Biophysical studies of human Aquaporin 1
Structural insights by Solid-State NMR and mechanism of inhibition by Mercury

by
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ABSTRACT

BIOPHYSICAL STUDIES OF HUMAN AQUAPORIN 1
STRUCTURAL INSIGHTS BY SOLID-STATE NMR AND
MECHANISM OF INHIBITION BY MERCURY

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University of Guelph, 2016
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Dr. Leonid S. Brown

Human Aquaporin 1 (hAQP1) is a membrane protein that transfers water across the membrane with a rate faster than that of the simple diffusion. This protein plays an important role in different tissues such as the kidney, the eyes and the skin in human body. Overexpression of this protein can be associated with different diseases such as cancer, glaucoma, and brain swelling. Thus, inhibition of hAQP1 is a promising approach to decrease the symptoms of such diseases and, ultimately, to cure them. Therefore, knowing the function and structure of the protein in its native environment is important in order to understand the inhibitory effects of different drugs. In this respect, solid-state NMR (ssNMR) is a promising technique as it can probe structure and dynamics of membrane proteins in lipids. In order to study hAQP1 with ssNMR, the most critical step is to overexpress isotopically labeled functional protein in its proper fold with good stability. In this project, the production of the doubly ($^{13}$C/$^{15}$N) isotopically labeled hAQP1 protein in yeast Pichia pastoris was established. The production of homogeneous labeled protein yielded an excellent resolution of the ssNMR spectra, which allowed running suites of multidimensional experiments yielding assignments for the majority of hAQP1 resonances. The assignments revealed a wealth of site-specific information, including the secondary structure, chemical environment, hydrogen bonding and water accessibility of the amino acid residues (via H/D exchange). The inhibition studies of hAQP1 by a mercurial compound were also conducted using different biophysical techniques such as ssNMR, Fourier transform infrared spectroscopy (FTIR), dynamic light scattering (DLS), small angle X-ray scattering (SAXS) and stopped-flow kinetic experiments. The novel mechanism of inhibition of hAQP1 by mercury chloride was shown to affect its conformation and to cause the tetramer and lattice disruption. Finally, novel promising organic compounds
were tested and shown to be effective as inhibitors in vitro, using proteoliposome systems.
Dedication

I dedicate my thesis to my family. A special feeling of gratitude to my loving parents and my brother. Thanks for your great support and continues care.
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## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>1D</td>
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<tr>
<td>2D</td>
<td>two dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>three dimensional</td>
</tr>
<tr>
<td>bAQP1</td>
<td>bovine Aquaporin 1</td>
</tr>
<tr>
<td>BMD</td>
<td>buffered minimal dextrose</td>
</tr>
<tr>
<td>BMM</td>
<td>buffered minimal methanol</td>
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<td>β-OG</td>
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<td>CARA</td>
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<td>channel-forming integral protein of 28 kDa</td>
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<tr>
<td>FTIR</td>
<td>Fourier-transform Infra-red spectroscopy</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>hAQP1</td>
<td>human Aquaporin 1</td>
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</table>
H/D                      hydrogen/deuterium
IgG                      Immunoglobulin G
MAS                      Magic angle spinning
MALDI-TOF                matrix-assisted laser desorption/ionization – time of flight
PAS                      principal axis system
PC                       phosphatidylcholine
PS                       phosphatidylserine
PE                       phosphatidylethanolamine
OD600                    optical density at 600 nm
rpm                      revolutions per minute
SAXS                     small angle X-ray Scattering
SPINAL64                 small phase incremental alternation heteronuclear dipolar decoupling scheme with 64 composite pulses
ssNMR                    solid-state nuclear magnetic resonance
UCN                      uniform $^{13}$C, $^{15}$N
UV-Vis                   ultraviolet-visible spectroscopy
YPDS                     yeast peptone dextrose sorbitol
Chapter 1

Introduction to Human AQP1 Structure, Function, and Inhibition
1.1 Membrane Proteins

The main objective of this work is a better understanding of human Aquaporin 1 (hAQP1), which is a eukaryotic membrane protein. Therefore, this project has employed the eukaryotic expression system and advanced structural techniques suitable for membrane proteins under native-like conditions, such as solid-state NMR.

The main structural elements of eukaryotic cells, such as mammalian or yeast cells, are the nucleus, cytoplasm with organelles, and lipid membrane that surrounds the cell. In order for cells to be able to communicate with the outside environment, they need to have receptor and transport proteins embedded in their lipid membranes. These proteins are called integral membrane proteins and they span the lipid bilayers and contain both hydrophilic and hydrophobic regions [1–4].

Membrane proteins have variety of functions in the cells. They function as the molecular transporters across the membrane and/or serve for communication between the cells. They also can play role as enzymes and receptors [5], control the shape of the organelles, and participate in energy transduction in the respiratory and photosynthetic systems [4, 6–8].

There are two different classes of integral membrane proteins. The first class is integral polytopic proteins or transmembrane proteins, which cross the lipid bilayer at least once. The integral polytopic proteins could be divided into two groups based on their tertiary structures. These groups are helical bundle and beta-barrel proteins. The helical bundle proteins contain several parallel or antiparallel helices in their structure [4,
6–8]. The beta-barrel proteins are usually located in the outer membranes of Gram-negative bacteria and membrane of some Gram-positive bacteria, as well as in the membranes of mitochondria and chloroplasts. The second class of membrane proteins is monotopic proteins. These proteins do not span the lipid bilayer and reside on one side of the membrane.

Membrane proteins are located in an asymmetric environment; on the one hand there are cytosolic and extracellular environments which are hydrophilic and most of the hydrophilic amino acids are located in these regions, on the other hand, there are hydrophobic regions inside the lipid membrane that require hydrophobic amino acid residues to be located there [8]. Thus, amino acid location in a transmembrane protein depends on its hydrophobicity, and most amino acids have preferred locations along the transmembrane axis. Aliphatic amino acids such as Phe, Ile, Val and Leu prefer regions inside the protein core. Arginines and lysines are more often found in the cytoplasmic domains compared to the periplasmic domains and this is called the positive-inside rule [9]. Aromatic amino acids such as Tyr, His and Trp prefer interfacial regions of membrane proteins [10].

Interactions of lipids with membrane proteins are important for the proper functioning of these proteins. The membrane proteins by their nature are hydrophobic and have a tendency to aggregate. Thus, for solubilization and purification of these proteins one may need to use different detergent treatments [11]. Proper choice of detergent for solubilization of membrane proteins is crucial, especially for protein crystallization. Detergent molecules can affect the behavior and stability of membrane proteins and result in protein inactivation or aggregations. The amphiphilic nature of
membrane proteins makes them difficult to be crystallized and the structures of many membrane proteins remain unknown [3, 4, 12, 13]. The structures of 1008 membrane proteins have been determined through X-ray and electron diffraction methods and this is very low given that membrane proteins constitute up to a third of all proteins (http://blanco.biomol.uci.edu) [14–16]. Some problems that exist in understanding the structure of membrane proteins with X-ray crystallography are described later in this chapter. Below, we first describe the discovery of AQP1 and then we further detail the structural studies of this protein.

1.2 Discovery of CHIP28 (AQP1)

Water molecules can pass through a lipid bilayer by simple diffusion at a limited slow rate. Some cells need to receive or export water at a much faster rate than that of a simple diffusion, for example, red blood cells and cells of renal proximal tubules [17]. Thus, observation of a faster rate of water transfer in these as well as some other tissues resulted in a proposal of the existence of specific channel proteins responsible for accelerated water transport through their lipid membranes [18, 19].

Studies of the red blood cells showed that when these cells were incubated with HgCl₂, water permeability was inhibited. Later, the water permeability was restored by addition of reducing agents to the red blood cells [19]. The inhibition of red blood cells led to the proposal of the existence of certain water channel proteins inside the lipid membrane and the presence of intrapore sulfhydryl groups that react with mercuric ion. The first hypothesis for such protein was the glucose transporter 1, but it was not
observed to pass water molecules with a higher rate than a simple diffusion of water molecules when expressed in *Xenopus* oocytes [20].

The water channel protein was discovered accidentally by Agre et al [21] in another project on the Rh(D) antigen, where a protein band with a molecular weight of 30-32 kDa appeared on SDS-PAGE gels, which was first assumed to be a degradation product of Rh(D). The newly discovered protein was named CHIP28, which stands for channel-forming integral protein of 28 kDa [21]. Later evidence showed that CHIP28 protein is part of the major intrinsic protein (MIP) family that was found in the lens fiber cell [22]. Preston et al. [23] introduced CHIP28 as a postulated red blood cell water channel after the expression of this protein in *Xenopus* oocytes. Thus, in 1992 the idea that a channel-forming integral protein of 28 kDa (CHIP28) transports water was established [23]. Later, the general name of aquaporins was introduced and CHIP28 was renamed as AQP1 and now AQP0 is the name of the MIP protein [24–28].

Expression of AQP1 in *Xenopus* oocytes was done in 1992 by Preston et al. [23] and oocytes were transferred from isotonic solution to a diluted solution for the osmotic swelling experiments. It was shown that upon expression of AQP1, osmotic water permeability increased in oocytes and resulted in their swelling and explosion [23], the control oocytes remained unchanged (Figure 1-1).

After this discovery, other homologs of this protein were found and it was shown that water channel proteins exist in many living organisms from bacteria to plants, yeast, and mammalian cells. There are so far 13 different kinds of AQPs known in mammalian cells (AQP0-AQP12) [2, 29–34].
Fig 1-1. Consequences of hAQP1 expression in oocytes. After transfer to hypotonic solution, injected oocytes showed swelling while control oocytes remained unchanged. Figure reproduced from Preston et al. [23] with permission from Science.

It has been proposed that water permeability of AQP1 is reversibly inhibited by mercury and later cysteine 189 was found to be the mercury binding site of the protein [35]. Consequently, mercury chloride became a traditional and well-known inhibitor of AQP1 and several other aquaporins [35] (see Chapter 5 for details).

1.3 Physiological roles of aquaporins and the importance of their study

The AQP s are categorized into two subgroups based on their main function. These subgroups include the orthodox aquaporins and the aquaglyceroporins. The orthodox AQP s are including AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8 and their main function is to transport water selectively and bidirectionally across the lipid membrane. The aquaglyceroporins, including AQP3, AQP7, AQP9 and AQP10 are transferring both water and glycerol [36]. In the native membrane, all AQP s are assembled into homotetramers and each subunit is a functional unit and contains a single
water channel. This creates a central pore in the middle of the four monomers, and it was suggested that ions and some small molecules can transfer through this pore [22, 37]. More specifically, it has been shown that AQPs such as AQP1, AQP3, AQP7, AQP9 and AQP10 can transport small polar molecules that are neutral, including urea, pyrimidines, glycerol, sugars, amino acids as well as some other small molecules such as hydrogen peroxide, nitric oxide, carbon dioxide and ammonia [34, 38–43]. Some AQPs, such as AQP0, AQP1 and AQP6, were suggested to pass ions through the pore between their four subunits and act as ion channels [44]. Interestingly, AQP6 is located in the intracellular vesicles of the renal collecting duct. It was shown by Yasui et al. [45] that AQP6 allows bidirectional anion conductance through its channel at a lower pH (such as 5.5). Thus, AQP6 is shown to be an anion-channel with low water permeability and high ion conductance [45]. AQP1 was suggested to act as a cGMP-dependent potassium channel transferring potassium ions through the pore in the middle of its four subunits [46]. The main function of AQP11 and AQP12 is also to transfer water [30, 47]. They both belong to the group named superaquaporins. The “super” name comes from the fact that these two AQPs were added into the AQPs category later and the AQPs family was expanded after addition of these two AQPs despite their low homology with other subfamilies [48]. Figure 1-2 shows the phylogenetic tree of 13 human aquaporins [36].
Tissues that contain AQPs in their plasma membranes were shown to have much higher osmotic water permeability compared to other tissues without this protein [49]. In order to increase membrane water permeability, AQPs must be present in the membrane at high density [50], i.e., about several thousand or more molecules per $\mu$m$^2$ of membrane, compared to other ion channels that are present in smaller numbers [51].

Due to the important physiological functions of AQPs, malfunction or overproduction of AQPs was shown to cause health problem in humans. Overproduction of AQPs can cause cells to lose or gain more water than needed. Thus, human diseases
such as fluid imbalances and edema are seen in tissues where AQPs are expressed. Furthermore, alterations in fluid transport and homeostasis in AQP-expressing tissues are targets of treatment for diseases such as edema in brain, glaucoma, cancer and renal dysfunction [52, 53]. Figure 1-3 shows the distribution of different AQPs in the human body [54].

Depending on the tissues that AQPs are expressed in, different diseases can occur upon failure in AQPs function. Figure 1-4 describes the important physiological processes dependent on AQPs functioning as water channels (a-e) and water and glycerol channels (f-h) in different tissues in the human body [55].

It has been proposed that AQP4 is responsible for the movement of water molecules across astrocyte cells in the brain [56]. This protein is responsible for the selective water transport in the central nervous system at the plasma membrane barriers of the astrocyte cells (neural cells) where the exchange between interstitial fluid and the blood happens [54].
Fig 1-3. Distribution of AQPs in human tissues. The names of the organs that AQPs are
distributed in are as follows: a) Retina, b) Olfactory epithelium, c) Inner ear, d) Brain that
includes astrocytes in choroid plexus, e) Spinal cord, f) Blood vessels, g) Heart, h) 
Kidney, i) Salivary glands, j) Gastrointestinal tract, k) Liver, l) Pancreas, m) Lung, n) 
Adipocytes, o) Female reproductive system, p) Male reproductive system. Figure is 
reproduced and adapted from Day et al. [54] with permission.
Fig 1-4. Functions of AQPs in different tissues as water channels including: a) AQPs located in kidney proximal tubules accelerate transfer of water to concentrate urine; b) AQPs facilitate secretion of different fluids such as saliva secreted from salivary glands, cerebrospinal fluid (CSF) secreted from choroid plexus of the ventricles in brain and aqueous humor that is secreted from the ciliary epithelium; c) Involvement of AQPs in migration of the normal and tumor cells and angiogenesis; d) AQPs function transporting water bidirectionally into the brain and out of the brain in the blood-brain and blood-CSF barrier. The location of these AQPs is in ependymal cells that are located in the ventricular system in the central nervous system, and in sagittal sinus in human brain; e) Function of AQP4 in astrocytes during excitation of neurons taking up potassium. AQPs function as glycerol and water channel including: f) AQP3 functions in skin hydration through facilitating glycerol transport in stratum corneum that is the outer layer of the epidermis; g) AQP3 functions in cellular proliferation through preserving the high level of glycerol inside the cells and helping in biosynthesis of lipids and ATP; h) AQP7 in adipocytes can limit fat accumulation by sending glycerol out of the adipocyte cells. Figure is adapted from Verkman et al. [55] with permission from Nature Publishing Group.

Human diseases related to mutations of several AQPs are known. Among these diseases is nephrogenic diabetes insipidus (NDI), which is related to the absence of AQP2 in the membrane of kidney cells. In NDI, the vasopressin-regulated water permeability in the collecting ducts of the kidney fails to function properly (Figure 1-4a).
The genes responsible for NDI disease are carried as autosomal recessive or dominant (hAQP2) or X-linked (vasopressin receptor) alleles and the expression of these genes affects the regulation of the membrane trafficking of AQP2. Mutant AQP2 is retained in the endoplasmic reticulum and is not present in the principal cells of the collecting duct in the kidney. The absence of AQP2 causes electrolyte imbalances due to insufficient water transfer across the membrane [32, 56–58].

The function of AQP3 is important for skin and cornea as water and glycerol transport within these tissues help them to stay healthy and not overly dry and also improves wound-healing processes [53]. Figure 1-4f shows the function of AQP3 in skin hydration. Overexpression of AQP3 was found to be involved in skin cancer [59]. The AQP3 function is also important in cell proliferation and tumor cell growth (Figure 1-4g). AQP1 was also shown to be involved in tumor cell growth as described later in this section.

Figure 1-4d presents functions of AQP4 in the human brain. In brain edema, due to the high osmotic driving forces, transfer of water through AQP4 in the blood-brain barrier increases significantly. Thus, in brain edema, AQP4 was observed to be upregulated and cause inflammations [52, 56, 60–65]. Accordingly, knock out mice that have no AQP4 in their plasma membrane were examined for brain edema and inflammation and showed a reduction of brain swelling and inflammations [56]. On the other hand, in hydrocephalus disease that is caused by excessive accumulation of water and fluid in the brain, AQP4 was shown to be down-regulated and less active. It has been shown that AQP4 is also involved in neuromyelitis optica (NMO) disease. This disease is in the category of autoimmune diseases and can cause blindness and paralysis. In the
NMO disease, the serum autoantibodies (IgG1) act against AQP4 (AQP4-IgG) that are located in the astrocyte cells. It was proposed that the development of the NMO disease symptoms is related to the level of the AQP4-IgG complex [59, 66, 67].

The function of AQP0, besides transferring water, is to act as a bridge between neighboring cells and make cell junctions [68]. Thus, the disease that has been proposed to be related to mutation of AQP0 is congenital cataract that causes problems in adhesion of neighboring cells to each other [8, 69, 70].

Human Aquaporin 1 (hAQP1) plays important roles in human physiology as well. Experiments that were performed on the knockout mice lacking hAQP1 in the kidney proximal tubule have suggested a dramatic decrease in the fluid absorption (Figure 1-4a) [31, 59, 71]. This protein is also expressed in high amounts in peripheral endothelial cells [72]. The main physiological function of hAQP1 in these tissues is to increase transepithelial osmotic water permeability. Thus, fluid secretion and absorption are the consequences of the hAQP1 function in this tissues [59]. Reduction in the epithelial osmotic water permeability causes problems in active fluid transport through the cells. One of the problem that is caused by overexpression or malfunction of hAQP1 protein in choroid plexus and in the ciliary epithelium is its impact on transepithelial osmotic equilibration [59]. The choroid plexus produces cerebrospinal fluid and hAQP1’s function in this tissue is very important to keep the transepithelial osmotic balance. It is also suggested that hAQP1 inhibitors might be useful in intraocular hypertension therapy in glaucoma. hAQP1 also functions in maintaining water balance in the cornea. Thus, up-regulation of hAQP1 results in reducing corneal and lens edema in the eye [59].
In the primary glioblastoma multiform tumours and many other tumors, hAQP1 expression is up-regulated [73], possibly enabling cancer cell volume regulation [74]. hAQP1 plays an important role in the renal proximal tubule cells migration and proliferation. The migrating cells contain more hAQP1 at their leading edge where an extended lamellipodium (with cytoskeletal protein actin) exist. The faster rate of water transfer at these edges promotes tumor angiogenesis (Figure 1-4c). Thus, the expression and localization of hAQP1 in the tumor cells showed an increase in tumor angiogenesis, including adhesion and endothelial cell proliferations [55]. These effects of hAQP1 in angiogenesis are used to estimate the relationship between the expression of this protein and the level of tumor development. Thus, hAQP1 inhibitors may be important in reducing the tumor spread and angiogenesis in tumor therapy [53], and it is possible that blockage of hAQP1 channels could slow down the proliferation and migrations of tumor cells. Consequently, inhibition of hAQP1 in tissues highly expressing this protein may be important, therefore it is necessary to study inhibition of hAQP1 and its functionality before and after inhibition. The implied role for hAQP1 expression in tumorigenesis at early stages makes hAQP1 an attractive target for potential therapeutic strategies.

The functions of AQPs in the human body are vital to preserve the adequate level of water or glycerol molecules inside cells. Consequently, the up-regulation and over-expression of hAQP1 and other AQPs have been implicated in several human diseases and their pharmacology is being actively explored [53]. Thus, the structure and function of hAQP1 need to be well studied and understood both in terms of basic science and as a pharmacological target.
1.4 Human AQP1 structure

Aquaporin 1 is the first discovered human aquaporin and it has been a focus of extensive structural studies by crystallographic methods: two medium-resolution cryo-electron microscopy structures and a medium-resolution X-ray crystal structure of hAQP1 are available [75–77], as well as a higher resolution structure of the homologous bovine AQP1 [78]. In addition, there is a refined structural model of hAQP1 [37] which is based on the EM structure of hAQP1 and the X-ray structure of its bacterial homolog GlpF [79].

