanine and valine side chains in the AQP1 mutants.
histidine and substitution of the cysteine by a second aromatic residue; there are also aquaglyceroporins carrying aliphatic residues as the lipophilic moieties (Beitz 2005).

4.2 Point Mutations in the Ar/R Constriction of AQP1

In many respects, mammalian AQP1 is the best-characterized water channel. Besides numerous physiological studies, permeability assays have shown its water specificity; further, over the years, the atomic channel structure has been obtained and improved down to 2.2 Å resolution (Sui et al. 2001). We asked whether pore specificity is indeed defined in the ar/R constriction and whether it is possible to modify solute permeability of AQP1 by systematic replacement of residues in the region (Beitz et al. 2006). The mutations aimed at increasing the diameter, changing the shape, and reducing polarity at this site. We replaced, alone or in combinations, Phe56 and His180 with the smaller alanine and Arg195 with valine (Fig. 3c). Mutation of Arg195 to valine (R195V) removed a positive charge, elongated the pore shape, and enlarged the pore area almost threefold. Removal of the imidazole ring (H180A) deleted a putative protonation site and, similar to the R195V mutation, shaped and enlarged the pore, but in the opposite direction. Combination of both mutations (H180A/R195V) led to a flat pore shape that was strongly elongated in one direction, but hardly any wider than the wild-type pore in the other dimension. Here, the bulky aromatic Phe56 is restrictive. Consequently, replacement of Phe56 (F56A) widened the ar/R constriction dramatically, forming a more round pore shape. Despite the major impact on the protein structure in *Xenopus* oocytes as well as in yeast cells, expression levels of all mutants appeared to equal the wild-type AQP1 as shown by Western blotting using a specific antiserum.

4.3 Water and Solute Permeability

In the *Xenopus* oocyte swelling assay, all AQP1 mutants passed water at similar rates as the wild-type channel (Table 2). Even the Arrhenius activation energy for water permeation of 5 kcal mol$^{-1}$ was unchanged compared with wild-type AQP1. Considering the pronounced differences in polarity in the mutant ar/R constrictions, especially in the H180A/R195V mutant, this came as quite a surprise and shows firstly, that hydrophilic residues at the pore mouth are not required for efficient water permeability and secondly, that formation of hydrogen bonds with the ladder of carbonyl oxygens suffices to compensate for energy costs of bulk water isolation.

Water permeability was unaltered, but would the generated changes in shape and diameter allow larger solutes to pass? Isosmotic oocyte swelling assays with urea and glycerol (Table 2) showed that the single mutants AQP1-R195V and AQP1-H180A were impermeable. The double mutants AQP1-F56A/H180A and AQP1-H180A/R195V, however, were excellently permeated by urea. Glycerol
Table 2 Permeability of AQP1 wild-type and the mutants H180A, R195V, H180A/R195V, and F56A/H180A for water, urea, glycerol, ammonia, and protons (Beitz et al. 2006)

<table>
<thead>
<tr>
<th></th>
<th>AQP1</th>
<th>AQP1-HA</th>
<th>AQP1-RV</th>
<th>AQP1-HA/RV</th>
<th>AQP1-FA/HA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water ((L_P)) ((\mu m s^{-1}))</td>
<td>0.61 ± 0.07</td>
<td>0.84 ± 0.11</td>
<td>0.54 ± 0.05</td>
<td>0.62 ± 0.09</td>
<td>0.78 ± 0.09</td>
</tr>
<tr>
<td>Urea ((P_{urea})) ((\mu m s^{-1}))</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.13 ± 0.02</td>
<td>0.50 ± 0.15</td>
</tr>
<tr>
<td>Glycerol ((P_{gly})) ((\mu m s^{-1}))</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.34 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>Ammonia ((I_{H^+} at 5 mM NH_4Cl, pH 7.4)) ((nA))</td>
<td>8.70 ± 2.45</td>
<td>96.20 ± 15.76</td>
<td>61.20 ± 16.30</td>
<td>58.70 ± 5.98</td>
<td>107.06 ± 16.85</td>
</tr>
<tr>
<td>Protons ((I_{H^+} at pH 5.6)) ((nA))</td>
<td>0.65 ± 0.35</td>
<td>0.60 ± 0.28</td>
<td>3.45 ± 0.48</td>
<td>8.60 ± 2.05</td>
<td>2.15 ± 0.29</td>
</tr>
</tbody>
</table>

permeability was seen only in AQP1-F56A/H180A and was clearly lower than that for urea. Elongation of the carbon chain to four methylhydroxyl groups, i.e., erythritol, had already abolished passage. This indicates that the ar/R constriction is indeed a major filter region in AQP1, limiting solute permeability by size. However, additional pore regions must exist that, although being wide enough for urea, restrict passage of larger solutes, such as glycerol. Theoretical analyses of the AQP1 mutants have very recently confirmed the findings in silico (Hub and de Groot 2008).