All structural data reveal a consensus tetrameric assembly of hAQP1 with 28.5 kDa molecular weight and 269 amino acids for each monomer [37, 75, 76, 78]. Figure 1-5 shows the structure of the AQP1 as well as its important regions. hAQP1 consists of two similar halves presumed to arise from the duplication of the gene encoding three α-helical transmembrane domains [80, 81]. Each subunit comprises six full α-helical transmembrane domains (M1-M6 in the figure, most often called H1-H6) and five loops connecting them (named LA, LB, LC, LD, LE) (Figure 1-5b). There are two additional short non-spanning helices inserted in the membrane, located in loops B and E (helices M3 and M7, most often called HB and HE). The non-spanning helices HB and HE fold into the bilayer from opposite sides of the membrane, and, together with other helices, form the aqueous pore, initially described in an “hourglass” model [74, 76, 78, 82–84]. Loops B and E both contain the signature motif for AQPs that is highly conserved among all AQPs and consists of asparagine, proline and alanine (NPA) [84, 85] (Figure 1-5d).
Human AQP1 contains a number of important regions responsible for passage of water through the channel including two wide vestibules on the extracellular and cytoplasmic sides, and a long selectivity filter, formed by a series of backbone oxygen’s of G188, C189, G190, I191 on the extracellular side, and G72, A73, H74, L75 on the cytoplasmic side. The selectivity filter contains two constrictions: the aromatic arginine (ar/R) region and the NPA motif region. The ar/R region consisting of the highly conserved R195, H180, F56, and C189 residues is the narrowest point within the selectivity filter (~2.8 Å diameter), responsible for physically excluding larger solutes [78] (figure 1-5d). The second constriction site is formed by two characteristic NPA motifs (N76-P77-A78 and N192-P193-A194) at the end of the non-spanning helices M3 and M7, which are located in close proximity to each other in the structure. This constriction, together with the ar/R filter, is thought to be responsible for the prevention of proton (and other cation) transport across the membrane [78]. Figure 1-5 shows AQP structure as well as ar/R and NPA regions [55].
Fig 1-5. Bovine Aquaporin 1 structure. **a)** The tetramer structure of AQP1 (based on bovine AQP1 X-ray structure, PDB code: 1J4N). Monomers are shown with different colors and numbered 1 to 4. **b)** Topology of AQP1 in the lipid bilayer. **c)** The monomer structure of bAQP1 with the transmembrane helices indicated as M1-M8 and their connecting loops as a-e. **d)** The arginine/aromatic (green) and NPA (orange) constriction regions of bAQP1. The orange color indicates Asn194, Pro195 and Ala196. The violet color shows the backbones of residues Ile192, Cys191, Gly190, and Gly189 that are hydrogen-bond acceptors in their interaction with water molecules, the black color is for the other non-polar side chain atoms. Figure is reproduced from Verkman et al [55] with permission from Nature Publishing Group.
Figure 1-6 shows the complete water channel with selectivity filter and all residues lining the water pore of AQP1 with the water molecules indicated as green spheres [78]. This figure is showing bovine AQP1 and its important residues such as N194, N78, C191, R197 and H76 (equivalent to N192, N76, C189, R195 and H74 in hAQP1, respectively). These residues are important for hAQP1 as they are part of the selectivity filter.

Fig 1-6. Structure of bovine AQP1 with residues important for water transport shown. The green spheres are water molecules as they pass through the AQP channel. The blue arrow shows the ar/R constriction region area and the black arrow shows the meeting point of the two half helices that contain NPA motifs. Image adapted from Sui et al [78] with permission from Nature.
1.5 Mechanism of water transport and proton exclusion from hAQP1 channel

As known from the earlier EM studies, the hAQP1 hourglass structure gives the protein a conical shape both in its intracellular and extracellular parts. The entrance of the channel is about 15 Å wide and is located on the upper part of the constriction region [32, 46, 78, 86–88]. Towards the middle of the channel, where ar/R and NPA regions are located, the width of the channel becomes narrower, which can limit the entry of molecules based on their size. The width of the ar/R region of about 3 Å can limit the entry of urea and glycerol due to the larger size of these molecules (> 3.4 Å) [88, 89]. A single water molecule has 2.8 Å van der Waals diameter allowing it to enter the channel. The diameter of the NPA region that is located under the ar/R constriction region is around 4 Å [88, 90, 91]. Water molecules that enter the channel form hydrogen bonds with Arg 195 that is located in the ar/R region. Water molecules participate in hydrogen bonding interactions through the Ne and η1 side chain nitrogens of Arg 195. As water molecules come down the channel, the special orientation of water molecules occurs in the NPA region through hydrogen bonding interactions of oxygen atom of water molecule with two hydrogens of the amide sidechains from Asn76 and Asn192. Also, other important residues that are forming hydrogen bonds with water molecules via their backbones are Gly188, C189, G190, and I191, allowing water molecules to pass through the channel. Figure 1-7 shows the amino acid residues in the selectivity filter and their hydrogen-bonding pattern with water molecules [87].
Fig 1-7. The selectivity filter in bAQP1 and the important amino acid residues in hydrogen bonding interactions with water. The distances (in Å) between heavy atoms are shown next to each interacting pair. The equivalent residues in hAQP1 from the top are Gly125, Arg195, Asn192, Asn76, His74, Ala73, Gly72, Gly188, His180, Gly190, Phe56, Val176, Ile60, Leu149, Ile172, Ala64 and Val53. Figure is reproduced from Ho et al. [87] with permission from Proc. Natl. Acad. Sci. USA journal.

It is important to emphasize that while hAQP1 channel transports water, it excludes the passage of cations, including protons [88]. Exclusion of protons and other cations is very important for the function of hAQP1 as it is needed to preserve the ionic transmembrane gradients. The mechanism of cation exclusion has been studied through
site-directed mutagenesis by Beitz et al [89], where the residues Arg195 and His180 that are part of the constriction region were replaced by Val and Ala. These point mutations allowed protons to pass through the hAQP1 channel, implying the role of ar/R filter in cation exclusion. In a later work, proton and cation conductivity through the channel were examined in more detail [88], where two residues in the NPA region, Asn76 and Asn192, were replaced with aspartate. The two short half-helices can generate positive electrostatic field at the NPA ends in the middle of the pore due to their helical dipole moments [76], which can affect passage of cations as well. Nevertheless, the single mutations of Asn76 and Asn192 to Asp did not change the water channel into a proton channel [88]. Later, the triple mutations of Asn76 and/or Asn192 to Asp, His180 to Ala and Arg195 to Val were performed on the AQP1, conferring cation permeability (Figure 1-8).
Fig 1-8. AQP1 constriction region consists of selectivity filter on top and NPA motifs in the middle. A) Constriction region of rat AQP1 without mutations. B) mutated constriction region. The gray ellipses indicate the shape and size of the aromatic/arginine and NPA regions. The diameter of the ar/R region increased after the triple point mutations allowing cation permeability. The figure is reproduced from Wu et al. [88] with permission from EMBO journal.

According to these experiments [88], the mutant AQP1 expressed in *Xenopus laevis* oocytes remains functional but becomes more leaky and permeable to protons after the triple mutation. The diameter of the ar/R constriction region increases and makes it possible for other cations to enter to the channel as well (Figure 1-9).
Fig 1-9. Selectivity filter and NPA region bear positive charge in AQP1. A) ar/R and NPA regions of AQP1 that contain positive charge and exclude passage of cations through the channel. B) From left, ND shows two single mutations of N76 and N192 to D that remove positive charge in NPA motif and allows the sodium to pass through the channel. HA/RV are two mutations in ar/R region that remove positively charged amino acids in this region and allow protons to pass through the channel. ND/HA/RV is a combination of these mutations in both NPA and ar/R region that changes the water channel to complete cation channel. Figure adopted from Wu et al [88] with permission from EMBO journal.

Figure 1-9 indicates that the presence of H180 and R195 in the entrance of the pore creates a positively charged environment that can exclude protons and cations [88]. Also, in the NPA region the dipoles of the two half-helices at Asn76 and Asn 192 create positively charged environments and repel protons and cations as well.
In addition to the electrostatic hypothesis of proton exclusion, the orientation of water molecules in the channel may play a role. In the NPA region, there is a proline-proline interaction which holds the N-termini of the two short α-helices in the center [76]. Based on the orientation of the Asn residues, hydrogen atoms of water molecules are not involved in hydrogen bonding interactions in the NPA region. Consequently, the oxygen atom of the water molecule forms hydrogen bonds with the side chains of Asn residues. Thus, the hydrogen atoms of the adjacent water molecules face each other and fail to participate in hydrogen bonding interactions as there are no oxygen atoms available [76]. Therefore, there is a central water molecule in the NPA region that breaks the pathway of hydrogen bond formation and prevents passage of protons [76]. Figure 1-10 shows the orientation of the water molecules in the center of the NPA region, which helps exclusion of protons [76].
Fig 1-10. Water orientation in the channel of AQP1. a) Orientation of two short α-helices in the water channel of AQP1. b) Hydrogen bonding interactions of water molecule with side chains of Asn residues. c) Position of water molecule in the center with its oxygen atom in the hydrogen bonding interaction with side chains of Asn residues. Figure adopted from Murata et al. [76] with permission from Nature.

### 1.6 Goals and experimental approaches of the thesis

hAQP1 is an important protein for the human body. As discussed above, the improper function, overexpression, or absence in certain tissues can contribute to various health problems. Thus, hAQP1 may contribute to several human diseases and it was
shown that hAQP1 inhibition could be a promising therapy. Therefore, pharmaceutical companies are seeking suitable drugs to inhibit the function of hAQP1. hAQP1 inhibitor, mercury chloride, is toxic for the human body, but understanding the mechanism of its inhibitory action could certainly help develop more suitable hAQP1 inhibitors. There are also other chemical compounds that showed inhibitory effects on hAQP1, such as tetraethylammonium (TEA), acetazolamide (AZA) and derivative of bumetanide (AqB013), as well as some novel blockers [35, 92–96], but their exact inhibitory mechanisms are still unknown. On the other hand, the available assays to investigate the activity of hAQP1 in different systems such as Xenopus oocytes, hAQP1-expressing epithelial cells, red blood cells, and proteoliposomes often showed contradictory results for the mechanisms of hAQP1 inhibition [92, 97–100]. Therefore, there is a need for a unique technique that can characterize the binding of inhibitors site-specifically and provide detailed information on the protein-ligand (in this case ligand is inhibitor) interactions and any conformational changes that occur to the protein in the process. Thus, in this thesis we studied structure of native and mercury-inhibited hAQP1 using solid-state NMR, and revealed the mechanism of mercury inhibition through other biophysical techniques.

X-ray crystallography, electron microscopy, and electron crystallography are common techniques to investigate membrane protein structure. These techniques were used before to report on the structures of hAQP1 [37, 75–77]. However, there are some limitations on how these techniques could be used to investigate the inhibition mechanism of hAQP1. The X-ray crystallography study of purified and crystallized hAQP1 [78] did not yield the structure of this protein under its native-like lipid
membrane conditions. Moreover, acquiring good X-ray diffraction and EM data requires cryo-temperatures that can affect dynamic motions of hAQP1 [1, 101]. At the same time, membrane proteins such as hAQP1 that contain many dynamic loops in their structure may not form ordered 3D crystals [102]. The flexible regions in hAQP1 such as loops A, B, C, and D can show a smeared electron density map that could result in an ambiguous prediction of the structure in these regions [75, 77, 78]. Therefore, the mechanism of inhibition of hAQP1 by mercury could not be understood properly due to the differences that can occur in the dynamic motions of the protein in 3D crystals. Moreover, our goal was to understand the mechanism of mercury chloride inhibition of hAQP1 in its lipid environment. The electron microscopy or electron crystallography on the other hand shows some limitations as well, due to limited spatial resolution and cryogenic temperatures. Thus, we cannot study the site-specific dynamics of the inhibitor binding sites using these structural techniques.

Another choice of a structural method to be applied to our study could be solution NMR. However, solution NMR may not be a good technique due to its own limitations. Solution NMR requires soluble protein or small protein-model membrane systems, but large protein-detergent micelle complexes do not tumble rapidly enough to average anisotropic interactions [1]. This is a serious limitation as hAQP1 protein exists in the membrane as a tetramer, which has a total molecular weight of 124 kDa. Thus, solution NMR is not suitable technique for our goals.

Solid-state NMR (ssNMR) is a promising technique that was used for this study. ssNMR can provide detailed information about the structure of hAQP1 in its native-like lipid membrane as in this method there is no need for crystallization. This method also
overcomes the size limitation problem of solution NMR [1, 103, 104]. Homogenous samples can provide well-resolved ssNMR spectra that can yield structures of membrane proteins, and structures of 18 alpha-helical membrane proteins have been obtained through ssNMR as of today [105–113].

ssNMR could provide direct observations of mercury chloride interactions with hAQP1. ssNMR is a powerful technique which can specifically characterize the binding site of the inhibitor of hAQP1. ssNMR can report on any rearrangements in the global structure of the protein as well as any conformational changes that can occur upon the binding of mercury. Finally, ssNMR can provide detailed information at the atomic level on the site-specific dynamics of the inhibitor-protein complex.

One of the biggest challenges in ssNMR studies of membrane proteins is to obtain a homogeneous natively folded sample giving high spectral resolution sufficient for structural studies. To achieve this goal, first one needs to produce the target protein in the appropriate expression system. Choosing an appropriate expression host is an important decision, as it can affect the quality and quantity of the final product. The expression level of the membrane protein could be very low for the following reasons: an incorrectly chosen expression system; the interference of the secondary structure of the mRNA with the host cellular machinery; inconsistency between the codon usage and the supply of the host strain’s tRNA; presence of the inclusion bodies and host cell toxicity [114]. Eukaryotic membrane proteins are especially difficult and expensive targets in this respect. Methylotrophic yeast *Pichia pastoris* is a reliable producer of eukaryotic membrane proteins for crystallography and a promising economical source of isotopically labeled proteins for NMR.
In this project, the first objective was to investigate the structure and conformational dynamics of wild-type hAQP1 reconstituted in its native-like lipid environment with ssNMR. Therefore, an optimal sample preparation protocol needed to be established in order to determine the structure of hAQP1. Thus, a part of this project was devoted to working on developing a protocol that is suitable for the production of the doubly ($^{13}\text{C}/^{15}\text{N}$) isotopically labeled hAQP1 suitable for ssNMR studies in this host (Pichia pastoris). The second objective was to reveal the changes that can occur to hAQP1 upon its inhibition by mercury chloride.

We showed that eukaryotic membrane protein hAQP1 can be doubly ($^{13}\text{C}/^{15}\text{N}$) isotopically labeled in this system and functionally reconstituted into phospholipids, giving excellent resolution of solid-state magic angle spinning NMR spectra (Chapter 3). We have been able to assign chemical shifts of a total of 192 out of 269 residues of hAQP1 using a variety of two- and three-dimensional NMR experiments. We also obtained unique information about the secondary structure of the hAQP1 from our NMR results. The information about solvent accessibility and hydrogen bonding strength of the backbone amides and nitrogen-bearing side chains of hAQP1 was also obtained from ssNMR H/D exchange data. At that stage, when we acquired enough information about the native hAQP1, it was time to investigate its inhibition mechanism by mercury chloride. Thus, the native reconstituted hAQP1 was inhibited with high concentration of mercury chloride for ssNMR studies, but, unexpectedly, the highly resolved NMR spectra lost the resolution, suggesting mercury-induced heterogeneity. As a consequence, the mechanism of mercury inhibition remained unanswered by NMR (Chapter 4).
The unanswered question about the mechanism of inhibition of hAQP1 by mercury chloride was investigated through other biophysical methods. Therefore, we studied the effects of mercury chloride on hAQP1 using different biophysical techniques such as Fourier transform infrared (FTIR) spectroscopy, small angle x-ray scattering (SAXS), stopped-flow kinetic experiments and dynamic light scattering (DLS).

Fourier transform infrared (FTIR) spectroscopy can provide us with the secondary structure of hAQP1 in its native form and in the presence of the inhibitor by following changes of certain peaks related to the protein (e.g., Amide bands). Looking at vibrations of lipid esters as well as lipid head groups we were able to follow the protein to lipid ratio and its changes upon the inhibition of hAQP1 by mercury chloride (Chapter 5).

We also examined the activity of hAQP1 in the absence and presence of the inhibitor by stopped-flow kinetic experiments [115, 116]. The stopped-flow technique is a type of flow injection analysis where reactants are rapidly mixed in a mixing chamber. This technique allows measurement of the interaction between the sample and a reactant [117]. We used this technique in order to follow the changes in the activity of hAQP1 upon its inhibition by mercury chloride and other hAQP1 blockers (Chapter 5).

Dynamic light scattering (DLS) was another important technique that we used in this work. This technique is used to determine the size distribution profile of particles in a solution [118–120]. We used DLS experiments to measure the size of the hAQP1 proteoliposomes in the absence and the presence of the inhibitors and to reveal possible protein aggregation (Chapter 5).

Finally, we used small angle X-ray scattering (SAXS) that provided information about the formation of the two-dimensional crystal lattice in our native and inhibited
reconstituted hAQP1 samples [121, 122]. The basic physical principles of ssNMR, FTIR, stopped-flow, SAXS, and DLS techniques are provided in Chapter 2.
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Chapter 2

Introduction to biophysical methods
2.1 Rationale for choosing methods for studying hAQP1

In this chapter we describe the basic physical principles of the biophysical techniques used to characterize hAQP1 structure and its interaction with Hg\textsuperscript{2+}. Although there are numerous structures of human AQP1 and its highly homologous bovine variant, the structural details of hAQP1 in the native-like lipid environment and near ambient temperature, as well as in the presence of inhibitors are not available. NMR is ideally suited for studying these aspects, as it does not require crystallization and can be applied to probe even weak protein-ligand interactions. As discussed in the Introduction, AQP1 is a relatively large membrane protein and is not amenable to solution NMR. Thus, we chose solid-state NMR to study hAQP1.

Studying hAQP1 required characterizing its function, oligomerization, as well as interactions with ligands by additional techniques. Thus, in addition to the basic ideas of solid-state NMR, the general principles of the stopped-flow kinetics, DLS, SAXS, and FTIR spectroscopy, which were used in this thesis, are outlined in this chapter.

2.2 Theory of NMR

Nuclear Magnetic Resonance (NMR) is one of the key techniques used to analyze molecular structure and dynamics. It uses the spin property of nuclei, and relies on detecting magnetization resulting from a Boltzmann population difference between the energy levels of nuclear spins resulting from the Zeeman interaction [1, 2]. The Zeeman interaction is based on the coupling of the magnetic moments to the magnetic field.
Biological samples such as proteins contain protons, and are typically isotopically enriched with $^{13}\text{C}$ and $^{15}\text{N}$, which are all spin-1/2 nuclei. The interactions of these spins with the external magnetic field, electrons, as well as with other spins are described by the Hamiltonian, $H(t)$, which includes both external interactions with the magnetic field, and internal molecular interactions:

$$H = H_Z + H_{CS} + H_D + H_J + H_{RF}$$ (2.1)

Internal interactions such as chemical shift interaction ($H_{CS}$), dipolar interaction ($H_D$) and J-coupling ($H_J$) depend on molecular structure, whereas external interactions are described by the Zeeman ($H_Z$) interaction and the radio-frequency ($H_{RF}$) term, and correspond to the interactions with the magnetic fields, and provide ways for manipulating the internal interactions [3]. In the Equation 2.1 above we restrict our consideration to spin-1/2 nuclei and neglect the quadrupolar interactions.

### 2.2.1 Zeeman Interaction

The Zeeman interaction is described by the Hamiltonian

$$H_Z = \hbar\omega_0 I_z$$ (2.2)
where \( \hbar \) denotes the Planck constant, \( I_z \) denotes the \( z \)-component of a spin \( \frac{1}{2} \) operator, and \( \omega_0 \) is the Larmor frequency of a nuclear spin placed in the magnetic field \( B_0 \) which is chosen to be along \( z \)-axis:

\[
\omega_0 = -\gamma B_0.
\] (2.3)

In the Eq. 2.3 \( \gamma \) is the nuclear gyromagnetic ratio, which is the ratio of the magnetic dipole moment of the nucleus to its angular momentum. The Zeeman interaction yields two energy eigenstates corresponding to two projections of the magnetic moment \( m_1 = \pm 1/2 \) onto the \( z \)-axis, which can be visualized as nuclear magnetic moments aligned with the external field (\( m_1 = 1/2 \) or \( \alpha \)-state, we assume positive gyromagnetic ratio), and opposite to the field (\( m_1 = -1/2 \), or \( \beta \)-state). The energies of these states are given as

\[
E_\alpha = -\frac{1}{2} \hbar \gamma B_0
\] (2.4)

\[
E_\beta = \frac{1}{2} \hbar \gamma B_0
\] (2.5)

At equilibrium, these states are differently populated according to the Boltzmann distribution:

\[
n_{\alpha,\beta} = \exp \left( -\frac{E_{\alpha,\beta}}{k_B T} \right)
\] (2.6)
$k_B$ is the Boltzmann constant and $T$ is the absolute temperature in Kelvin. By NMR convention, the energy and magnetic field are measured in frequency units. Under typical experimental conditions, the population difference is small and results in a small but detectable net magnetization $M_0$ for a sample containing $N$ nuclei:

$$M_0 \approx \frac{N\gamma^2B_0}{4k_BT}$$

(2.7)

It is proportional to the strength of the magnetic field $B_0$, the number of spins $N$, and to the square of the gyromagnetic ratio, and inversely proportional to temperature. Therefore, the equation 2.7 shows the importance of working at high $B_0$ fields.

### 2.2.2 Chemical Shift Interaction

In diamagnetic biological solids containing nuclei with spin-1/2 only, the Zeeman interaction is the strongest and dominant among all nuclear spin interactions. Thus, the parts of the internal interactions that do not commute with the Zeeman Hamiltonian can be neglected (the so-called secular approximation).

The external magnetic field experienced by a nuclear spin is shielded by the electron density cloud surrounding the nucleus. This shielding effect is generally anisotropic (i.e., it depends on the relative orientation of the cloud with respect to the external magnetic field) [3, 4]. In addition, as the structure of the electronic orbitals around each nucleus depends on the chemical environment (i.e., chemical bond structure, interactions with other molecules, solvent conditions, etc.), chemically non-equivalent
spins would experience different shielding effects and would have different Larmor frequencies. The chemical shielding effect is described by a second rank tensor denoted here as $\tilde{\sigma}$. The induced field can be expressed in terms of this tensor and the external magnetic field:

$$\vec{B}_{induced} = \tilde{\sigma} \cdot \vec{B}_0 = \begin{bmatrix} \sigma_{xx} & \sigma_{xy} & \sigma_{xz} \\ \sigma_{yx} & \sigma_{yy} & \sigma_{yz} \\ \sigma_{zx} & \sigma_{zy} & \sigma_{zz} \end{bmatrix} \cdot \vec{B}_0$$

(2.8)

The chemical shift tensor in Eq. 2.8 can be expressed in terms of its principal components $\sigma_{11}, \sigma_{22}, \sigma_{33}$ through a series of rotations described by Euler angles $\Omega_{PL} = (\phi, \theta, 0)$ from the Principal Axis System (PAS) in the which the chemical shift tensor is diagonal to the laboratory (LAB) frame associated with the external Zeeman magnetic field. Figure 2-1 shows the relation of the Principle Axis System to the laboratory frame.
The chemical shift Hamiltonian can be written in terms of these principal components $\sigma_{11}, \sigma_{22}, \sigma_{33}$ as follows:

$$H_{CSA} \approx \omega_0 S z (\sigma_{11} (\sin \theta \cos \phi)^2 + \sigma_{22} (\sin \theta \sin \phi)^2 + \sigma_{33} (\cos \phi)^2)$$

(2.9)

In solution phase, fast isotropic molecular tumbling averages this interaction to its isotropic value, $\omega_{iso} = \omega_0 \frac{1}{3} (\sigma_{11} + \sigma_{22} + \sigma_{33})$. In the absence of motions (e.g., in samples prepared as powders, or in immobilized samples containing randomly oriented molecules) chemical shift interaction contributes to the anisotropic line broadening. We will discuss below how it can be averaged out using the magic angle spinning technique.
2.2.3 Dipolar Interactions

Dipolar interaction is the direct through-space interaction between the magnetic dipole moments associated with two spins[5]. The secular part of the dipolar Hamiltonian for two interacting spins \( I, S \) can be expressed as

\[
H^{II} = b_{IS} \frac{3 \cos^2 \theta - 1}{2} \left[ 2 I_z S_z - \frac{1}{2} (I_+ S_- + I_- S_+) \right]
\]  

(2.10)

for the homonuclear spin pair, and

\[
H^{IS} = b_{IS} \frac{3 \cos^2 \theta - 1}{2} 2I_z S_z
\]

(2.11)

for the heteronuclear spin pair. \( \theta \) is the angle between in the internuclear vector and the external magnetic field, and \( b_{IS} \) is the dipolar interaction strength given as

\[
b_{IS} = -\hbar \frac{\mu_o \gamma_I \gamma_S}{4\pi r_{IS}^3}
\]

(2.12)

where \( \mu_o \) is the magnetic constant, \( \gamma_I, \gamma_S \) are gyromagnetic ratios of spins \( I \) and \( S \), respectively, and \( r_{IS} \) is the internuclear distance. Similar to the chemical shift interaction, the dipolar interaction is anisotropic and causes line broadening in the spectra, which can be suppressed by magic angle spinning.
2.2.4 Scalar Interactions

The $J$-coupling or scalar coupling is the indirect through-bond interaction between two spins. Only the isotropic (dominant) part needs to be considered for practical applications. Typical $J$ coupling strengths vary from 20 to 50 Hz for $^{13}\text{C}^{13}\text{C}$ bonds to $\sim140$ Hz for the directly bonded $^{13}\text{C}^{1}\text{H}$ spin pair. The secular part of this interaction is

$$H_J = 2\pi J I_x S_x$$  \hspace{1cm} (2.13)

In the applications discussed below, heteronuclear $^{13}\text{C}^{1}\text{H}$ and $^{15}\text{N}^{1}\text{H}$ $J$-couplings are suppressed by applying heteronuclear decoupling, while homonuclear $^{13}\text{C}^{13}\text{C}$ $J$-couplings result in line splitting and/or line broadening in the NMR spectra [6, 7].

2.2.5 Interaction of spins with radio-frequency field

The final term in the Hamiltonian of Eq. 2.1 is due to the interaction of a nuclear spin with the applied radio-frequency (RF) field $B_{RF}(t)$. Assuming $B_{RF}(t)$ to be periodically time-dependent and along x-axis, $B_{RF}(t) = (2\,\vec{B}_1\cos(\omega_{RF}t + \varphi), 0, 0)$, the RF Hamiltonian is represented as

$$H_{RF} = -\gamma I \cdot B_{RF}(t) = 2\omega_1 I_x \cos(\omega_{RF}t + \varphi)$$  \hspace{1cm} (2.14)
where $\omega_1 = -\gamma B_1$ is the strength of the RF field expressed in frequency units, $\omega_{RF}$ is the frequency of the applied field, and $\varphi$ is the phase of the pulse. Following the usual treatment, the linearly polarized oscillatory field could be visualized as a sum of two circularly polarized components rotating at $\omega_{RF}$ clockwise and counterclockwise. Only the component rotating with the frequency close to the Larmor frequency $\omega_0$ and in the same direction would have a strong effect on the nuclear magnetization and must be retained. In the Rotating Frame of reference (i.e., in the frame rotating with the resonant RF component), the RF Hamiltonian becomes time-independent:

$$H_{RF} = \omega_1 (I_x \cos\varphi + I_y \sin\varphi) \tag{2.15}$$

All other interactions remain the same in the Rotating Frame, with the exception of the Zeeman interaction, which is shifted by $-\omega_{RF}$. This shift does not affect the apparent results of the NMR experiments as those are carried out in the rotating frame, where interactions are detected as offsets from the carrier frequency.

### 2.2.6 Magic Angle Spinning

As discussed above, NMR spectra of partially immobilized or immobile molecules are broadened by the anisotropic dipolar and chemical shift interactions. This broadening can be removed through the application of the magic angle spinning (MAS) technique [8, 9]. In a typical MAS experiment, the sample is spun around an axis directed at an angle of 54.7° with respect to the external magnetic field (Figure 2-2).
Fig 2-2. Magic angle spinning (MAS) technique. The sample is packed in a rotor which is spun about an axis pointing at 54.7° with respect to the external magnetic field $B_0$.

MAS averages out chemical shift and dipolar anisotropies and results in high resolution spectra as shown in Figure 2-2. As a result of sample rotation, the CSA and dipolar Hamiltonians become time dependent [10]

\[
H_{\text{MAS}}^{\text{CSA}} = \omega_{\text{CSA}}(t) S_z \\
H_{\text{MAS}}^{\text{D}} = \omega(t)(2I_z S_z - \frac{1}{2}(I_+ S_- + I_- S_+))
\]

The coefficient $\omega_{\text{CSA}}(t)$ gains periodic time dependence, which can be written as:

\[
\omega_{\text{CSA}}(t) = -\omega_{\text{ISO}} - \omega_I \{ g_{1}^{\text{CSA}} \cos(\omega_R t + \phi_1) + g_{2}^{\text{CSA}} \cos(2\omega_R t + \phi_2) \}
\]
\( \omega_R = 2\pi \nu_r \) is the angular frequency of the rotation (expressed in radians per seconds), \( \omega_{ISO} \) is the isotropic shift, and \( \omega_I \) is the anisotropy strength:

\[
\omega_I = \omega_0 \sigma_{33} - \omega_{ISO}
\]

(2.19)

The orientation dependent coefficients \( g_1^{CSA}, g_2^{CSA} \) and phase angles \( \phi_1, \phi_2 \) are:

\[
g_1^{CSA} = \sqrt{\frac{3}{2}} \frac{\sin 2\theta_m \sin \beta}{\eta} \times \left[ (\eta \cos 2\gamma + 3)^2 \cos^2 \beta + \eta^2 \sin^2 2\gamma \right]^{1/2}
\]

(2.20)

\[
g_2^{CSA} = \sqrt{\frac{3}{2}} \frac{\sin^2 \theta_m}{2} \times \left\{ \left[ \frac{3}{2} \sin^2 \beta - \frac{\eta}{2} \cos 2\gamma (1 + \cos^2 \beta) \right]^2 + \eta^2 \cos^2 \beta \sin^2 2\gamma \right\}^{1/2}
\]

(2.21)

\[
\phi_1 = \alpha + \psi_1
\]

(2.22)

\[
\phi_2 = 2\alpha + \psi_2
\]

(2.23)

\[
\tan \psi_1 = \eta \frac{\sin 2\gamma}{(\eta \cos 2\gamma + 3) \cos \beta}
\]

(2.24)

\[
\tan \psi_2 = -\eta \frac{\cos \beta \sin 2\gamma}{\left( \frac{3}{2} \right) \sin^2 \beta - \frac{\eta}{2} \cos 2\gamma (1 + \cos^2 \beta)}
\]

(2.25)
In Equations 2.20-2.25, $\alpha, \beta, \gamma$ are the Euler angles defining the orientation of the chemical shift anisotropy tensor in the laboratory frame, $\theta_m$ is the magic angle, $\theta_m = \cos^{-1}\left(\frac{1}{\sqrt{3}}\right) \approx 54.7^\circ$, and $\eta$ is the asymmetry parameter:

$$\eta = \frac{\sigma_{11} - \sigma_{22}}{\sigma_{33} - \sigma_{\text{iso}}} \tag{2.26}$$

The dipolar coefficients can be expressed in a similar form [11]:

$$\omega_D(t) = b_{IS} (g_1^D \cos(\omega_R t + \phi) + g_1^D \cos(2\omega_R t + 2\phi)) \tag{2.27}$$

with the orientation dependent coefficients defined as:

$$g_1^D = \frac{3}{2} \sin2\theta_m \sin2\theta_{IS} \tag{2.28}$$

$$g_2^D = -\frac{3}{2} \sin^2\theta_m \sin^2\theta_{IS} \tag{2.29}$$

$\theta_{IS}$ describes the orientation of the internuclear vector $\vec{r}_{IS}$ in the rotor frame. Figure 2-3 shows the effect of MAS on the powder spectra. Without spinning, the spectrum is broadened by the effects of chemical shift anisotropy and dipolar couplings. The oscillatory time dependence of the $\omega_{CSA}(t)$ and $\omega_D(t)$ terms in Eqs. 2.18 and 2.27 results in general line narrowing and in the appearance of the spectral sidebands occurring at multiples of spinning frequencies. The sideband intensities are reduced at higher MAS
rates exceeding the anisotropy strengths and this results in nearly complete averaging of anisotropies [12–15]. The MAS ssNMR has been extensively used to study protein structure and provided important insights into their function [16–24].

\[ \nu_R = 4000 \text{ Hz} \]

\[ 2000 \text{ Hz} \]

\[ 1000 \text{ Hz} \]

\[ 583 \text{ Hz} \]

\[ 0 \text{ Hz} \]

50 40 30 20 10 0 -10 -20 -30 -40 ppm

Fig 2-3. Effect of magic angle spinning on the NMR linewidth. $^3\text{P}$ spectra of ammonium dihydrogen phosphate collected at a field strength of 200 MHz and shown as a function of spinning frequency $\nu_R$. Figure is a courtesy of Dr. Glenn A. Facey [25].

**2.2.7 Chemical shift correlation spectroscopy and spectroscopic assignments**

As was pointed out above, chemical shift interactions experienced by nuclear spins are exquisitely sensitive to the chemical environment and molecular structure around a nucleus. Even small differences in torsion angles, local hydrogen bonding...
properties, charge distribution, etc., can result in detectable chemical shift differences. Conversely, site-specific chemical shifts, obtained using chemical shift assignment experiments can be used to predict secondary structure [26, 27] and estimate backbone angles [28], monitor conformational changes occurring in a process of protein function[29, 30]. As the large number of spins results in a significant spectral overlap and spectral degeneracy in proteins, resolving resonances requires application of multidimensional spectroscopy methods [29–34].

In protein structure determination, the first step is the chemical shift assignment. In Figure 2-4 we show examples of three-dimensional pulse sequences [35] used in this thesis to resolve spectral degeneracy in hAQP1, and to site-specifically assign resonances. A typical experimental suite consists of three three-dimensional correlation experiments, NCACX, NCOCX, and CANCO. CANCO correlates chemical shifts of CA[i], N[i] , CO[i-1] recorded independently in the F1, F2, F3 dimensions. That is, a CANCO spectrum can be visualized as a 3D box in which a spectral peak would have three frequency coordinates corresponding to CA[i], N[i] , CO[i-1] chemical shifts. Similarly, NCOCX correlates N[i], CO[i-1], CX[i-1] chemical shifts (CX = CA, CB, CG, etc), while NCACX establishes intrar residue correlation between N[i], CA[i], CA[i] chemical shifts (CX = CO, CA, CB, CG, etc).
Fig 2-4. Experimental pulse sequence for A) 3D NCACX/NCOCX correlation experiments. Hollow and filled bars are representing $\pi$ and $\frac{\pi}{2}$ pulses respectively. For carbon mixing dipolar assisted rotational resonance (DARR) [36] is used. Phase cycling is $\Phi_1 = 0, \Phi_2 = 3, \Phi_3 = 0.2, \Phi_4 = 1, \Phi_5 = 0, \Phi_6 = 0, \Phi_7 = 1, \Phi_8 = 1, \Phi_9 = 1, 1, 3, 3; \Phi_9 = 3, \Phi_{rec} = 0, 2, 2, 0$. TPPI phase sensitive detection in $t_1$ and $t_2$ is accomplished through incrementing $\Phi_5$ and $\Phi_8$. B) Pulse sequence of 3D CANCO experiment. Phase cycling includes $\Phi_1 = 1, \Phi_2 = 0, 2, \Phi_3 = 1, 3, \Phi_4 = 0, \Phi_5 = 0, \Phi_6 = 0, 0, 2, 2 \Phi_7 = 0, \Phi_8 = 0, \Phi_9 = 0; \Phi_{10} = 0, \Phi_{rec} = 0, 2, 2, 0$. TPPI phase sensitive detection in $t_1$ and $t_2$ is accomplished through incrementing $\Phi_3$ and $\Phi_4$. This figure is adopted from Shi and Ladizhansky [37] with permission from Elsevier.
In Figure 2-5 we show an example of 2D planes extracted from 3D experiments that we recorded on our hAQP1 sample. Nearly complete backbone resolution is achieved in the CANCO spectrum, and this facilitates the assignment of the chemical shifts of the protein.

![2D plane of the 3D CANCO experiment. First contour is taken at 5×σ, with each additional level multiplied by 1.2. Assignments are shown according to the CO[i-1] shifts.](image)

The CANCO, NCOCX, and NCACX spectra can be co-analyzed to yield site-specific assignments. The procedure of data analysis and assignment is shown in Figure
2-6. The NCACX N[i]-CA[i]-CX[i] correlations share N[i]-CA[i] sets of shifts with the CA[i]-N[i]-CO[i-1] correlations obtained in the CANCO experiment. Similarly, the NCOCX N[i]-CO[i-1]-CX[i-1] correlations share N[i]-CO[i-1] sets of shifts with the CA[i]-N[i]-CO[i-1] correlations of the CANCO experiment. By comparing and matching of the N[i]-CA[i] and N[i]-CO[i-1] pairs of shifts, one can build an extended spin system CX[i-1]-CO[i-1]-N[i]-CA[i]-CX[i] which incorporates both the backbone and side chain shifts of two consecutive residues (Figure 2-6b) [38]. The sequential assignment through ssNMR was used first on peptides [39, 40] and later on used for larger proteins [41, 42].

To determine secondary structure of a protein we use chemical shift indexing (CSI) based on the $^{13}$C chemical shifts. Positive values of CSI indicate an $\alpha$-helical backbone structure, while the negative values suggest that the structure contains $\beta$-turns or $\beta$-strands. TALOS (Torsion Angle Likeliness Obtained from Shift and Sequence Similarity) is another method which predicts backbone torsion angles based on the assigned chemical shifts.
Fig 2-6. Assignment procedure through three NCOCX, CONCA and NCACX correlation spectroscopies. A) From top, NCOCX experiment could relate chemical shift values of nitrogen, N[i], with the preceding CO[i-1] and Cα[i-1] values. In the middle CONCA correlation spectroscopy relates chemical shift values of Cα[i] to N[i] and CO[i-1]. At the bottom there is the NCACX experiment that shows the correlation between N[i] with Cα[i] and CO[i] in the same residue. B) linking fragments strategy based on the same backbone and side chain chemical shift values. Figure reproduced from Shi and Ladizhansky [37] with permission from Elsevier.
2.3 Fourier Transform Infrared (FTIR) spectroscopy

2.3.1 Vibrational Spectroscopy

Vibrational spectroscopy is a technique that produces infrared (IR) and Raman spectra of molecules in the solid, liquid, and gas phases. Infrared and Raman spectroscopy can provide information about composition and interactions within a sample. Changes in both rotational and vibrational states can lead to absorption of infrared photons. The frequency of electromagnetic waves associated with this emission or absorption of photons is proportional to the difference in energy levels, which in turn reports on the frequencies of various molecular vibrations (stretches, bends, wags, etc.). Infrared spectroscopy can be used to determine the conformation and orientation of proteins and lipids. They can provide structural characterization of the protein based on the frequencies of the molecular vibrations. The excitation of vibrations of the atoms in a molecule is caused by absorption of IR photons characterized by a wavenumber $\tilde{\nu}$ in $cm^{-1}$. The infrared range is divided into different regions with respect to the visible range [43, 44]:

1) Near-infrared (NIR) is from 14,000 to 4,000 $cm^{-1}$, that is the closest to the visible region. This region contains higher harmonics of molecular vibrations (overtones).

2) Mid-infrared ranges from 4,000 to 400 $cm^{-1}$ and contains the main molecular vibrations.
3) Far-infrared ranges from 400 to 10 cm\(^{-1}\) and contains lattice vibrations and rotational transitions.

### 2.3.2 Fourier transform infrared (FTIR)

Fourier transform (FT) of IR method is called FTIR and is useful to study protein secondary structure and conformational changes [45]. The difference between FTIR and traditional dispersive spectroscopy techniques is that in FTIR a beam containing many frequencies of light hits the sample at once and thus all the spectral elements are measured simultaneously. In dispersive spectroscopy, the monochromatic light beam illuminates the sample and the absorbed light is to be measured for each wavelength separately. Additional advantage of this is that interferometer-based FTIR machines have higher light throughput, which improves signal-to-noise ratios of the measurements.

Spectral acquisition is based on the applied interferometric pattern of IR radiation that passes through the sample and reaches the detector [46]. The Fourier transform spectrometer consists of several main elements (Figure 2-6). The beamsplitter (BS) splits (transmits and reflects) the infrared light that comes from the light source. The fixed mirror (FM) reflects the transmitted light that came from the beamsplitter while the movable mirror (MM) reflects the original reflected light that came from the beamsplitter. Then, both reflected lights from fixed and movable mirrors combine together and make a new beam that hits the beam splitter again [47]. This newly reflected beam again splits into two beams, one goes to the light source and one hits the sample.
and is detected by the detector (Figure 2-7A). The re-combination of the beams reflected from the two mirrors creates an interference pattern (interferogram), with the maximum intensity at the center. This intensity is reduced by changes in the distance between the movable mirror and the beamsplitter. The final radiation intensity, \([I(X)]\) is measured as a function of the distance \((X)\) by the detector (Figure 2-7B).

There is also the He-Ne calibration beam that follows the same direction as the infrared radiation. This laser light is a monochromatic beam and thus, the interferogram of the laser beam is a sine function. The equation for the resulting intensity, \(I(X)\) is given as [47]:

\[
I(x) = S(\nu)\cos(2\pi\nu x) \tag{2.30}
\]

where \(x\) is the position of the mirror, \(S(\nu)\) is the intensity of the monochromatic spectral line and \(\nu\) is the frequency.
Fig 2-7. The diagram of a time-resolved FTIR spectrometer: A) Interferometer composed of fixed mirror (FM), movable mirror (MM), beam splitter (BS), combined with HgCdTe detector (MCT), analog-digital converter (A/D), personal computer (PC), Nd-YAG laser for sample excitation at 1064 nm or 532 nm, mirror (M), infrared light source (ir-source). B) The interferogram. C) example of the interaction followed by intensity versus time at fixed mirror position. The figure is reproduced from Jung et al [47] with permission.
The digitized interferogram or \(I(n\Delta x)\) is the result of data acquisition. The counting number for the distance difference between the two zero crossing points of the He-Ne laser is shown as \(n\). The Fourier transformation of the interferogram to the spectrum, \(S(k\Delta \nu)\), presents as follows [47]:

\[
S(k\Delta \nu) = \sum_{n=1}^{N} I(n\Delta x) \exp\left[\frac{2\pi nk}{N}\right]
\]

\(\Delta \nu = 1/(N\Delta x)\) \hspace{2cm} (2.31)

where the single channel spectrum is shown as \(S(k\Delta \nu)\), \(N\) shows the total number of digitized points, \(\Delta \nu\) is the frequency increment and \(k\) is the counting number of the \(\Delta \nu\).

Thus, the final transmission spectrum is obtained through the division of this single beam spectrum by that of the empty sample compartment (\(S_{sample}/S_{reference}\)), which can be further converted to the absorption spectrum.

In principle, FTIR is a very simple method that provides valuable molecular information in cases where detailed atomic structure of the protein cannot be obtained or may not be needed [48, 49].

One of the advantages of using FTIR is the suitability of this technique for studying membrane proteins under their native environment as the presence of phospholipid does not disturb the spectra but even provides details about the quality and quantity of the lipid [48]. It is possible to acquire FTIR absorbance spectra of proteins in aqueous solutions, organic solvents, detergents, lipids, and micelles or in single crystals. Among other
advantages of FTIR, it is worth mentioning that the amount of protein needed for this method could be as low as 10 μg and the size of the protein does not matter [49].

As mentioned above, FTIR spectrum is based on absorption of infrared light by various vibrational modes of the chemical bonds [44, 48, 50]. For proteins, two major bands dominate FTIR absorbance spectrum, i.e., amide I, amide II and amide III bands [51]. Amide I reports on related to \( C = O \) stretching vibration and is located at about 1600-1700 cm\(^{-1}\) in the spectrum. Amide II band is N-H and C-N stretch at around 1550 cm\(^{-1}\) and less intense amide III is around 1300 cm\(^{-1}\) and is related to C-N and N-H bends. Figure 2-8 shows a typical FTIR spectrum of the protein. Water has strong absorbance in the same regions as the protein, which creates technical problems that can be bypassed by using thin samples, reflectance modes, replacement of water with D\(_2\)O as well as a background subtraction.