4.4 Ammonia Permeability

The requirements of an aquaporin pore layout that permits ammonia passage are far less clear. AQP1 effectively excludes ammonia, whereas other aquaporins, such as plant TIP2;1 and human AQP8, have been reported to conduct ammonia (Jahn et al. 2004; Holm et al. 2005). How can these channels discriminate between water and ammonia? Size exclusion cannot account for the selectivity against ammonia, because it has the same dimension as a water molecule and a dipole moment close to that of water (1.5 vs. 1.8 Debye). The most obvious difference lies in the number of hydrogen bond acceptor sites (two in water vs. one in ammonia), donor sites (two in water vs. three in ammonia), and the greater lipophilicity of ammonia. At the ar/R constriction of TIP2;1 and of AQP8, the position of the histidine is switched to that of phenylalanine in AQP1, resulting in a more hydrophobic edge in juxtaposition to the positive pore arginine. The AQP1 mutants described above are also more hydrophobic than the wild-type channel, providing a suitable system to ask whether the reduced polarity of the AQP1 mutants would enable ammonia or its derivative methylamine to pass.

Both phenotypic yeast assays, i.e., ammonia uptake in the Δmep1–3 strain as well as methylamine release using the Δfps1 strain, showed growth-sustaining
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permeability of all generated AQP1 mutants, whereas yeast cells expressing the wild-type channel did not survive and therefore did not conduct physiologically meaningful amounts of ammonia or methylamine (Beitz et al. 2006). As expected, yeast growth was pH-dependent, indicating that only the uncharged forms of ammonia and methylamine passed the mutant aquaporins.

These findings were confirmed by an independent electrophysiological setup using *Xenopus* oocytes (Beitz et al. 2006). Yet, uncharged ammonia molecules are electroneutral, and the influx into oocytes cannot be measured directly with electrodes. However, inside the oocyte at pH 7.4, about 99% of the entering ammonia molecules get protonated in the process of establishing the pH-dependent chemical equilibrium. The oocytes will not tolerate a shift in internal pH, and a secondary proton influx is initiated through an as yet undefined transport entity that is measurable as a whole-cell inward current. In a range of pH 5.6–8.4 (corresponding to ammonia concentrations of 1.12 μM to 1.12 mM), the AQP1 variants carrying an H180A mutation showed particularly high ammonia permeability (Table 2).

### 4.5 Proton Exclusion

In the case of AQP1 H180A/R195V toward more acidic pH, i.e., at lower availability of ammonia, an unexpected increase of the inward current was detected (Beitz et al. 2006). This indicated an ammonia-independent pH-effect. When the pH-shift experiments were repeated in the absence of ammonia, the pH-induced currents were still observed (Table 2). The currents were inward directed in the acidic range and outward in the alkaline range, with a reversal pH of 7.0 clearly calling for proton permeability of the AQP1 mutant. The AQP1 R195V mutant also showed some, although weaker, proton leakage, whereas the wild-type channel was impermeable. This established the ar/R constriction as a part of the proton exclusion mechanism of AQP1, which was not predicted before in silico. Subsequently adapted theoretical models based on the AQP1 ar/R mutants led to the same conclusion (Chen et al. 2006). Calculation of the proton flux rate of AQP1 H180A/R195V gave approximately 50 protons per channel per second, i.e., a rather low value, three orders of magnitude smaller than that of typical proton channels (Beitz et al. 2006). The major proton barrier may, therefore, indeed reside in the NPA region at the pore center. If, however, one considers the high copy number of aquaporin channels in a cell membrane (up to 200,000 in a red cell), this supporting proton barrier at the ar/R region seems physiologically relevant.

### 5 Conclusion

Today there are numerous assay systems in place for the characterization of aquaporin permeability properties. They yield quantitative biophysical parameters or show the impact of aquaporin function on the phenotype of living cells under certain
conditions. We are looking at a rapidly growing number of novel aquaporin sequences that are produced by genome sequencing. Many of those depict low similarity to prototypical channels, and theoretical deliberations alone cannot clarify their functionality. Hence, in vitro assays are instrumental to establish their function, to identify novel physiological permeants, and to analyze gating mechanisms and selectivity filters. Here, iteration between in silico and in vitro methods will provide optimal advancement. The continuous improvement of established assays and the installation of novel test systems mirror an increasing diversification in the field and will progressively reveal the complex relations between water, solutes, and their conducting channels.

References

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