The lipid ester band is located around 1741 cm\(^{-1}\) in the FTIR absorbance spectra. Other characteristic frequencies of lipids are symmetric CH\(_3\) stretch at 2870 cm\(^{-1}\), CH\(_2\) symmetric stretch at 2850 cm\(^{-1}\), COO\(^-\) antisymmetric stretch at 1623 cm\(^{-1}\). Also, there is a band at 820 cm\(^{-1}\) for P-O stretching vibration and C-O stretching vibration at 1200 cm\(^{-1}\). Based on the relative intensity of lipid ester bands and amide I band, the lipid to protein ratio can be obtained through FTIR absorbance spectra [48, 52, 53].
Fig 2-8. FTIR spectrum of a water-soluble protein in H$_2$O. A) The transmittance spectra of H$_2$O and the protein solution. B) The absorbance spectrum of the water-soluble protein. The absorbance of water was subtracted digitally from the spectrum. The figure is reproduced from Haris et. al [49] with permission.

Generally, $\alpha$-helical proteins have their amide I band at 1650-1655 $cm^{-1}$ [46, 54]. Based on the known protein structures, the $\beta$-sheet structure has strong amide I band between 1612 and 1640 $cm^{-1}$ and also sometimes weaker band at 1685 $cm^{-1}$ [45, 46, 51]. This band is mostly (about 80%) related to $C = O$ stretching vibration and less than 20% related to NH bend [51]. For random coil structures, amide I band frequency is
around 1652-1660 cm$^{-1}$ [48, 51]. Amide II band is used much less frequently for distinguishing between $\beta$–sheets and $\alpha$-helical structure of the protein, but it is known that strong amide II bands at 1530 cm$^{-1}$ imply parallel $\beta$-sheet structure of protein [46]. Thus, the amide bands can be used to determine structure and conformational changes of protein backbone.

FTIR studies of proteins in deuterated water are also possible and important in order to investigate the backbone proton exchangeability when D$_2$O replaces H$_2$O. The amide I and amide II band positions will be shifted from 1650-1657 cm$^{-1}$ for amide I in H$_2$O to 1647-1654 cm$^{-1}$ in D$_2$O and for amide II band, the frequency of the band decreases by around 100 cm$^{-1}$ [48]. The extent of this large shift of amide II upon H/D exchange is used to evaluate the degree of folding and solvent accessibility of the protein core, and also reports on the fraction of stable helices, whose backbone amides are usually not exchangeable. Thus, analysis of secondary structure of proteins with FTIR provides an easy way to understand their native structure and folding [55].

The vibrational bands of amino acid side chains are often close to amide I and amide II regions of the spectrum. The only side chains that are not overlapping with any other groups are carboxyl groups and sulfhydryl groups. The deprotonated $COO^{-1}$ side chain of two amino acids such as Asp and Glu, have band position at 1540-1620 cm$^{-1}$ for asymmetric vibrations and at 1300-1420 cm$^{-1}$ for symmetric vibrations [56]. For the protonated state of the $COOH$ groups of Asp and Glu, CO stretching vibrations show band positions at 1710-1760 cm$^{-1}$ in H$_2$O and 1700-1750 cm$^{-1}$ in D$_2$O [57, 58].
The vibration of $S-H$ group of Cys is found around 2400-2700 $cm^{-1}$ in H$_2$O and at 1845-1860 $cm^{-1}$ in D$_2$O [59, 60]. The hydrogen bonded $S-H$ groups have band at lower position around 2290 $cm^{-1}$ [44, 47].

Absorption of Arg side chain ($CN_3H_5^+$) is at 1652 and 1695 $cm^{-1}$. This absorption is around the same region as amide I and in order to differentiate between amide I and Arg side chain, H/D exchange experiments need to be performed. Upon the exchange of hydrogen with deuterium, Arg peak shifts by about 50 to 70 $cm^{-1}$ and thus it will be separated from amide I peak [43].

The vibrational band of Tyr $C-C$ ring is at 1515 $cm^{-1}$ and its isolated and sharp bandwidth makes this peak distinguishable in FTIR spectrum. Protonated Tyr has band at 1235-1270 $cm^{-1}$ for its ($C-O$) group and band position at 1169-1260 $cm^{-1}$ for its (COH) group in D$_2$O. Also ionized Tyr with ($C-O^-$) group has an absorption band at 1270 $cm^{-1}$.

Side chains of His amino acid including ($CN$) groups have band position at 1439 $cm^{-1}$ and ($C-H$), ($C-N$) and ($N-H$) groups show their band positions at 1217, 1229 and 1199 $cm^{-1}$ in H$_2$O for His$^-$, HisH and HisH$_2^+$ respectively. The hydrogen bonding for His residue could be investigated as well because the positions of these three peaks change upon protonation [43]. Table 2-1 shows a summary of assignments of these peaks.
<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Groups</th>
<th>Wavenumber (cm(^{-1})) in H(_2)O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>COO(^{-1})</td>
<td>1540 – 1620 (asymmetric vibration)</td>
</tr>
<tr>
<td>Glu</td>
<td>COO(^{-1})</td>
<td>1300 – 1420 (symmetric vibration)</td>
</tr>
<tr>
<td></td>
<td>COOH</td>
<td>1710 – 1750</td>
</tr>
<tr>
<td>Cys</td>
<td>S – H</td>
<td>2400 – 2700</td>
</tr>
<tr>
<td>Arg</td>
<td>(CN(_3)H(_5))</td>
<td>1652 – 1695</td>
</tr>
<tr>
<td>Tyr</td>
<td>C – C</td>
<td>1515</td>
</tr>
<tr>
<td></td>
<td>(C – O)</td>
<td>1235 – 1270</td>
</tr>
<tr>
<td></td>
<td>(C – O(^{-}))</td>
<td>1270</td>
</tr>
<tr>
<td></td>
<td>(C – OH)</td>
<td>1169 – 1260</td>
</tr>
<tr>
<td>His</td>
<td>His(^{-})</td>
<td>(C – H)</td>
</tr>
<tr>
<td></td>
<td>HisH</td>
<td>(C – N)</td>
</tr>
<tr>
<td></td>
<td>HisH(^{2+})</td>
<td>(N – H)</td>
</tr>
</tbody>
</table>

Table 2-1. Infrared absorption signals of amino acid side chains.

As shown in Figure 2-7, FTIR can be also used in time-resolved mode to study kinetics of light-induced reactions or interactions with ligands. In these types of measurements, the signal acquisition should be synchronized with the initiation of the reaction.
2.4 Stopped-flow

In order to measure reactions on a time scale of seconds or faster, we need to use kinetic (time-resolved) techniques. The stopped flow technique was first demonstrated by Chance in 1940 [61]. In the stopped-flow experiments, the reactants are mixed in a chamber and then the kinetics of the reactions is measured right away, as soon as the mixing is finished (usually takes a few ms). Thus, stopped flow measurement is good for reactions that occur at a sub second rate. In contrast to this method, in continuous flow experiment, reactants flow and mix continuously [62–65]. Figure 2-9 shows schematics of the stopped-flow apparatus.

Fig 2-9. Stopped flow apparatus diagram. Sample and other reactant or solutions are injected from the two separate drive syringes of the apparatus. The figure is reproduced from Mottola [65] with permission from Wiley.
In this method, the reactant and samples are injected simultaneously into the mixing chamber and the reaction starts. This operation is often carried out in UV-Vis spectrophotometer cells to measure ensuing absorbance changes, but is suitable for other measurements such as circular dichroism (CD), scattering, or fluorescence. In this work, the scattering mode of this technique was used.

Final storage of the reactants is in the stopping syringe. The recording starts when the mixing ceases. The stopping syringe drives its plunger against the stopping block and thus it stops the flow and triggers the activation of a data acquisition on the computer [64]. The time that is required for the sample and its reactant to travel to the spectrophotometer is called “dead time” [63].

Among the advantages of this method are small sample volume (i.e., 500 μL) and wide temperature range [63, 64]. Different kinds of reactions such as enzyme substrate reactions, ligand binding, redox reactions, catalysis reaction such as polymerization, coordination reactions or protein folding could be measured using stopped-flow. Kinetic traces acquired through the stopped-flow measurements can yield rate constants of reactions or velocities of transmembrane transport.

Monitoring changes in the intrinsic fluorescence of the protein is very common application of stopped-flow measurement as well. Thus one can acquire data about folding and unfolding of the protein using chemical denaturants.

Stopped flow traces can be fitted to single or double exponential equations that could give us apparent rate constants of the interactions. The following equation is an example of a double exponential function for fluorescence signal:
\[ F = F_0 + F_1 (1 + e^{-K_1 t}) + F_2 (1 + e^{-K_2 t}) \]  

(2.33)

where F is a relative intensity of fluorescence at time t, \( F_0 \) is fluorescence at time t=0 and \( K_1 \) and \( K_2 \) are fitted rate constants. Thus, through the capturing and analyzing time courses of stopped flow-induced spectral changes at a single wavelength one can obtain the kinetics of reactions or rates of folding.

2.5 Dynamic Light Scattering (DLS)

Dynamic Light Scattering (DLS) is a technique to measure the size of particles and molecules in suspension. The interaction of light with the electric field of a molecule results in light scattering. The electric field of light induces an oscillating polarization of electrons in the molecules [66–68]. The molecule then acts as secondary source of light and as a result it scatters light. DLS measures variations in scattered light intensity with time at a fixed scattering angle that is typically 90°. Therefore, DLS measures real-time intensities \( i(t) \) which reflect the dynamic properties of the particles. However, static light scattering measures time-averaged intensities dependent on the molecular mass.

This technique is based on the Brownian motion that is the random movement of particles [69]. DLS relates these Brownian motions to the size of the particles. Brownian motion is characterized by the translational diffusion coefficient (D). Using the translational diffusion coefficient, the size of the spherical particle can be obtained through the Stokes-Einstein’s equation:
In this equation, $d_H$ indicates hydrodynamic diameter, $k$ is the Boltzmann’s constant, $T$ is the absolute temperature, $\eta$ is viscosity and $D$ is diffusion constant \[69, 70\]. The movement of a dispersed particle in a liquid medium causes a thin layer of the solvent to adhere to the particle’s surface. The addition of this layer to the true geometrical size of the particle creates what is called the hydrodynamic diameter.

What is actually being measured in DLS is autocorrelation function of the light intensity. In DLS, the time dependent signal is recorded, where Brownian motion causes intensity fluctuations, and autocorrelation function is constructed as follows:

$$G(\tau) = \frac{\langle I(t)I(t+\tau) \rangle}{\langle I(t) \rangle^2}$$  \hspace{1cm} (2.35)

In this equation, $G(\tau)$ is the autocorrelation function with a delay time of $\tau$, $I$ is the intensity and $t$ is the time.

In DLS measurements, the intensity of the signal at time equal to $t$ is compared to the intensity that is acquired after some delay time or $\tau$. Thus the correlation between two signals at different time is measured. The correlation between the measured intensities decreases as the delay time increases. Thus there will be no correlation between signal intensities that were measured at initial time and at time $t = \infty$. For larger particles, the signal is changing slowly and thus it takes longer time for the autocorrelation function to decay because correlation will persist for longer time (Figure 2-10).
Fig 2-10. Relation between correlation coefficient and size of particles. Larger particles have slower decay of autocorrelation function. The figure has been reproduced by kind permission of Malvern Instruments Ltd [70].

The smaller the particles are, the faster they are moving and thus the correlation will reduce more rapidly.

Correlation function can be fit with exponential functions:

\[ G(\tau) = B + A \sum e^{-2q^2D\tau} \]  

(2.36)

where B is the baseline at infinite time, A is the amplitude, D is diffusion coefficient and q is the scattering vector and it is equal to:
$$q = \left( \frac{4\pi n}{\lambda_0} \right) \sin \frac{\theta}{2}$$  \hspace{1cm} (2.37)

In this equation $n$ is representing dispersant refractive index, $\lambda_0$ is the laser wavelength and $\theta$ is the angle.

The correlation function here includes the diffusion coefficient information which can be used in Stokes-Einstein equation (2.34). Thus, diffusion coefficients are obtained through fitting the autocorrelation function and hydrodynamic radii can be further calculated from them.

Two methods of analysis are important in interpreting DLS results and these methods are as follows:

1) Cumulants analysis that is based on a mean size ($z$-averages) and polydispersity index (PdI).

2) Distribution analysis that is based on real size distribution of suitable data.

The size distribution is based on the intensity-weighted distribution obtained data through cumulants analysis. The $z$-average diameter is sensitive to aggregates in the solutions. The polydispersity index (PdI) is an estimate of the width of the size distribution and it is dimensionless (chapter 5, section 5.7). Figure 2-11 shows the information that could be extracted from the DLS correlation coefficient curve.
Fig 2-11. Information extracted from correlation coefficient curve. The figure has been reproduced by kind permission of Malvern Instruments Ltd [70].

Thus, DLS experiments can provide important information about the size and the distribution width of the particles in the sample as well as the aggregation of the sample if it occurs. The DLS technique can provide information about the protein-protein associations or associations of proteins with other ions or molecules. The changes of these associations could be investigated under different conditions such as changes in pH, concentration or ionic strength [71]. As an example, the polymerization(depolymerization) of the myosin filament in the presence of Mg$^{+2}$ was studied by DLS [72]. Another example of the applications of DLS technique in biology is related to the studies of regulatory protein RecA from *Escherichia coli* [73]. In this work, the size distribution of
the particles were measured after self-association of the RecA protein upon the addition of MgCl$_2$ [73].

### 2.6 Small Angle X-ray scattering to detect sample crystallinity

Small Angle X-ray Scattering (SAXS) is a method to analyze the structure of the molecules based on the averaged particle size or shapes. Liquid and solid samples can be analyzed with SAXS instruments. An X-ray beam irradiates the sample and the detector measures the scattered radiation that is emitted from the sample [74–76]. The scattered x-rays are detected at a specific range of angles from approximately 0.04° to 10° for small angle x-ray scattering [74, 76]. Figure 2-12 shows a diagram for SAXS measurement [74].

![Collimated X-ray beam Sample X-ray detector](image)

Fig 2-12. Schematics of the SAXS experiment. Figure adapted from Putnam et al [74] with permission from Quarterly Reviews of Biophysics journal.

In SAXS, the whole sample volume is investigated through the scattered intensity at varying angles recorded by the detector. Consequently, the averaged values of the structure parameters of the particles in the sample as well as their overall dimensions are
obtained by SAXS. Therefore, SAXS is very sensitive to the presence of big particles and aggregations in the solution.

2.6.1 Theory of SAXS

For X-ray crystallography one of the requirements is the presence of crystal structure in molecules. Crystals are defined as an arranged order of atoms in one dimension such as fibers, two dimensions such as sheets and three dimensions such as lattice. Comparing X-ray crystallography to SAXS it is worth to mention that SAXS does not need crystals for the measurement and thus it is not a crystallographic method even though it can detect crystallinity [74, 77].

X-ray crystallography and SAXS both are based on coherent X-ray scattering [74]. Coherent scattering happens when electrons are oscillating under the electric field and emitting X-rays with the same wavelength as the incident beam with an angle of about 180° relative to the direct beam. The measured intensity of the scattered beam is proportional to \((1 + \cos^2 2\theta)\). The angle \(2\theta\) is the angle of the scattered beam with regard to the incident beam [74].

SAXS results are usually presented in terms of distance in reciprocal space, momentum transfer or \(q\), expressed as

\[
q = \frac{2\sin \theta}{\lambda} \times 2\pi
\]  

(2.38)

Scattering results can be presented in the form of intensity plot against \(2\theta\) or as an
averaged function of intensity \( I(q) \). In this representation, \( I(q) \) is a function of momentum transfer and its unit is \( A^o^{-1} \) or \( nm^{-1} \).

Guinier approximation [78] is an important plot of scattering results and it is defined as follows:

\[
I(q) = I(0)\exp\left[-\frac{q^2R_G^2}{3}\right] \tag{2.39}
\]

where \( R_G \) is radius of gyration which can be obtained from the slope of the plot \( \ln[I(q)] \) versus \( q^2 \). Guinier plot is used to derive \( I(0) \) approximation and it can predict if the sample is aggregated or not by giving \( R_G \). When there is an aggregation in the protein sample, the \( q^2 \) decreases, therefore, the Guinier plot becomes non-linear and thus, the aggregation can be followed using SAXS measurements.

Figure 2-13 that is adopted from Putnam et al [74] shows Guinier plot and the extraction of \( R_G \) and \( I_0 \) from this plot. Using this plot one can identify the aggregated proteins or protein denaturation in the sample.

![Guinier plots](image)

Fig 2-13. Guinier plots, from which \( R_G \) and \( I_0 \) could be calculated. The non-linearity of the plots indicates aggregation. Figure adapted from Putnam et al. [74] with permission from Quarterly Reviews of Biophysics journal.

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Another representation of SAXS data is Kratky plot [79] of $I(q)q^2$ versus $q$ that can be obtained from the scattering results. Folded proteins have a plot with Gaussian shape at small $q$ values and decay approximately as $q^{-4}$ at high $q$. However, the random coil structures have a plot that decays approximately as $q^{-2}$. The unfolded proteins present a plateau at the higher range of $q$. Therefore, the plot for folded proteins is bell-shaped. But for random coil or unstructured peptides, the bell-shaped plot is changed to linear one at larger values of $q$. Figure 2-14 shows a Kratky plot example.

Fig 2-14. Kratky plot of folded, partially folded and unfolded protein. While globular proteins have bell-shaped curves, unfolded proteins show increasing values of $I(q)q^2$ at larger q values. Figure adapted from Putnam et al. [74] with permission from Quarterly Reviews of Biophysics journal.
In crystal structure, there are multiple parallel planes that are diffracting X-rays and thus the path of each diffracted X-ray is different based on the planes. If these path differences correspond to an integral number of wavelengths of the diffracted X-rays, the constructive (in-phase) interference of diffracted X-rays can be detected.

When radiation is scattered from a crystalline system, Bragg diffraction occurs. Bragg’s law can provide information about the distances $d$ between atomic planes based on the following formula:

$$2dsin\theta = n\lambda$$

(2.40)

In this formula, $n$ is positive integer and $\lambda$ is the wavelength of the incident wave, $\theta$ is Bragg angle or the angle of incidence and reflection. Thus, crystal lattice reflections are given based on Bragg angle.

Figure 2-15 shows SAXS plots for a non-ordered sample as well as for near range order and a crystal (ordered) samples. Thus, by using SAXS one can not only investigate folded and unfolded proteins in solution, but also detect crystallinity of proteins in membranes and other media.
Fig 2-15. SAXS intensities for different systems from dilute and not ordered sample to the well-ordered crystal structure. Figure adapted by kind permission from Anton Paar [80].
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Chapter 3

Expression of isotopically labeled human AQP1 in yeast *Pichia pastoris*
Statement of the contribution

All the experiments, results, tables and figures presented in this chapter have been performed by me except for:

- Transformation of the plasmid into protease-deficient *P. pastoris* strain SMD1168H by Dr. Ying Fan.
This work was published in J. Biomol. NMR (2013) 55:147–155,

‘Yeast-expressed human membrane protein aquaporin-1 yields excellent resolution of solid-state MAS NMR spectra’

Sanaz Emami, Ying Fan, Rachel Munro, Vladimir Ladizhansky and Leonid S. Brown

3.1 The importance of sample preparation for ssNMR studies

The key prerequisite of any successful NMR study is the production of natively folded isotopically labeled homogeneous protein, which requires robust overexpression and functional reconstitution into a membrane-like environment. Eukaryotic membrane proteins are particularly challenging targets, in view of the difficulties of achieving their high expression levels in combination with the native fold and post-translational modifications in heterologous systems. Thus, much effort has been spent on experimentation with various expression methods and suitable membrane mimics [1–5]. Expression in Escherichia coli remains the easiest and the cheapest method of production of eukaryotic membrane proteins for NMR. Even though it yielded several structures [6, 7] and some promising samples [8], the problems of non-native fold or poor expression are often encountered. The alternatives such as cell-free expression, as well as mammalian and insect cell cultures, used to be costly, considering that milligram quantities of uniformly labeled protein are required, but the progress in this area is obvious [9–11]. These techniques yet have to produce full NMR structures of eukaryotic membrane proteins, even though there are recent examples of backbone structure determination [12] and experiments based on specific labeling [13]. An alternative, which
has not yet been extensively utilized for isotopic labeling of membrane proteins is expression in yeast, especially the methylotrophic yeast *Pichia pastoris* [14], despite the fact that it has been actively explored for production of eukaryotic membrane proteins for crystallographic studies [15–17].

### 3.2 Double isotope labeling and functional lipid reconstitution of hAQP1

*Pichia pastoris* is a promising candidate for isotopic labeling of eukaryotic membrane proteins due to the powerful combination of two factors. First, *Pichia* showed its promise in NMR studies of soluble proteins, and the protocols for their efficient and inexpensive isotopic labeling are well developed [18–20]. Second, there is a vast literature on crystallographic studies of *Pichia*-expressed mammalian membrane proteins, including a number of X-ray structures of channels, transporters, and G-protein coupled receptors (GPCRs) [21–25]. Even though it seems that amalgamation of these two streams of knowledge should have produced a number of membrane protein samples suitable for high-resolution NMR, such examples are still very rare.

Present work describes a protocol for the double isotope labeling and functional lipid reconstitution of mammalian multi-spanning membrane protein, human aquaporin 1 (hAQP1), which yielded MAS SSNMR spectra of an exceptional resolution amenable to the detailed structural analysis.

Previously, expression of hAQP1 in *P. pastoris* was shown to produce exceptionally high yields in shake-flasks and especially in fermenters, and has been thoroughly optimized [16, 26].
We used the expression plasmid developed earlier elsewhere [26], while the isotope labeling protocol for hAQP1 in *P. pastoris* mainly followed our protocol developed for *Leptosphaeria* rhodopsin (LR) [27] with some modifications. The modifications introduced for hAQP1 mainly relate to the protein solubilization and reconstitution steps, similar to those employed earlier [26].

The expression vector pPICZB-hAQP1-Myc-His6 (kindly provided by Frederick Öberg and Kristina Hedfalk, Göteborg University, Sweden) encodes full-length hAQP1 with a C-terminal Myc and 6xHis tags and allows for selection of the transformants based on resistance to zeocin. The plasmid was linearized using PmeI (New England Biolabs), desalted by the QIAquick nucleotide removal kit (Qiagen), and transformed into protease-deficient *P. pastoris* strain SMD1168H (Invitrogen) by electroporation (MicroPulser, Bio-Rad) as described previously [27]. The transformants were grown on yeast peptone dextrose sorbitol (YPDS) plates containing two different concentrations of zeocin (Cedarlane, 100 and 500 µg/ml). To screen the transformants for the highest level of hAQP1 production, eight colonies were selected for small-scale growth. The colonies were inoculated in 5 mL buffered minimal dextrose (BMD) media (BMD, pH 5 (per 1 L): mix 100 mL of potassium phosphate solution (pH 5), 25 mL of 10 × YNB solution, 2 mL of 500 × (0.02% Biotin), 25 mL of 10 × (20% Dextrose), 100 mL of (NH₄)₂SO₄ and 750 ml of sterile MilliQ water) and grown in shake-flasks at 300 rpm and 30°C overnight. An additional 20 mL of BMD media was added to the cultures when OD₆₀₀ (optical density) exceeded 2 and the cultures were shaken at 300 rpm and 30 °C for 24 hours, or until OD₆₀₀ reached 10. The 2.5 mL of culture were taken and the cells were collected by centrifugation at 1500×g, 4°C for 10 minutes, gently resuspended in 25 mL buffered
minimal methanol (BMM) (BMM, pH 5 (per 1 L): mix 100 ml of potassium phosphate solution (pH 5), 25 mL of 10 × YNB solution, 2 ml 500 × (0.02% Biotin), 10 mL of methanol, 100 mL of (NH₄)₂SO₄ and 765 mL of sterile MilliQ-water) media in sterile 250 mL flasks, and grown for 24 hours at 240 rpm and 28°C. The cells were harvested and resuspended in cell resuspension buffer (CRB) containing 20 mM Tris–HCl, pH 7.6, 100 mM NaCl, 0.5 mM EDTA (Ethylenediaminetetraacetic acid), and 5% (w/v) glycerol (Nyblom et al. 2007).

To digest the cell walls, 15 mg of lyticase (from *Arthrobacter luteus*, Sigma) were added to the pellets and incubated with slow shaking for 3 hours at room temperature. The cells were centrifuged at 1,500×g for 5 min at 4°C and immediately resuspended in one pellet volume of CRB buffer, the same volume of ice-cold acid-washed glass beads (Fisher, 420–600 µm diameter) was added, and the cells were disrupted using vigorous vortex mixing. The cell debris were removed by centrifugation at 700×g for 5 min at 4°C and the cell lysate was collected. This step was followed by repeating vortexing and centrifugation steps (normally, 8 times in total) to achieve complete breakage of the cells.

All cell supernatants containing the membrane fraction were combined and centrifuged at 40,000×g for 30 min at 4°C. The relative hAQP1 content of these membranes was monitored by immunoblotting [26] (using anti-His₆ primary antibody, Clontech and anti-mouse IgG HRP Conjugate secondary antibody, Bio-Rad) and the colony giving the most intense band corresponding to the His-tagged protein (MW about 30 kDa) was chosen for the large-scale growth and isotope labeling.

Before proceeding with the large-scale expression, we performed additional optimization of the post-induction growth length (the time between transfer to BMM and
harvesting) in the small-scale cultures, using a colony with the highest level of hAQP1 expression. The cells were harvested after 6, 12, 24, 36, and 44 hours after the induction (additional 0.5% of methanol was added after 24 hours) and the level of expression was monitored by immunoblotting. As a result of this screening, we found that the best time for harvesting the cells is 24 hours, as the longer incubation does not produce appreciable increase in the hAQP1 yield but requires addition of extra methanol. In fact, the amount of hAQP1 substantially decreases upon prolonged induction (barely detectable at 44 hours). Similar trends were observed earlier for puroindoline-a [28] and for LR Leptosphaeria rhodopsin, but the peak yield for LR was shifted to about 40 hours [27], which stresses the need for thorough individual screening for each protein.

For the large-scale expression and isotope labeling of hAQP1, the best producing colony was used to inoculate 50 mL of $^{13}$C,$^{15}$N-BMD containing 0.8% ($^{15}$NH$_4$)$_2$SO$_4$ and 0.5% $^{13}$C glucose (Cambridge Isotope Laboratories) in a sterile 250 mL baffled flask. The cells were grown at 300 rpm and 30 °C for 18-24 hours and transferred to a sterile 2 L baffled flask with an additional 200 mL of isotope-labeled BMD. The cells were then shaken at 275 rpm and 29°C for 18-24 hours before being spun down at 1500 ×g at 4 °C for 10 minutes. The cells were resuspended in 1 L of $^{13}$C,$^{15}$N-BMM (0.5% $^{13}$C-methanol and 0.8% ($^{15}$NH$_4$)$_2$SO$_4$) in a sterile 2.8 L flask and grown for 24 hours at 240 rpm and 28°C. The cells were collected by centrifugation at 1500×g, 4°C for 10 minutes and washed twice with MilliQ water before storing the pellet at -20°C for later use.

The cells were broken and the membrane fraction was collected as described above, and the total protein concentration was determined by Bradford assay with bovine serum albumin standard (Bio-Rad DC Protein assay)[26]. hAQP1 was solubilized by
resuspending the membrane fraction from 1 L culture in 60 mL of solubilization buffer (20 mM Tris–HCl pH 8.0, 100 mM NaCl, 20% glycerol, 10 mM imidazole, protease inhibitor cocktail (for general use, Sigma), and 5% n-octyl-β-D-glucopyranoside (β-OG, Fisher)) [26]. The mixture was stirred at 4°C for 2 hours and unsolubilized material was removed by centrifugation at 160,000×g for 30 min at 4°C. The supernatant containing solubilized hAQP1 was incubated with 2 mL of Ni$^{2+}$-NTA resin (Qiagen) for 3 hours at 4°C. The resin was placed in a column and washed with washing buffer (30 mM imidazole, 20 mM Tris–HCl pH 8.0, 100 mM NaCl, 20% glycerol, protease inhibitor cocktail, 1% β-OG). Next, hAQP1 was eluted using elution buffer with increasing concentrations of imidazole (150 mM, 250 mM, or 500 mM in 20 mM Tris–HCl pH 8.0, 100 mM NaCl, 20% glycerol, protease inhibitor cocktail, 1% β-OG) [26]. Immunoblotting was used to verify hAQP1 presence throughout the purification process and the completeness of binding to the resin. The final yield of purified hAQP1 was estimated by Bradford assay as well as by UV-Vis spectroscopy (Cary 50, Varian) after the removal of imidazole, using estimated absorbance of 0.1% (1g/L) hAQP1 of 0.944 (PROTPARAM), as well as by the amplitudes of FTIR and NMR signals after lipid reconstitution. The total yield of ~6 mg of isotope-labeled protein per liter of culture is similar to that obtained for LR [27] and represents a very economical protocol, considering that no second addition of $^{13}$C-methanol was required. The purity of the final preparation was assessed by Coomassie SDS-PAGE (Figure 3-1) and MALDI TOF (Figure 3-2) mass spectrometry (University of Guelph Advanced Analysis Center) [26]. Figure 3-1 shows Western blot result of purified hAQP1. We can see purified hAQP1 band at 28 kDa.
Fig 3-1. Western blot of purified hAQP1 protein. From left to right: purified hAQP1, purified hAQP1, Western marker, Western marker. We can see purified hAQP1 band at 28 kDa. The reason that the band was observed at lower mass is because the hAQP1 was not fully unfolded in SDS, typical for membrane proteins.

Figure 3-2 shows mass spectrometry results for the hAQP1 sample. The main peak of the mass spectrum corresponds to the molecular weight of 31,257 ± 10 Da, which is close to that expected for sodium adducts of non-glycosylated natural abundance His- and Myc- tagged hAQP1 (MW of 31,205 Da estimated from PROTPARAM).
3.2.1 Reconstitution of hAQP1

The egg phosphatidylserine (PS) and brain phosphatidylcholine (PC) lipids were used to perform reconstitution of hAQP1. The choice of lipids is important in order to
have a functional reconstituted protein in its native-like environment. PC and PS lipids are common main constituents of the eukaryotic plasma membranes. PC lipids constitute more than 50% of the total phospholipid composition of the eukaryotic plasma membrane [30]. Thus, reconstitution of hAQP1 as a eukaryotic membrane protein in these lipids provides the native-like environment. To perform lipid reconstitution of hAQP1 for solid-state NMR, the lipid stock (egg PC:brain PS = 9:1 w/w, Avanti polar lipids) was prepared as described earlier [27], in 50 mM KH$_2$PO$_4$, 100 mM NaCl, pH 7.5, at ~11 mg/ml. Purified hAQP1 in 30 mL of elution buffer at 0.2 mg/mL concentration was concentrated to 5 mL volume with concurrent removal of imidazole (Amicon centrifugal concentrator, 10 kDa cut-off, Fisher) and mixed with the lipid stock at a protein/lipid ratio of 2 (w/w). After 2 hours of incubation with slow stirring at 4°C, the sample was placed into the dialysis bag (12-14 kDa cut-off, Spectra/Por, VWR) in a container with 10 mL of buffer (50 mM KH$_2$PO$_4$, 300 mM NaCl, pH 7.5) and 8 g of Bio-beads SM-2 (BioRad). The sample was dialyzed for 7 days at 4°C, changing the buffer every 24 hrs. The proteoliposome suspension was withdrawn from the dialysis bag and the proteoliposomes were collected by centrifugation at 300,000×g for 1 hour at 4°C. The pellet was washed several times by centrifugation at 300,000×g for 1 hour at 4°C in 10 mM NaCl, 25 mM Tris-HCl, pH 7, and the proteoliposomes were finally concentrated by ultracentrifugation at 900,000×g for 9 h. The pellet obtained this way was ready for SSNMR rotor packing and kept at -20°C until further use.

In order to verify the native fold, desired protein/lipid ratio, amount, and isotope labeling of hAQP1 in reconstituted samples, Fourier-transform infrared (FTIR) spectroscopy was employed. The proteoliposome pellets corresponding to ~0.05-0.2 mg
of hAQP1 (taken before 900,000×g centrifugation step to avoid excessive aggregation) were resuspended in MilliQ water and dried on a CaF$_2$ window (Harrick). The dry film was covered by another CaF$_2$ window with a 6 micron Teflon spacer and placed in a Bruker IFS66vs machine with a temperature-controlled sample holder (Harrick). Absorbance spectra were averages of 100 scans at 2 cm$^{-1}$ resolution, with the spectra of two empty windows as a reference, the baseline distortions were corrected by OPUS software (Bruker).

Figure 3-3 shows a FTIR spectrum of natural abundance hAQP1 taken in the mid-infrared range, containing protein backbone amide bands and lipid esters vibrations. The typical position and narrow linewidth of the Amide I band (at 1657 cm$^{-1}$) agrees well with mainly α-helical structure of the protein, as previously determined by FTIR [31] and crystallography [32–34]. The ratio of the amplitudes of the Amide I band and the band corresponding to the lipid ester vibrations (at 1738 cm$^{-1}$) is consistent with the low lipid content (1/2 by weight) employed at the reconstitution step and suggests that no significant amount of yeast lipids is retained during purification.
Fig 3-3. FTIR spectrum of hAQP1 reconstituted in PC/PS liposomes. Protein to lipid ratio is 2.0 (w/w) [35]. With permission from the Springer’s Journal of Biomolecular NMR.

### 3.3 Functional assay of hAQP1

To verify the functionality of hAQP1 in the lipids chosen for NMR experiments, we employed water permeability assays [26, 29], mostly following the protocol for *S. cerevisiae*-expressed hAQP1 [29]. As it is known that high protein content can make proteoliposomes leaky [36], the water permeability experiments had to be conducted at a substantially lower protein/lipid ratio (1/10 w/w) than that used for NMR experiments (2/1 w/w). Egg PC/Brain PS liposomes (9/1 molar ratio) were prepared by reverse-phase evaporation [37] and extruded once through polycarbonate Isopore filters (0.4 and 0.2 microns) consecutively. The resulting liposome stock (15 mg/mL of lipids) was mixed
with 0.2 mg/mL solution of purified hAQP1 in the imidazole-free elution buffer (1% OG) to produce lipid/protein ratio of 10 (w/w), with addition of 0.8% of Triton X-100 and 0.56% of OG (Pitard et al. 1996a). After 2 hours of incubation with slow stirring at 4°C, the sample was placed in the dialysis bag in a container with 10 mL of buffer (150 mM KCl, 1 mM KH$_2$PO$_4$, pH 6.8) and 8 g of Bio-beads SM-2, and dialyzed for 48 h at 4°C. As a control, the identical procedure was performed with protein-free liposomes, where addition of hAQP1 was replaced by the same volume of the buffer with 1% OG. The proteoliposomes (and protein-free liposomes) were collected by centrifugation at 300,000×g for 1 hour at 4°C and resuspended in liposome buffer (20 mM Tris-HCl, pH 7.0, 150 mM NaCl) at 0.2 mg lipid/ml. Average diameter of the liposomes (170 nm) and proteoliposomes (179 nm) was estimated by Dynamic Light Scattering (DLS) (Malvern Zetasizer). Water permeability measurements were performed using a stopped-flow spectrophotometer (SX20, Applied Photophysics), by following kinetics of the increase in light scattering (at 480 nm) upon vesicles shrinking induced by hypertonic shock (180 mM sucrose at 22 °C), which reports on the rate of water efflux. The observed trends (Figure 3-4) are very similar to those reported earlier [26, 29], where hAQP1 proteoliposomes demonstrated much faster shrinking compared to the liposome controls. After the correction for the baseline drift (verified by mixing with isotonic solution lacking sucrose), the kinetics of water efflux from protein-free liposomes could be fitted by a single exponential with a characteristic time of 141±3 ms, while that for the proteoliposomes was markedly biphasic (the major phase 23±1 ms, 76% of the amplitude, and the minor phase 197±13 ms). Such a biphasic character for hAQP1 proteoliposomes was observed before and the slow kinetic phase was interpreted as
corresponding to the protein-free population (Nyblom et al. 2007). An additional control was performed using specific inhibition of hAQP1 (achieved by a 15-min pre-incubation with 0.6 mM HgCl$_2$), which showed very significant suppression of the efflux (a single exponential with a characteristic time of 103±1 ms). Taken together, these data indicate that hAQP1 is functional in the PC/PS lipid mixture employed in our study.

Fig 3-4. Stopped-flow measurements of water transport of hAQP1 reconstituted in PC/PS liposomes. Water efflux was induced by a 225 mOsm/kg inwardly directed sucrose gradient at 22°C in a stopped-flow machine. Cyan - control liposomes lacking protein, red - proteoliposomes with lipid to protein ratio of 10.0 (w/w), green - proteoliposomes pretreated with the 0.6 mM HgCl$_2$ inhibitor.
3.4 One- and two-dimensional solid-state NMR

For the SSNMR measurements, the proteoliposomes were hydrated with 10 mM NaCl, 25 mM Tris–Cl, pH 7.0. Approximately 6 mg of UCN (uniform \(^{13}\)C, \(^{15}\)N) hAQP1 sample was transferred and center-packed in a thin wall 3.2 mm rotor. All the SSNMR experiments were performed on a Bruker Avance spectrometer operating at 800 MHz equipped with 3.2 mm E-free \(^1\)H-\(^{13}\)C-\(^{15}\)N probe. The MAS frequency was 14.3 kHz and the temperature was set at 5°C in all experiments. The obtained one-dimensional (1D) \(^{15}\)N and \(^{13}\)C spectra are shown in Figures 3-5 and 3-6 [35].

Both spectra show very good dispersion and excellent resolution (line width of \(~0.5\) ppm for carbon and \(~0.5\) ppm for nitrogen). One dimensional \(^{13}\)C spectrum (Figure 3-6) confirms the lack of glycosylation of hAQP1, as no sugar signals are observed in the 70–80 ppm range. The sharpness of NMR peaks suggests good sample homogeneity and gives hope that \textit{Pichia}-expressed hAQP1 is fit for structural and functional studies by MAS SSNMR in the native-like lipid environment.
Fig 3-5. One-dimensional $^{15}$N MAS NMR spectrum of $^{13}$C,$^{15}$N-labeled hAQP1 reconstituted in PC/PS liposomes recorded at 800 MHz, at 5°C, and at a spinning rate of 14.3 kHz. The $^{15}$N spectrum was collected with 400 scans.
Fig 3-6. One-dimensional $^{13}$C spectrum of lipid-reconstituted hAQP1 averaged over 96 scans. SPINAL64 decoupling of 83 kHz was used during detection [38].

Two-dimensional $^{13}$C–$^{13}$C DARR (dipolar assisted rotational resonance, also known as radiofrequency assisted diffusion (RAD)) [39] chemical shift correlation spectra of $^{13}$C,$^{15}$N-hAQP1 proteoliposomes at 800 MHz and at a spinning frequency of 14.3 kHz and at 5°C were also collected. Figure 3-7 shows two-dimensional $^{13}$C–$^{13}$C DARR spectra collected with 14.95 ms of the indirect $t_1$ acquisition with TPPI (time-proportional phase incrementation) phase-sensitive detection, and 20.53 ms of the direct $t_2$ acquisition. 8 scans per point were collected with a recycle delay of 1.7 s. The total experimental time was 11 h. Data were processed with 40 Hz of Lorentzian line narrowing and 80 Hz of Gaussian line broadening in both dimensions.
Fig 3-7. a) Two-dimensional $^{13}$C–$^{13}$C DARR spectra, b) is enlarged Ala Cα/Cβ region of the same spectrum. Residues showing non-helical conformations are boxed. c) Enlarged Thr and Ser Cα/Cβ regions. Residues showing non-helical and helical conformations are boxed. The first contour level is cut at $6 \times \sigma$, with each additional level multiplied by 1.1 [35]. With permission from the Springer.

Next, 2D NCACB correlation experiments were done on the $^{13}$C,$^{15}$N-hAQP1 proteoliposomes. These experiments together with $^{13}$C–$^{13}$C experiments helped us in identification of the groups of peaks corresponding to intrareside correlations of several amino acid types, such as Ala, Ser, Thr, Gly and Pro. Figure 3-8 shows the 2D NCACB spectrum of $^{13}$C,$^{15}$N-hAQP1 proteoliposomes at 800 MHz collected at a spinning
frequency of 14.3 kHz and at 5°C. Both $^{13}$C–$^{13}$C (Figure 3-7 a) and NCACB (Figure 3-8) spectra showed an excellent resolution.

It is possible to estimate the spectral coverage by counting well-resolved peaks of one type and estimating integrated intensity of the unresolved peaks. From our previous experience with microbial rhodopsins, we can expect that the peaks from residues in the transmembrane helices should be mostly visible, while those in the loops and tails may be weak (or even invisible) due to the unfavorable dynamics [27, 40]. For example, we estimate that at least 18 Ala (out of the expected 22 TM and 8 extramembrane residues) and 20 Gly (out of the expected 18 TM and 9 extramembrane residues) are visible. Four Pro peaks are identifiable from the NCA spectra (there are 4 TM prolines), which should include the prolines of the functionally important conserved NPA motives in the water pore [33, 34]. As all of these Pro CA signals are found above 65 ppm, they must originate from the TM helices.
Fig 3-8. Two-dimensional NCACB spectrum of $^{13}$C,$^{15}$N- hAQP1 proteoliposomes recorded at 800 MHz, at a spinning rate of 14.3 kHz, and at 5°C. Glycine resonances are shown in the box. 2D spectrum was collected with 14.95 ms of the indirect $t_1$ acquisition with TPPI phase-sensitive detection, and with 20.53 ms of the direct $t_2$ acquisition. 24 scans per point were recorded with a recycle delay of 1.7 s. The total experiment time was 4 h. Data were processed with 40 Hz of Lorentzian line narrowing and 80 Hz of Gaussian line broadening in the C$\alpha$ direct dimension, and with 24 Hz of Lorentzian line narrowing and 40 Hz of Gaussian line broadening in the $^{15}$N indirect dimension. The first contour is cut at $5\times \sigma$, with each additional level multiplied by 1.2 [35]. With permission from the Springer.
One interesting observation is that some peaks clearly correspond to non-helical conformations [41], such as those boxed in Figure 3-7 b and c for Ala and Ser/Thr, respectively. The Cα/Cβ Ala resonances at around (51–52)/21 ppm (Figure 3-7 b) have chemical shifts close to those typical for β-strands, as do Ser resonances at (56–58)/(63–64) ppm and Thr resonances at (61–62)/70 ppm (Figure 3-7 c). As the available structures of human and bovine AQP do not show any β-strands, it is likely that these signals belong to the residues in β-turns, present according to FTIR [42] and crystallographic studies [32–34, 43]. In agreement with this, the Amide I band in the FTIR spectrum of hAQP1 shown in Figure 3-3 displays a shoulder at 1,682 cm\(^{-1}\) (clearly seen as a peak in the second derivative of the spectrum, not shown). In the absence of the corresponding strong shoulder at 1,630 cm\(^{-1}\) (signature of β-strands), this spectral feature can be safely interpreted as belonging to β-turns [44].

3.5 Conclusions

In summary, we demonstrated expression and isotope labeling in P. pastoris, combined with functional reconstitution in native-like lipids that could produce samples of relatively large α-helical human membrane protein suitable for high-resolution multidimensional MAS SSNMR spectroscopy. These samples represent perfect targets for the in-depth structural and functional analyses, including those for mechanisms of interactions with pharmacological agents and other modulators.
References


41. Wang Y, Jardetzky O: Probability-based protein secondary structure


Chapter 4

Solid-State NMR studies of doubly isotope-labeled hAQP1 protein
Statement of contribution

All the experiments, results, tables and figures presented in this chapter have been performed and prepared by me except for:

Experiments:

- ssNMR experiments on the NMR spectrometer were performed by Dr. Vladimir Ladizhansky and Dr. Shenlin Wang
- Analyzing the HD (Hydrogen/Deuterium) exchange results by me and Dr. Shenlin Wang together.
- ssNMR experiments on the NMR spectrometer for the mercury inhibition on the doubly isotope labeled hAQP1 was performed by Dr. Shenlin Wang
- SAXS was performed by Hongjun Liang and coworkers

Figures:

- Figure 4-16 and table 4-1 was prepared by Dr. Vladimir Ladizhansky
- Figure 4-13 was prepared by Dr. Shenlin Wang and Dr. Vladimir Ladizhansky
- Figure 4-14 was prepared by Dr. Shenlin Wang
4.1 Importance of Solid-State NMR studies of hAQP1

A number of studies of hAQP1 have been performed to date, including structural, functional, pharmaceutical and medical investigations [1–6]. As described in Chapter 1, possible involvement of hAQP1 in several human diseases encouraged researchers to seek suitable drugs to inhibit this protein. Thus, structure and dynamics of native hAQP1 in the lipid membrane should be well understood in order to be able to design its effective inhibitors.

Structure of hAQP1, including the transmembrane domains and the water channel, is well understood based on the available crystallographic, biochemical, and molecular dynamics simulations data [7–11]. At the same time, the native structure of hAQP1 inside the lipid membrane could be somewhat different from the structures observed crystallographically. Specifically, the conformations and dynamics of less structured and/or more mobile loops of hAQP1 are not well studied, as they are not readily available from the X-ray and EM structures obtained at cryogenic temperatures. At the same time, the biochemical data and MD simulations show that these flexible regions of hAQP1, in particular loops A, B, C, D and E (Figure 1-5 in Chapter 1), may be important for the regulation of water transport, folding, oligomerization, and possibly other processes [12–14]. For instance, N-linked glycosylation of Asn42 in loop A in hAQP1 is important for initial folding and topology of hAQP1 in the ER membrane [15–17]. At the same time, it has been proposed that the connecting loops in AQP1 can control the movement of the protein by interacting with the Sec61 translocon and thus affect the initial topology of AQP1 in ER and lipid plasma membrane [18–20]. Therefore,
hAQP1 should be studied under more native conditions (in lipids and at non-cryogenic temperatures) that can preserve the mobile loops in their native folds. Additionally, as described in Chapters 1 and 5, no structural details of the mechanism of inhibition of hAQP1 by mercury chloride or any other compound are known, so that the native structure of the protein is required as a foundation for the rational drug design.

Among different structural techniques that could be applied to the lipid reconstituted hAQP1, solid-state NMR (ssNMR) is a strong candidate as it can provide detailed information on the structure and conformation of hAQP1 in its native-like environment (see Chapters 1 and 2 for details). After obtaining the native structure of hAQP1 in the lipid membrane, the detailed structure of the inhibited hAQP1 should be studied and compared with the native one.

The well-known inhibitor of hAQP1 is mercury chloride and despite its toxicity for the human body, understanding its inhibitory effects is important in order to design better drugs inhibiting hAQP1. The inhibitory effects of mercury on hAQP1 have been studied in a number of papers [7, 21–24]. The mechanism of inhibition of hAQP1 by mercury was shown to be related to the binding of Hg to Cys189 residue that is part of the ar/R constriction region [21]. The recent MD simulations of inhibition of hAQP1 by mercury showed that the conformational changes of the ar/R region could be the main mechanism of inhibition, although the pore occlusion mechanism was not excluded in this work [7]. Other structural results that were derived from the X-ray data on the mutant AqpZ (a bacterial homolog of hAQP1) showed the possibility of multiple binding sites for mercury [22, 25]. However, the amino acid sequence of AqpZ is different from that of hAQP1 and thus, these results might not reflect true inhibitory mechanism of mercury on
hAQP1. Therefore, the inhibition mechanism of hAQP1 by mercury and the basic mechanism of the blockage of the water transport through the channel pore still remain ambiguous and need to be understood with an alternative technique.

ssNMR is a strong candidate for the method to study the inhibition effects of mercury. The detailed information on the hAQP1-Hg interactions can be obtained through the site-specific chemical shift changes of the individual amino acids in hAQP1. ssNMR can report on the site-specific dynamics of the mercury binding site as well as identify any structural changes that can possibly occur in hAQP1 upon its inhibition. Thus, in this work, we aim to solve the ambiguity of the mechanism of mercury inhibition of hAQP1 embedded in its native-like lipid membrane.

In this Chapter, we present MAS ssNMR study of hAQP1 and demonstrate that an important class of membrane-embedded water channels aquaporins is amenable to structural analyses by MAS ssNMR. Furthermore, the sequential resonance assignment of hAQP1 is described, as one of the prerequisites for understanding the secondary structure, dynamics, and ligand interactions of hAQP1. We report nearly complete resonance assignments of the protein reconstituted in lipid membranes using two-dimensional (2D) and three-dimensional (3D) NMR.

We also present the chemical shift indexing (CSI) of the data to derive the secondary structure of hAQP1. The program TALOS+ was used to obtain the backbone torsion angles \( \phi \) and \( \psi \) of the protein. Although these results indicated no major structural differences in the secondary structure of the transmembrane (TM) domains between the NMR data and the crystallographic structures, we found significant deviations for loop regions. Site-specific detection of H/D exchange of nitrogen-bearing
side chains and backbone amides provided additional information on hydrogen-bonding patterns and topology of the protein. Finally, we present the effects of mercury chloride treatment on the ssNMR spectra of hAQP1, which unexpectedly caused significant changes in the spectral resolution, implying promotion of sample heterogeneity.

4.2 Materials and Methods

4.2.1 Pulse sequence and optimization parameters

Proteoliposomes containing approximately 6 mg of uniformly $^{13}$C, $^{15}$N labeled (UCN) hAQP1 (sample preparation for UCN hAQP1 was described in chapter 3) were center packed in a 3.2-mm thin wall rotor for NMR experiments. All ssNMR experiments were performed on a Bruker Avance III spectrometer operating at 800.230 MHz equipped with 3.2-mm EFREE HCN probe (Bruker USA, Billerica, MA) at a spinning frequency of 14.3 kHz. The sample temperature was maintained at ~5 °C in all experiments. Three-dimensional NCOCX, NCACX, and CANCO chemical shift correlation experiments were performed using previously described pulse sequences and optimization procedures (chapter 2) [26].

Typical 90° pulses were 2.5 μs for proton, 4 μs for carbon, and 6 μs for nitrogen. The $^1$H/$^{15}$N cross-polarization (CP) contact time was 2 ms, with a constant radio-frequency (RF) field of 42 kHz applied on nitrogen, and with a proton field ramped linearly around ~55 kHz (10% ramp, optimized experimentally). The $^1$H/$^{13}$C CP contact time was 2 ms, with the constant RF carbon field of 64 kHz, and with the proton field
ramped linearly around \(~7\text{ kHz}\) (10\% ramp). $^{15}\text{N}/^{13}\text{C}$ and $^{15}\text{N}/^{13}\text{C}'$ band-selective CP steps \cite{27} were implemented with a contact time of 6 ms. For NCA CP, a constant lock field of $2.5 \times \nu_r$ ($\nu_r$ is the spinning frequency) field strength was applied on $^{15}\text{N}$, while the carbon field ramped linearly (10\% ramp) around $1.5 \times \nu_r$. For the NCO transfer, a constant lock field of $3.5 \times \nu_r$ field strength was applied on $^{13}\text{C}$, while the $^{15}\text{N}$ field was ramped linearly (10\% ramp) around $2.5 \times \nu_r$. The CW (continuous wave) decoupling during CP steps was always 90 kHz. SPINAL-64 decoupling \cite{28} of 86 kHz was used in both direct and indirect chemical shift evolutions.

### 4.2.2 Three dimensional CANCO experiment

In this experiment, 8 scans were recorded with a recycling delay of 1.8 s, resulting in a total acquisition time of 78 h. Data were processed with Lorentzian-to-Gaussian apodization function. 16 Hz of Lorentzian line narrowing and 40 Hz of Gaussian line broadening were applied in the indirect dimensions, and 40 Hz of Lorentzian line narrowing and 80 Hz of Gaussian line broadening were applied in the direct dimension.

### 4.2.3 Three dimensional NCACX experiments

To perform this experiment, two experiments with DARR mixing times of 20 ms and 50 ms were recorded. 8 scans per point were recorded, with a recycle delay of 1.7 s. Data were processed with Lorentzian-to-Gaussian apodization functions, with 16 Hz of Lorentzian line narrowing and 40 Hz of Gaussian line broadening applied in the nitrogen
indirect dimension, and 40 Hz of Lorentzian line narrowing and 80 Hz of Gaussian line broadening applied in the carbon dimensions.

**4.2.4 Three dimensional NCOCX experiments**

Two experiments with DARR carbon-carbon mixing times of 50 ms and 100 ms were recorded, with 8 scans per point and with a recycle delay of 1.7 s. Data were processed with Lorentzian-to-Gaussian apodization with 16 Hz of Lorentzian line narrowing and 40 Hz of Gaussian line broadening in the t₁ indirect dimension, and with 40 Hz of Lorentzian line narrowing and 80 Hz of Gaussian line broadening in the t₂ and t₃ dimensions.

**4.2.5 Two dimensional NCOCX experiment**

Two NCOCX experiments with different DARR carbon mixing times of 50 and 100 ms were recorded. The spectrum was collected with 14.95 ms of the indirect t₁ acquisition with TPPI phase-sensitive detection, and with 20.53 ms of the direct t₂ acquisition. 24 scans per point were recorded with a recycle delay of 1.7 s. The total experiment time was 4 h. Data were processed with 40 Hz of Lorentzian line narrowing and 80 Hz of Gaussian line broadening in the CO direct dimension, and with 24 Hz of Lorentzian line narrowing and 40 Hz of Gaussian line broadening in the \(^{15}\text{N}\) indirect dimension.
4.2.6 Procedures for analyzing ssNMR experimental data

Carbon chemical shifts were indirectly referenced to 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) by adjusting the position of the $^{13}$C adamantane downfield peak to 40.48 ppm [29]. Nitrogen chemical shifts were referenced indirectly by using the ratio of gyromagnetic ratios $\frac{\gamma_N}{\gamma_C} = 0.402979946$, taken without a temperature factor correction. Experimental data were processed with NMRPipe [30]. Peak picking and noise analysis were performed with the CARA software [31].

4.2.7 Inhibition of hAQP1 by mercury chloride

To directly detect interaction between hAQP1 and Hg$^{2+}$ by ssNMR we incubated UCN hAQP1 sample in NMR buffer (10 mM NaCl, 25 mM Tris–Cl, pH 7.0) with 0.5 $\mu$M mercury chloride (Hg/Protein molar ratio of 1). After 24-hour incubation with mercury chloride, the excess of mercury was removed by washing the sample in the NMR buffer. The first incubation led to no observable changes in ssNMR spectra. Thus, we increased the concentration of mercury chloride and this time incubated the sample with 20 $\mu$M mercury chloride (Hg/Protein molar ratio of 40). Again, the ssNMR spectra remained unchanged. The mercury chloride concentration was further increased to 80 $\mu$M (Hg/Protein molar ratio of 160).
4.3 Characterization of hAQP1 sample

Aquaporins are generally well-structured, predominantly α-helical proteins with high propensity to form tetramers and assemble into functional two-dimensional square arrays in phospholipids at high protein-to-lipid ratios [32]. We have reported a sample preparation protocol for hAQP1 in chapter 3, showing that most residues of the protein are visible in the dipolar correlation spectra, and give rise to sharp lines with typical line widths on the order of 0.5 ppm in both $^{15}$N or $^{13}$C dimensions [33]. We have further examined our NMR sample preparations for the presence of 2D order. Small angle X-ray scattering measurements indicate that hAQP1 in our samples forms small 2D crystals with tetragonal lattice of 6.5 nm periodicity (Figure 4-1). This high degree of macroscopic 2D order goes hand in hand with the high atomic level order, which is reflected in high resolution of ssNMR spectra of hAQP1 (see chapter 3, section 3.4), while the presence of most resonances in the spectra indicates limited dynamics. The resonances from residues with higher dynamics such as loops and tails become weak or even invisible due to the motional averaging of dipolar couplings.
Fig 4-1. SAXS results for hAQP1 reconstituted in the PC/PS liposomes with the protein to lipid ratio of 2 w/w. The labels present indices for different lattice reflections.

4.4 Three-dimensional heteronuclear correlation spectroscopy experiments

The multidimensional correlation experiments allow for site-specific resolution of many residues. In these experiments, magnetization is transferred between different nuclei so that the observed peaks spread into multiple dimensions. In Figure 4-2 we show an example of a 2D NCOCX correlation spectrum, which contains many well-resolved cross peaks, even in the NCO region, which is generally heavily congested due to the
limited dispersion of the carbonyl resonances. Still, there is considerable degeneracy and spectral crowdedness due to the large number of residues, and this necessitates the use of 3D spectroscopy for spectroscopic assignments.

Fig 4-2. 2D NCOCX spectrum of hAQP1 at 800 MHz. The spectrum was recorded with carbon-carbon DARR mixing of 50 ms. First contour is cut at 5σ with multiplication factor of 1.2. Selected assignments shown in the NCO region of the spectrum are according to the CO[i] shift.

An improvement of resolution in 3D spectra is demonstrated in Figure 4-3 where we show 2D planes of the three-dimensional NCACX. Additional examples of the 2D plane of the 3D CANCO spectra are shown in Figure 2-4 of Chapter 2.
Fig 4-3. 2D plane of the 3D NCACX spectrum recorded with 50 ms DARR carbon-carbon mixing. First contour is cut at $4.5\times\sigma$ with each additional level multiplied by 1.2. Assignments are shown according to the CX[i] shifts.

Nearly complete backbone resolution in the CANCO spectrum and availability of side chain resonances in both NCACX and NCOCX spectra is essential to facilitate spectroscopic assignments. Specifically, the 2D CACX plane of NCACX spectrum shown in Figure 4-3 is chosen at the $^{15}$N shift, which is close to the shifts of I29 and K36. Accordingly, we observe a complete set of side chain correlations for I29, and nearly complete set of side chain resonances for K36.
4.4.1 Resonance assignments of hAQP1

To obtain resonance assignments of hAQP1 we employed a combination of two- and three-dimensional homo- and heteronuclear correlation MAS ssNMR spectroscopy techniques. Our approach was described in detail in chapter 2 and here we recapture the basic ideas.

The 3D CANCO experiment establishes interresidue correlations between the backbone nuclei CA[i], N[i] and CO[i-1], and provides nearly full backbone resolution. The 3D CANCO is complemented by a set of two intraresidue 3D NCACX correlation experiments recorded with DARR carbon-carbon mixing times of 30 ms and 50 ms, and two interresidue NCOCX experiments recorded with DARR carbon-carbon mixing times of 50 ms and 100 ms. In both sets, the use of shorter mixing times favours shorter one- and two-bond correlations (e.g., N[i]-CA[i]-CO[i]/CB[i]/CG[i] or N[i]-CO[i-1]-CA[i-1]/CB[i-1] in NCACX and NCOCX, respectively), whereas longer mixing times result in longer-range intraresidue inter-carbon transfers within the carbon chains. It also allows observing interresidue correlations, e.g., N[i]-CA[i]-CO[i-1], or N[i]-CO[i-1]-CA[i] in the NCACX and NCOCX experiments, respectively. Additional information on the side chain shifts was obtained from 2D \(^{13}\)C-\(^{13}\)C correlation spectra shown in Chapter 3, Figure 3-7 previously.

In Figures 4-4 and 4-5 we demonstrate examples of a sequential backbone walk for C189-N192 and for R126-D131 residues. The availability of nearly complete side chain resonances in the 3D NCACX, NCOCX and 2D \(^{13}\)C-\(^{13}\)C correlation experiments allows for an unambiguous identification of the amino acid type, and also spin systems
according to their side chain carbon shifts.

Fig 4. An example of a sequential assignment walk for residues C189-N192 in the ar/R constriction region of hAQP1. The lowest contour is cut at $5\times\sigma$. NCOCX and NCACX strips are labeled by residue number according to the nitrogen shift, and C$\alpha$ and C$'$ chemical shifts are given in each strip. Vertical lines link spin systems detected in the NCOCX and NCACX experiments.
Fig 4-5. An example of a sequential assignment walk for residues R126-D131 in the loop C region of hAQP1. The lowest contour is cut at $5 \times \sigma$. NCOCX and NCACX strips are labeled by residue number according to the nitrogen shift, and $C\alpha$ and $C'$ chemical shifts are given in each strip. Vertical lines link spin systems detected in the NCOCX and NCACX experiments.
Using these approaches, we have been able to assign chemical shifts of a total of 192 out of 269 residues from the dipolar-driven correlation spectra (Appendix 1.1). Graphical summary of assignments is shown in Figure 4-6.

Fig 4-6. Topological model of hAQP1 with helices, loops, and purification His$_6$-tag and Myc-tag labeled. Residues shown in green have been assigned in the solid-state NMR spectra.

In particular, transmembrane regions have been completely assigned with an exception of the beginning of helix H1 and Ile111. Most unassigned residues are found in the termini or in the loops. To further investigate the potential flexibility of these regions,
we extracted cross-peak intensities from a 3D CANCO experiment, which are shown in Figure 4-7.

Fig 4-7. Site-specific S/N ratios of the cross peaks in the CANCO 3D experiment for the assigned residues. The reduced signal amplitudes indicate the higher mobility of these regions and such reduction was mostly observed in loop regions. Secondary structure of hAQP1 is indicated on top.

Reduced signal intensities in the dipolar correlation spectra typically correlate with increased mobility – sub-microsecond time scale motions of larger amplitudes result in more efficient averaging of dipolar couplings and, as a result, reduced cross peak intensities. We observe a number of regions where the signal is either attenuated or missing, and most of them occur in the loops. In addition to the terminal regions not observed in the NMR spectra, we see a reduction of intensity in LA, with the G40-Q43 stretch completely missing in the spectra. No signal was detected for residues L83-F92 in HB, including the end of half-helix HB and the part connecting HB and the following
helix H3. Likewise, there was no signal detected in loop LD for residues R160-G165, while reduced signal intensities for the surrounding residues indicate an increased mobility of this loop as well. In contrast, the 19-residue long loop C comprising residues T116-N134 appears to be less mobile as the signal intensities for most residues in this loop are comparable to those in helices H3 and H4 (Figure 4-7), suggesting that this loop is either structured or immobilized by interactions with other structural elements (or both).

### 4.5 Secondary structure analysis of hAQP1

#### 4.5.1 Chemical Shift Index analysis

NMR chemical shifts are sensitive reporters on secondary structure [34, 35]. We used $^{13}$C shifts to analyze the backbone conformation of hAQP1 and compare it with the available crystallographic structures for both the human and for the homologous bovine AQP1. Chemical shift index (CSI) [34] constructed of $C_\alpha$ and $C_\beta$ shifts is shown as a function of residue number in Figure 4-8. We observe six long stretches of positive secondary chemical shifts corresponding to membrane spanning helices, H1-H6, and two shorter positive regions, HB and HE, attributed to the non-spanning half-helices found in loops LB and LE, respectively.
Fig 4-8. Chemical shift index (CSI) for hAQP1. Positive secondary shifts correspond to \(\alpha\)-helical structure, while negative shifts indicate beta structure and other deviations from helicity. Secondary structure with designations of helices and loops is according to the NMR data where available, and using X-ray data for regions with incomplete assignments, e.g., helices H1 and HB, and is shown on top to guide the eye. The two half-helices HB and HE are located in loops LB and LE.

In Figure 4-9 we compared the secondary structures of hAQP1 and bAQP1 (bovine AQP1) determined by EM and X-ray techniques. Apart from some discrepancies between the helical boundaries, there is a good agreement on the helical secondary structure between the NMR data and the crystallographic data available for hAQP1 and for bAQP1 (Table 4-1). The agreement is especially good with the EM structure of lipid-embedded hAQP1 (PDB 1FQY).
Fig 4-9. Amino acid sequence alignment of hAQP1 and bAQP1. The secondary structures of hAQP1 obtained through EM and X-ray crystallography [9–11, 36] with their PDB codes (4CSK for X-ray, 1FQY, 1H6I, and 1IH5 for EM structure of hAQP1, and 1J4N for X-ray structure of bAQP1) indicated in front of each row and the secondary structure of the bAQP1 obtained through the X-ray crystallography [1] are shown on top and bottom of the amino acid sequence respectively.
Table 4-1. Comparison of secondary structure elements in aquaporin 1 derived from NMR, X-ray and EM studies. Note: all boundaries are given as defined in the PDB files.

<table>
<thead>
<tr>
<th></th>
<th>NMR data (1FQY)</th>
<th>EM (1H55)</th>
<th>EM (1J4N)</th>
<th>X-ray bAQPI(1) (1H6I)</th>
<th>Refined (1H6I)</th>
<th>X-ray (4CSK)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>A13-F35</td>
<td>K8-Y37</td>
<td>W11-G34</td>
<td>F5-Y37</td>
<td>L9-Y37</td>
<td>F5-L33</td>
</tr>
<tr>
<td>H2</td>
<td>N49-H69</td>
<td>D48-V67</td>
<td>D48-T62</td>
<td>D50-G74</td>
<td>N49-I70</td>
<td>D48-G68</td>
</tr>
<tr>
<td>HB</td>
<td>P77-G82</td>
<td>N76-L85</td>
<td>N76-C87</td>
<td>N78-S88</td>
<td>N76-S86</td>
<td>N76-S86</td>
</tr>
<tr>
<td>H3</td>
<td>R93-I115</td>
<td>A94-T116</td>
<td>L95-T120</td>
<td>S92-T118</td>
<td>S90-G114</td>
<td>F92-T116</td>
</tr>
<tr>
<td>H4</td>
<td>S135-T156</td>
<td>G136-T156</td>
<td>L139-R159</td>
<td>G138-T159</td>
<td>N134-T157</td>
<td>G138-T156</td>
</tr>
<tr>
<td>HE</td>
<td>P193-V201</td>
<td>N192-V201</td>
<td>P193-I202</td>
<td>N194-T205</td>
<td>N192-H204</td>
<td>N192-G198</td>
</tr>
<tr>
<td>H6</td>
<td>S207-I226</td>
<td>S207-F229</td>
<td>W210-D228</td>
<td>W212-F231</td>
<td>W210-L231</td>
<td>W210-F229</td>
</tr>
</tbody>
</table>

1Bovine numbering. Human AQP numbering is shifted down by 2 residues starting from A45.

In addition to the helical boundaries refined at nearly physiological temperatures, NMR chemical shifts reveal a number of distortions within helices. While most of them are located close to the protein-solvent interface (e.g., L33 in helix H1, K51 and V67 in helix H2), some occur in the middle of the TM regions. We observe low CSI for A108 in H3, and at W213 in the middle of H6. The latter distortion is likely a proline kink caused by P216.
The largest discrepancies between the NMR data and crystallographic structures are observed for loop regions. We point to the fact that the crystallographic temperature factors are generally higher for these regions, indicating their higher static disorder and/or higher degree of mobility, so that the crystal structures do not give reliable information about these structural elements. In contrast, through the chemical shift indexing (CSI) (Figure 4-8), we have detected backbone conformation of native hAQP1 for the majority of its residues. As mentioned earlier, the negative values of CSI indicate deviation from the helicity or presence of beta structure (section 4.4). Therefore, negative CSI value in loop LA indicate that this loop may contain a short beta-structure consisting of two short beta strands, comprising residues K36-P38 and W44-D48, connected by a flexible linker. These two stretches of residues are too far from each other to form the hairpin, so that the strands must be stabilized by interactions with some other structural elements. The proposed structure of loop LA correlates with the reduced intensity of the NMR signals towards the ends of the two strands observed for V39, and W44 and A45, with residues in the G40-Q43 being undetectable in the NMR spectra (Figure 4-7). Loop LD may have an architecture similar to that of loop LA, but with a higher mobility, which is interesting in view of the proposal that loop LD may be a homolog of LA as a result of ancient gene duplication reflected in the internal symmetry of aquaporins [37].

Interestingly, the first short strand contains proline residue P38, which lacks an amide proton and cannot act as a hydrogen bond donor to stabilize beta turn. Furthermore, the large 10.8 ppm difference between the chemical shifts of P38 $\text{C}_\alpha$ and $\text{C}_\gamma$ atoms indicates that the Y37-P38 peptide bond is in $\textit{cis}$ conformation. The $\textit{cis}$ Xaa-Pro motifs are known to facilitate bends (i.e., regions of high curvature [38]) in protein loops,
which may be important in creating conformation of loop LA that allows it to interact with LE and with other monomers [17, 39]. The presence of cis Y37-P38 bond is a new structural feature detected in the NMR data, as no other published structure of hAQP1 or bAQP1 contains cis Xaa-Pro bond at this position.

As described above, loop LC appears to be less mobile than other loops in hAQP1 (Figure 4-7). Further examination was done with the site-specific CSI plot shown in Figure 4-8, which indicates a potential ordered secondary structure in this loop. Secondary chemical shift values tend to alternate between negative and positive values suggesting the presence of a number of turns and possibly short beta-strands in the structure.

To gain additional insights into the hAQP1 secondary structure, we extracted backbone torsion angles using TALOS+ (Torsion Angle Likeliness Obtained from Shift and Sequence Similarity) program [40]. TALOS angles shown in Figure 4-10 indicate well-ordered transmembrane helices and membrane-embedded half-helices (with a typical values of -57 and -47 for the average phi and psi values), with other regions showing deviations from helicity, such as loops LA and LC. Minor potential deviations in the transmembrane regions indicated by the CSI analysis are too small and cannot be resolved by the TALOS+ program.
Fig 4-10. Analysis of the secondary structure of hAQP1 using backbone dihedral angles \( \psi, \phi \) derived from the chemical shifts using TALOS+ program. Only values classified as “good” are shown.

As expected, loop LC is found to contain a \( \beta \)-structure for residues L129-N134 with torsion angles \( (\phi, \psi) \) varying between \((-56\%\,\text{to}\,100\%, \, 120^\circ\,\text{to}\,178^\circ)\). The L129-N134 fragment is preceded by a potential type II \( \beta \)-turn, formed by R126-N127 with their \((\phi_{i+1}, \psi_{i+1}), (\phi_{i+2}, \psi_{i+2})\) angles being close to \((-60^\circ,120^\circ), (80^\circ,0^\circ)\) of an ideal type II \( \beta \)-turn [41]. The L129-N134 fragment should be stabilized through interactions with other hAQP1 monomers or loops.

TALOS+ predictions for loop LA are consistent with a beta turn structure and further corroborate the existence of a short beta strand for residues A45-N49, which may
be stabilized through intramonomer contact with D185 [17], while the \((\phi, \psi)\) values for the Y37-P38 fall in the range typical of a polyproline II helix.

We further compared the TALOS angles with the X-ray structure of bAQP1, which is the highest resolution structure available for close homologs of hAQP1 (Figure 4-11). There is a good agreement for the TM regions including the half-helices, but there are large deviations for the loop regions. It should be noted, that sequence homology for certain loop regions, e.g., loop C is quite low, which may account for some differences, the other factors being crystal constraints and cryogenic temperatures.

Fig 4-11. A comparison of torsion angles derived from X-ray structure of bAQP1 and from TALOS analysis of the NMR chemical shifts of hAQP1. Secondary structure derived from the NMR data is shown on top to guide the eye.
4.6 Hydrogen-deuterium exchange and water accessibility

To probe the solvent accessibility and hydrogen bonding strength of the backbone amides and nitrogen-bearing side chains of hAQP1, we carried out NMR-detected site-specific hydrogen/deuterium (H/D) exchange experiments. In an H/D experiment we compare signal intensities in 2D NCA or 3D NCACX experiments obtained from the same protein sample first prepared in an H$_2$O-based buffer and then incubated for 24 hrs in a D$_2$O-based buffer. Because the NMR signal is generated from the amide protons in these experiments, residues with protons that exchange with D$_2$O will yield reduced signal, while signals from inaccessible residues or from the residues protected by hydrogen bonds would be less attenuated. In Figure 4-12 we show representative $^{15}$N/$^{13}$C planes of the 3D NCACX experiment collected in H$_2$O and D$_2$O buffers, where the effect of the H/D exchange is readily visible, e.g., the signals of L33 and A221 clearly detectable in Figure 4-12A completely disappear in D$_2$O (Figure 4-12B), whereas signals of A171, L75, and A108 are strongly attenuated.
Fig 4-12. H/D exchange experiments recorded at 800 MHz. (A) Two-dimensional N-C plane of the three-dimensional NCACX experiment recorded on hAQP1 in the H$_2$O-based buffer. The plane corresponds to C$\alpha$ shift of 56.0 ppm. (B) The same plane recorded in the D$_2$O-based buffer. Many peaks corresponding to residues with exchangeable amide protons have attenuated intensities, or completely disappeared.
A site-specific comparison of the NMR backbone signals detected in H\textsubscript{2}O and D\textsubscript{2}O is shown in Figure 4-13A. We observed an overall reduction of signal for the entire protein, which may indicate partial low-level exchange and/or signal reduction due to the sample losses during incubation in D\textsubscript{2}O. Figure 4-13B shows the 3D structure of the exchangeable residues mapped on the structure of bAQPI which is highly homologous to hAQPI.

As expected, the amide protons of residues located in TM helices are protected and do not exchange except in the exposed flanks of some of the helices. In contrast, the exposed loops LA, LC and LD are generally exchangeable. Notable exceptions are residues A130, V133, and N134 in loop C, which are likely protected by strong hydrogen bonding, either within the loop or with some of the side chains. Interestingly, the homologous loop of bovine AQPI shares the AxxVN motif, and its X-ray structure shows hydrogen bonding of backbone amide nitrogens of the alanine and valine to carbonyl oxygens of the respective i-2 residues [8].
Fig 4-13. A) comparison between cross peak intensities from hAQP1 incubated in H$_2$O (grey) and D$_2$O (red) based buffers. Signal intensity reduction indicates exchangeable backbone amides. B) Map of the exchangeable amino acids (blue color) of hAQP1 on the bAQP1-based structural model with the cytoplasmic site at the bottom.
In Figure 4-14 we compared the intensities of nitrogen-bearing side chain signals.

Fig 4-14. A comparison of cross peak intensities corresponding to nitrogen-bearing side chain signals detected in the H/D exchange experiments. Grey bars represent intensities in H$_2$O, blue bars are intensities detected after 24 h incubation in D$_2$O. All intensities are measured in the 2D NCOCX experiment.

Exchangeability of nitrogen-bearing side-chains shown in Figure 4-14 provides interesting information on their solvent accessibility and hydrogen-bonding, some of which could not be deduced from the available structures of hAQP1. While some of the solvent-exposed sidechains found on the cytoplasmic (His74) and extracellular (Asn49, Asn122, Arg126, Gln137, His180, Asn205) sides of the protein are exchangeable as expected, several other residues are fully or partially protected from the H/D exchange. These residues are indicated with green color in Figure 4-15 using the known structure of the hAQP1 (PDB code: 1I5).
Fig 4-15. The 3D structure of hAQP1 (PDB code: 1IH5) with indicated location of residues with exchangeable side chains. Red arrows indicate residues that are fully or partially protected from H/D exchange. The cytoplasmic side is at the bottom.

Except for Gln148, which is buried facing the middle of the bilayer and is non-exchangeable, several other non-exchangeable or partially exchangeable residues are expected to be solvent-accessible, as they are located either on the extracellular surface or in the water-filled pore. In particular, the two asparagine residues of the NPA motifs in the pore are either not exchangeable (Asn76) or exchange on a very slow time-scale (weeks, Asn192). This is surprising, considering that they are found in contact with water molecules in the X-ray structure of bAQP1 [8]. This may correlate with the fact that aquaporin-1 does not conduct protons [42–44], suggesting that water molecules do not dissociate while in the channel. Additionally, the side-chain nitrogen of Asn127 in loop C and one of the three side-chain nitrogens of Arg195 in the ar/R filter of the water pore are
not exchangeable. This agrees with their hydrogen-bonding suggested by various structural models, e.g., hydrogen bond of Asn127 to Ser196, and Arg195 to the backbone carbonyl of Gly125 are suggested from the structure of bovine AQP1 [8], while hydrogen bond between Arg195 and Asn127 and Asp128 has been proposed from one of the EM models [10]. Figure 4-16A shows the H/D exchange pattern of the ar/R constriction region and Figure 4-16B shows the possible interactions of Arg195 with its neighbouring amino acids.

The exchangeability of only one of the terminal nitrogens of Arg195 is puzzling, considering their similar (different by ~1 ppm) chemical shifts, as it is believed that strong asymmetry in hydrogen bonding should result in large differences in chemical shift values between the two nitrogens [45].
Fig 4-16. The H/D exchange pattern of the ar/R constriction region and its possible interaction with loop C. A) The extracellular surface of the ar/R constriction region (model based on the bAQP1 structure) with the residues that are exchangeable (blue) and non-exchangeable (grey). The side chain of R195 is partially exchangeable. B) **Left:** The position of the R195 in the ar/R constriction region and its possibility to participate in the hydrogen bonding interaction with S196 located in the loop C, based on the X-ray structure of bAQP1 [10]. **Right:** Possible interaction of the R195 with the N127 and D128 suggested by the EM structure of hAQP1 [8, 13].
Additional information on hydrogen bonding of some side chains can be inferred from their chemical shifts. In particular, chemical shifts of terminal carbons of carboxylic side chains (Asp and Glu) correlate with their protonation state and hydrogen-bonding, with protonated and buried deprotonated carboxylic acids having lower values of chemical shifts than exposed deprotonated ones [46, 47]. In this respect, it is interesting to compare the available chemical shifts of two symmetrically located glutamates (Glu17 and Glu142), each interacting with the backbone of the respective half-helices [48]. Glu142 has much lower value of the chemical shift of the delta-carbon than Glu17 (179.5 vs. 182.6 ppm), suggesting much stronger hydrogen bonding of Glu142, which may also correlate with anomalously high chemical shift of amide nitrogen of Gly190, to which Glu142 is hydrogen-bonded. Assigned aspartic acids show chemical shifts of gamma-carbons ranging from those typical for exposed carboxyls (Asp158, Asp185, Asp48) to buried hydrogen-bonded ones (Asp128). The latter may correlate with the proposed interaction of Asp128 with Arg195 [10].

4.7 Inhibition studies of hAQP1 by mercury chloride through ssNMR

Based on the previous site-directed mutagenesis, MD, structural and biochemical studies [7, 21, 22, 49, 50], it was proposed that Cys189 residue in hAQP1 serves as a mercury binding site and thus mercury chloride inhibits the protein function through the covalent interaction with this Cys189 residue that is located in the ar/R filter [21]. The MD studies of the inhibited bovine AQP1 showed that reorientation of several amino acid residues in the ar/R region occurs upon binding of Hg to Cys191 of bAQP1 [7]. These reorientations of the backbone oxygen atoms of the Gly190, Cys191, and Gly192 caused
conformational changes in the ar/R constriction region and block the passage of water molecules through the channel. In free AQP1, the position of the backbone carbonyl oxygen atoms of Gly190, Cys191, and Gly192 are toward the inside of the channel. Glu144 makes several hydrogen bonds with Gly192 and Ile193 and these hydrogen bonds help stabilize the conformation of ar/R region. In Hg-AQP1, it was shown that when the positive charge of the mercury is located on Cys191 in the vicinity of Arg197, the two positive charges repel each other so that the backbone carbonyl oxygens of Gly190, Cys191 and Gly192 face toward the outside of the channel. The repulsion between Arg197 and mercury would also destroy the hydrogen bonding interactions of Glu144 and Ile193, which are used to stabilize the ar/R constriction region. While this MD study proposed mercury-induced disruption of the conformation of the ar/R region and consequent occlusion of the water channel, there is still no direct experimental evidence in a native-like environment confirming or disproving this mechanism.

NMR is ideally suited to study such interactions through chemical shift perturbations: binding and structural changes typically cause chemical shift changes that can be readily observed site-specifically in the 2D or 3D NMR spectra.

The 2D $^{13}\text{C}-^{13}\text{C}$ spectrum collected following the first and second incubations with 0.5 and 20 $\mu$M mercury chloride showed no observable chemical shift changes. When mercury chloride concentration was further increased to 80 $\mu$M (Hg/Protein molar ratio of 160), we observed intense changes of the ssNMR spectra: peaks became broader and the resolution of the spectra decreased significantly (Figure 4-17), precluding detect of any site-specific changes in the spectra.
Fig 4-17. 2D $^{13}$C-$^{13}$C spectra of left) native hAQP1 with excellent resolution and right) hAQP1 inhibited with 80 $\mu$M mercury chloride (Hg/Protein molar ratio of 160) with deteriorated resolution.

Such drastic changes in the spectra were not expected based on the previously proposed inhibitory mechanisms of mercury. According to the proposed inhibitory mechanisms [7, 21, 22] one would expect changes in the chemical shift of Cys189 residue and some other residues located in the ar/R regions. Whereas we did not detect changes in the overall cross peak chemical shift distribution, site specific chemical shift perturbations could not be detected because of the broadening. The severe peak broadening makes it impossible to further investigate the inhibition of hAQP1 by mercury through ssNMR and we therefore applied other biophysical methods to reveal the underlying causes of the spectral broadening and to investigate the inhibitory effects of mercury on hAQP1 as described in the next chapter.
4.8 Conclusion

In this chapter, we showed that excellent resolution of ssNMR spectra obtained from highly ordered 2D-crystalline hAQP1 led to the resonance assignments of 192 amino acid residues, including all transmembrane regions (except for one amino acid in helix 3 and the beginning of helix 1). We obtained and analyzed the secondary structure of hAQP1 in its native-like lipid environment. The secondary structure of hAQP1 obtained through chemical shift index (CSI) and backbone torsion angles derived from TALOS+ indicated the presence of the short β-structures in loops A, D, and C. Furthermore, we found the presence of the cis conformation for the Y37-P38 peptide bond in the loop LA that creates special conformation, which possibly allows interactions with helix E of the same or another monomer. Except for the presence of β-structures in the loop regions and slightly different helical boundaries, our results were in good agreement with the X-ray crystallographic data. However, due to the more native sample conditions, we believe that our results are closer to the native structure of hAQP1 in the human body [8, 11].

The H/D exchange experiments yielded unique information about the water accessibility and hydrogen bonding strengths of the backbone and selected side chain atoms of hAQP1. The residues that had exchangeable backbone amide or side chain nitrogens that interacted with water and also were not protected by strong hydrogen bonding showed a suppressed NMR signal after incubation in D$_2$O. The amino acid residues with exchangeable sidechains were found in the important hAQP1 motifs including ar/R regions, such as Arg195 (with two exchangeable and one non-
exchangeable nitrogens) and His180. Surprisingly, some residues in the water channel, most strikingly in the NPA motif, showed no exchangeability, e.g., for Asn76, and very slow exchangeability for Asn192 residue. We suspect that this may come from the fact that water does not dissociate in the channel, consistent with the lack of proton conductance.

Further structural and conformational studies of the inhibited protein through ssNMR turned out to be impossible due to the effects of HgCl₂ on the sample and severe broadening of the NMR spectra. Thus, we decided to investigate the inhibitory mechanism of mercury chloride through other biophysical methods, aiming to understand what happened to the sample that resulted in the heterogeneity and peak broadening in the NMR spectra. The next chapter provides results of the mercury chloride inhibition studies on hAQP1 using techniques other than ssNMR.
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Chapter 5

Novel Mechanism of hAQP1 Inhibition by Mercury
Statement of the contribution

All the experiments, results, tables and figures presented in this chapter have been performed and prepared by me.
5.1 Medical importance of AQP blockers and review of experiments leading to determination of the mercuric ion binding sites

Besides their main role in water transport, aquaporins are associated with other important processes in the human body such as angiogenesis, cell development and cell migration [1]. Human diseases such as cancer and neuropathological disorders often occur in tissues in which hAQP1 is overexpressed [1]. It has been shown that inhibition of hAQP1 in tissues affected by cancer or neuropathological diseases helps to decrease the symptoms. Thus, AQPs are attractive targets for drug discovery towards human diseases such as cancer, edema, and fluid imbalances, and inhibition of this protein by drugs could be vital to cure such diseases [1]. Mercuric ion is the classical blocker of hAQP1 [2].

In early 1920s, mercury was found to be an antisyphilitic agent, which showed an additional diuretic effect during the treatment [3]. At that time, diuretic effects of mercury were observed in patients that were using a mercurial compound named Novasurol for syphilis treatment. Those patients were reported to produce an increase in their urinary output [4], which may be related to inhibition of renal aquaporins.

It has been shown that mercury can also have some effects on membrane permeability [5]. According to Aduayom et al. mercury can interact with membrane thiol and its toxicity may be related to the increase in membrane permeability, which allows other toxic metals to enter [5].

Although mercuric ion is toxic for the human body, understanding its inhibitory mechanism is necessary to be able to design other AQP blockers. Mercury ion
specifically binds to a cysteine residue in most AQP\(\text{s}\) (except for AQP4) [6]. This reagent also blocks human Aquaporin 1 (hAQP1) and inhibits its function [2]. The free sulfhydryl group of Cys189 in hAQP1 interacts with \(\text{Hg}^{2+}\) ion covalently and reversibly. The C189 residue is located inside the hAQP1 water pore in the vicinity of the NPA motif in loop E [7, 8]. The inhibition mechanism of hAQP1 was revealed through site-directed mutagenesis experiments [2]. hAQP1 contains four cysteine residues (Cys87, Cys102, Cys152, Cys189) in its amino acid sequence. Figure 5-1 shows the structure of hAQP1 based on the electron crystallography model (PDB code: 1FQY) [9], with Cys residues in space-filling representation. Cysteines 102 and 152 are inside the transmembrane domain and thus they serve as internal providers of free sulfhydryl groups [2], and are likely to be inaccessible for reaction with mercury ion.

![Fig 5-1. Electron crystallography structure of hAQP1 with its four cysteine residues shown in space-filling representation. PDB code: 1FQY [9]. The cytoplasmic side is at the bottom.](image)
These cysteine residues (Cys 87, 102, 152 and 189) were substituted by serines and the four mutants were examined through oocyte swelling assay to measure their osmotic water permeability coefficient [2]. The osmotic water permeability coefficient of oocytes (Pf in cm/s) was calculated using the following parameters: relative volume changes in time, $\frac{dV}{dt}$, and oocytes surface area (S) [2], and according to the following formula:

$$Pf = \frac{V_0 \left[ \frac{dV}{dt} \right]}{S \times V_w \times (Osm_{in} - Osm_{out})}$$  \hspace{1cm} (5.1)

By using phase contrast microscope connected to video camera, osmotic swelling of oocytes was monitored after changing Barth’s buffer from 70 mosM (osm$_{in}$) to 200 mosM (osm$_{out}$). All four mutants showed increased osmotic water permeability compared to the control oocytes, close to that induced by the wild type CHIP28 RNA [2]. Consequently, replacing Cys residues did not affect osmotic water permeability and thus hAQP1 function [2]. Next, all four mutants were incubated with 0.3 mM HgCl$_2$ and osmotic water permeability was examined by oocytes swelling assay. Among the four mutants, three of them, including C87S, C102S and C152S, as well as the wild type CHIP28 were inhibited by 0.3 mM HgCl$_2$. Thus, only the C189S mutant sample showed no inhibition effects of mercury chloride. These studies showed that Cys189 in CHIP28 is the Hg$^{2+}$ sensitive residue. Different concentrations of HgCl$_2$ ranging 0.1-3 mM were examined and the complete inhibition was achieved at 3 mM HgCl$_2$ [2].
5.2 Mercury chloride inhibition and activation effects on different AQPs through cysteine and non-cysteine related processes

In order to study the effects of mercury on AQPs and compare them to the existing data, one needs to be aware that stopped-flow experiments in the presence of HgCl$_2$ have been performed under varying conditions. For example, to verify functionality of reconstituted hAQP1 in liposomes, Laize et al. [10] measured the water permeability of control and inhibited proteoliposomes with HgCl$_2$ using stopped-flow apparatus. In this set-up, proteoliposomes are subjected to a 225 mOsm/kg gradient that is inwardly directed, created by sucrose at 20$^\circ$C. Water permeability of vesicles was measured following changes in 90$^\circ$ scattered light intensity at $I_{ex}=480$ nm as a function of time [10]. Changes in light intensity are induced by shrinkage of vesicles after they are exposed to hypertonic solution of 180 mM sucrose. Permeability coefficient is calculated through the following formula:

$$P_f = k/[\left(\frac{S}{V_0}\right) \times V_w \times \Delta \text{osm}]$$

(5.2)

In this formula, $k(s^{-1})$ represents initial rate constant of light scattering changes, $S/V_0(cm^{-1})$ stands for the ratio of surface area to osmotically active volume at $t=0$, $\Delta \text{osm}(mol/cm^3)$ is the osmolality difference between the initial intra and extravesicular concentrations. $V_w$ is partial volume of water (18 cm$^3$/mol) [10].

This group used a concentration of 0.5 mM HgCl$_2$ to monitor the inhibitory effects of mercury chloride on hAQP1. It was shown that 0.5 mM HgCl$_2$ could strongly
inhibit the hAQP1 protein and decrease water permeability of the proteoliposomes [11]. The permeability coefficient was measured and compared for both inhibited and not inhibited proteoliposomes [11]. Pf value of 0.05 cm/s was found for proteoliposomes that were not inhibited, 0.0039 cm/s for protein-free liposomes and 0.0051 cm/s for proteoliposomes inhibited by 0.5 mM HgCl$_2$ at 21°C [11]. In a similar fashion, in the studies by Nyblom et al. [12], 1 mM mercury chloride was used to inhibit hAQP1. In all these studies, the permeability coefficient decreased upon inhibition of the proteoliposomes with mercury chloride becoming close to that for protein-free liposomes and indicating no activity of hAQP1 in the proteoliposomes after the treatment with mercury.

Similar to AQP1 [2], it has been shown that other mammalian AQPs including AQP2 [13] and AQP3 [14] could be inhibited with mercury chloride through the interaction of their Cys residues with Hg$^{2+}$. In the meantime, other reports showed that mercury could activate some AQPs, such as AQP6 [15, 16]. Mercury could bind to AQP6 through Cys155 and Cys190 (Cys190 is the homolog of Cys189 in AQP1), but despite of the existence of such Cys, AQP6 was not inhibited by mercury chloride [16].

On the other hand, it has also been discovered that mercury chloride could activate AQPs via non-Cys processes [17]. In a work on spinach AQP SoPIP2:1, it was shown that mercury could enhance AQP function as judged from a faster rate of water transport through the channel [17]. These effects are known not to be related to Cys residues in the protein [17]. One idea for the mechanism of activation of some AQPs via non-Cys processes is through secondary effects on other factors such as lipid properties, proteoliposomes size and mechanical stimulation [17, 18]. It was shown that secondary
effects such as changes in lipid bilayer properties are involved in mediating the activation effects of mercury on spinach AQP SoPIP2:1. Mercury could bind to the amine head group of phosphoethanolamine which is the major lipid that was used in these studies [17]. It was shown that lipid fluidity decreased as a result of binding of mercury and this affected the rate of water transport as well as other compounds through the membrane [17]. Decrease in the membrane fluidity was suggested to change the conformational state of SoPIP2;1 to an open conformation and result in a faster rate of water transport through the membrane [17].

Different sizes of proteoliposomes produce different lipid curvatures and thus exert different pressure on the AQP channel to be open and transfer water with a higher rate [17, 18]. For instance, when AQY1 from *P. pastoris* is reconstituted in liposomes, smaller proteoliposomes show faster rate of water transport compared to the larger spheroplasts [18]. On the other hand, composition of lipids and lipid bilayer fluidity may affect the rate of water transport through AQY1 by changing the conformational state of AQY1 from closed to open and thus causing more water molecules to transfer through the channel [18].

5.3 Known mercury chloride effects on hAQP1 and their suggested mechanisms

As already mentioned, mercury chloride can inhibit hAQP1 through its interaction with free sulfhydryl group of Cys189 [2]. Savage *et al*. [19] studied mutant bacterial aquaporin AQPZ as a model to understand the effects of mercury on AQP1. In this study, T183C mutant of AQPZ was constructed to emulate Cys189 of AQP1 and the X-ray
crystal structure of the mutant was obtained in the presence of mercury. The structure of the inhibited mutant AQPZ shows that mercury occludes the channel pore and inhibits the protein. Thus, the inhibition mechanism of mercury was suggested to be based on the occlusion of the water channel upon its binding to the protein [19].

Hirano et al. [20] calculated molecular dynamics simulations of mercury inhibition of bAQP1 and suggested two hypotheses for the inhibition of AQP1 by mercury. The first mechanism was a closure of the channel pore by mercury atom while the other mechanism was related to the conformational changes of the ar/R region that happened due to the binding of mercury to the Cys residue in the pore of AQP1. Molecular dynamics results show that when Cys-SHg\(^+\) replaced Cys191 residue (equivalent to Cys189), water permeation was blocked. This binding did not affect the whole structure of the protein but rather led to a collapse in the ar/R region. Residues Gly190, Cys191 and Gly192 in bAQP1 (equivalent to Gly188, Cys189 and Gly 190 in hAQP1) are part of the ar/R region and have their backbone oxygen atoms oriented toward the entrance of the pore where water is entering the channel. Upon addition of mercury, the orientation of backbone oxygen atoms of Cys-SHg\(^+\) changed and they pointed toward the outside of the water channel pore. Based on changes in the orientation of backbone oxygen atom of Cys-SHg\(^+\), displacement in position of backbone oxygen atoms of Gly190, Cys191 (which is now Cys-SHg\(^+\)) and Gly192 residues occurs and as a result the ar/R region collapses. In this work, the construction of Cys154-SHg\(^+\) (equivalent to Cys152 in hAQP1) was also made. The MD simulation results confirm that the ar/R constriction region in the Cys154-SHg\(^+\) construct remains the same as in free AQP1. This result proves that only Cys191-SHg\(^+\) in bAQP1 (equivalent to Cys189 in
hAQP1) shows conformational changes in the constriction region, reconfirming that the inhibitory binding site of mercury is Cys189 in hAQP1 [20].

Hirano et al. [20] also showed that there are four hydrogen bonds between Glu144 sidechain and Gly192 backbone (equivalent to Glu142 and Gly190 in hAQP1) and between Glu144 sidechain and Ile193 backbone (equivalent to Glu142 and Ile191 in hAQP1) in free bAQP1 protein. However, hydrogen bonds in Hg-AQP1 were reduced to two, and only between Glu144 and Gly192 residues. Thus, in free AQP, these hydrogen bonds help residues Gly190, Cys191 and Gly192 (equivalent to Gly188, Cys189 and Gly190 in hAQP1) to form a straight line through the channel in which this backbone carbonyl chain guides the movement of water molecules across the channel toward the NPA motif. Consequently, water molecules form hydrogen bonds with side chains of Arg197 and His182 and backbone oxygen atoms of Cys191 and also side chains of Asn78 and Asn194 (equivalent to Arg195, His180, Cys189, Asn76 and Asn192 in hAQP1). In contrast, in Hg-AQP1, the ar/R region is not big enough in order for water molecules to be able to permeate through the channel. On the other hand, three residues of Gly190, Cys191, and Gly192 are also not forming a line in Hg-AQP and as a result water molecules cannot make hydrogen bonds and are disordered. Thus, it is concluded that conformational changes in ar/R region induced by mercury are the cause of the inhibition of AQP1 by mercury ion [20]. Figure 5-2 shows the orientation of these important residues in free and inhibited AQP1 [20].
Fig 5-2. Orientation of residues and hydrogen bonding with water in a) free and b) inhibited AQP. Orientation of water molecules in a) free AQP showing hydrogen bonding with Asn residues in NPA motif and backbone oxygen atoms of Cys 191 as well as side chains of Arg197 and His182 in ar/R region. b) In Hg-AQP1 no hydrogen bonding occurs because of the misorientation of residues in ar/R region and NPA motif. Figure adopted from Hirano et al [20] with permission from Biophysical Journal, Elsevier.
5.4 Controversies on mercurial effects on AQPs and necessity for further studies

As already mentioned, even though it has been shown that Cys residues are responsible for inhibition of AQP1, AQP2 and AQP5 by mercury [21], structural studies of other AQPs such as AQP6 [15, 16] and plant homolog γ-AQP [22] showed a different mechanism of inhibition. These AQPs were shown to have inhibition not dependent on cysteine residues (and γ-AQP does not even have Cys residues in its amino acid sequence). Another interesting fact is related to AQP4, which is mainly expressed in the brain. Two isoforms of AQP4, M1 and M23, are insensitive to mercury. In a work by Agre et al. [21], it was reported that AQP4 expressed in oocytes shows no inhibition upon the addition of mercury chloride. In a work by Jung et al. [23], insensitivity of the AQP4 is said to be related to the lack of cysteine residue at the location equal to the position of Cys189 of AQP1.

The insensitivity of AQP4 was proved through measurements of permeability coefficient (Pf) of inhibited and not inhibited AQP4 that was expressed in oocytes and other types of cells. Stopped flow analysis of AQP4 M1-reconstituted proteoliposomes was used to determine the insensitivity of AQP4 to mercury [23–26].

In contrast to these results, Yukutake et al. [15], showed that rat AQP4 (rAQP4) M23 isoform could be inhibited by mercury chloride when it was reconstituted in proteoliposomes but not when it was expressed in oocytes. The reason for this is related to the orientation of AQP4 in both systems as described below. In this work, the difference of inhibition of AQP1 and AQP4 reconstituted into proteoliposomes was discussed. AQP4 could be inhibited by 5 μM HgCl₂ up to 50% while in the case of
AQP1, 100 μM HgCl$_2$ was required to inhibit AQP1 up to 50% of its activity. These results also showed that time dependencies of AQP1 and AQP4 to be inhibited by mercury are different. The 50% inhibition of AQP1 was achieved after 5 minute incubation with 300 μM HgCl$_2$, while 50% activity of AQP4 was inhibited 30 s after incubation with the same concentration of mercury chloride.

Site-specific mutagenesis studies by Yukutake _et al._ [27] revealed that Cys178 is the mercury binding site in AQP4. rAQP4 M23 contains six cysteines in its amino acid sequence. The location of Cys178 is on the intracellular side of the channel and far from the constriction region where water molecules are passing through the channel. Yukutake _et al._ suggested that AQP4 could only be inhibited by mercury through the dynamic conformational changes that are induced by binding mercury to that Cys residue.

As mentioned earlier, the orientation of AQP4 in proteoliposomes versus oocytes was the main factor in inhibition of AQP4 in proteoliposomes. In order to check the orientation of AQP4 in both systems, two antibodies against AQP4 were used, anti-human NMO (neuromyelitis optica)-IgG with the recognition site at the extracellular region of AQP4 and rabbit anti-AQP4 that binds to the intracellular side of the AQP4. Results showed that both antibodies could bind to AQP4 proteoliposomes while only NMO-IgG could bind to oocytes expressing AQP4. Thus, when incubated with mercury chloride, Hg$^{2+}$ could bind to Cys178 that was oriented toward the outside of proteoliposomes. But, in oocytes, because only one orientation of AQP4 exists, binding of Hg$^{2+}$ to Cys178 is not possible (Figure 5-3).
Fig 5-3. A) Structure of the monomer of AQP4 showing the location of Cys178. B) Orientation of AQP4 in proteoliposomes and oocytes, figure is adapted from Yukutake et al [27] with permission from Wiley.

There were some more contradictory results regarding mercury binding sites in AQP1. It was shown that when cysteine residue was introduced in AQP0 and AQP4 in the vicinity of their second NPA motif at the position homologous to Cys189 of AQP1, AQP0 and AQP4 both remained insensitive to mercury chloride. Even though the introduced Cys was expected to be accessible to mercury from the extracellular side, no inhibition was seen [23, 28].

Earlier in this chapter (Section 5.2), we also discussed results related to activation of some AQPs by mercury chloride through their cysteine residues or through changes in physical properties of proteoliposomes. These findings suggest that there may be more
complexity in the effects of mercury on AQPs, which need to be addressed. Such complex interactions of mercury with AQP1 may be in line with the results presented in chapter 4, where we tried to study the conformational changes of hAQP1 upon inhibition by mercury chloride. There, as we increased the mercury chloride concentration, the ssNMR spectra became broader, suggesting heterogeneity of the sample. The quality of the NMR spectra decreased significantly making it impossible to follow the conformational changes of the protein through NMR spectra. Consequently, we decided to investigate effects of different concentrations of mercury chloride through other biophysical and biochemical methods including stopped flow, Fourier transform infrared (FTIR) spectroscopy, dynamic light scattering (DLS), SDS-PAGE and crosslinking.

5.5 Materials and Methods

5.5.1 Stopped-flow measurements

The stopped-flow spectrophotometer (SX20, Applied Photophysics) was used for stopped-flow measurements. The kinetics of the increase in light scattering due to the vesicles shrinkage was measured at the 90° angle to the incident beam at 480 nm at 22 °C upon the addition of the hypertonic solution (180 mM sucrose). The water permeability experiments were conducted with protein/lipid ratio (1/10 w/w). The lipid and proteoliposomes preparation was already described in section 3.3 in chapter 3. Brain PS lipids were omitted from our lipid stock when performing the mercury inhibition experiments. However, the Egg PC/Brain PS liposomes (9/1 molar ratio) were used for
the stopped-flow experiments that were performed to follow the effects of the putative inhibitors on hAQP1 by the novel compounds. The lipid stock was prepared by reverse-phase evaporation and extruded 13 times through the polycarbonate Isopore filters (0.4 and 0.2 microns). The preparation steps for proteoliposomes were exactly the same as described in section 3.3. The control liposomes and proteoliposomes were diluted to 0.2 mg lipid/ml in the liposome buffer (20 mM Tris-HCl, pH 7.0, 50 mM NaCl). Incubation time of proteoliposomes and liposomes (as a control experiment) with mercury chloride was about 30 minutes at 4 °C prior to the stopped-flow measurements. All the stopped-flow experiments were repeated three times and, in each measurement, 15 traces were recorded and the reproducibility of each result was confirmed by getting similar results. The absolute amplitudes of the stopped-flow results were 7 to 8 V.

5.5.2 Dynamic Light Scattering (DLS)

Average diameter of the liposomes and proteoliposomes, as well as inhibited proteoliposomes, were obtained by Dynamic Light Scattering (Malvern Zetasizer). The control liposomes and proteoliposomes were diluted to 0.2 mg lipid/mL in the liposome buffer (20 mM Tris-HCl, pH 7.0, 10 mM NaCl) prior to each measurement. Incubation time of proteoliposomes with mercury chloride was about 30 minutes at 4 °C prior to the measurements. To perform DLS on the proteoliposomes that were inhibited with mercury chloride, the white precipitates (formed only at higher mercury concentrations) were resuspended with the supernatant prior to the measurement. After resuspending the white sediments, we measured DLS two times, first time was right after the resuspension of the
white sediments and second time, one minute after resuspending white sediments. All DLS experiments were repeated three times and reproducibility of each experiment was confirmed by obtaining similar results.

### 5.5.3 Fourier Transform Infrared spectroscopy (FTIR)

To perform FTIR measurements on the inhibited proteoliposomes, the proteoliposomes were diluted to 0.2 mg lipid/ml in the liposome buffer (20 mM Tris-HCl, pH 7.0, 50 mM NaCl) and were incubated with mercury chloride for 30 minutes. The inhibited proteoliposome pellets corresponding to ~0.05-0.2 mg of hAQP1 were obtained by centrifugation at 900,000 × g to remove extra non-bound mercury chloride. The pellets were then resuspended in MilliQ water and dried on a CaF₂ window (Harrick). The dry film was covered with another CaF₂ window with a 6 micron Teflon spacer and placed in Bruker IFS66vs machine with a temperature-controlled sample holder (Harrick). The setup of the instrument is already mentioned in section 3.2.1 in chapter 3. All FTIR experiments were repeated three times and reproducibility of each experiment was confirmed by obtaining similar results.
5.5.4 SDS-PAGE experiments on the cross-linked inhibited hAQPI

In this set of experiments, we made proteoliposomes with a lipid to protein ratio of 10 w/w with hAQPI reconstituted into PC lipids (Avanti polar lipids) and inhibited by 0.83 μM, 18.7 μM, and 60 μM HgCl₂ after reconstitution in liposome buffer (20 mM Tris-HCl, pH 7.0, 50 mM NaCl). The incubation time of the hAQPI with mercury chloride was 30 minutes at 4 °C.

Extra mercury chloride was removed (after incubating for 30 minutes) by centrifugations at 300,000×g to avoid excessive aggregation. For crosslinking the hAQPI, glutaraldehyde was used. Glutaraldehyde has two aldehyde groups per molecule with a flexible hydrocarbon chain. Both aldehyde groups of a single glutaraldehyde molecule react with proteins. Glutaraldehyde can specifically bind to the ε amino group of Lys side chain [29]. A reaction mixture containing 50 μg to 100 μg of lipid-reconstituted hAQPI in phosphate buffer (pH 7.5) was mixed with 5 μL of 2.3% (v/v) freshly prepared solution of glutaraldehyde at 37 °C for 2 to 5 minutes. The reaction was terminated upon addition of 10 μL of 1 M Tris-HCl, pH 8.0 [30].

The SDS-PAGE gels were prepared by using a Bio-Rad vertical electrophoresis system based on the manufacture guide. Gel solutions were prepared based on materials shown in table 5-1 and poured between two glass plates that were separated by a 1.0 mm comb. The separating gel was poured first, and in second step the stacking gel solution was poured on top of the separating gel between the glass plates. After polymerization of the gel, the glass cassette was used in an electrophoresis apparatus. The running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS, pH 8.3) was used and the electrophoresis
apparatus was submerged in this buffer and the 2×SDS gel-loading buffer (60 mM Tris-HCl, pH 6.8, 25% glycerol, 4% (w/v) SDS, 14.4 mM β-mercaptoethanol and 0.1% bromophenol blue) were used.

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stacking Gel 5%</td>
</tr>
<tr>
<td>Water</td>
<td>2.32 mL</td>
</tr>
<tr>
<td>30% (w/v) acrylamide/0.8% (w/v) bisacrylamide</td>
<td>0.67 mL</td>
</tr>
<tr>
<td>Stacking Gel Buffer (0.5 M Tris/0.4% SDS, pH 6.8)</td>
<td>1 mL</td>
</tr>
<tr>
<td>Separating Gel Buffer (1.5 M Tris/0.4% SDS, pH 8.8)</td>
<td>-</td>
</tr>
<tr>
<td>10% (w/v) ammonium persulfate</td>
<td>12 μL</td>
</tr>
<tr>
<td>TEMED</td>
<td>6 μL</td>
</tr>
<tr>
<td>Total</td>
<td>4 mL</td>
</tr>
</tbody>
</table>

Table 5-1. Solutions used to prepare SDS-PAGE gels.
The sample preparation for SDS-PAGE was slightly different depending on the nature of samples. The cross-linked samples were treated with 2× SDS gel-loading buffer. After mixing the samples with the loading dye, they were loaded on the gel after boiling the samples for 5 minutes. White sediment sample was treated with 2× SDS gel-loading buffer containing 4% (w/v) SDS, and, after mixing with loading dye, loaded on the gel without boiling. Samples were run under 100 V for 3 h until they reached the mark 1 cm from the gel bottom. All the cross-linking experiments were repeated three times and reproducibility of each experiment was confirmed by obtaining similar results.

5.5.5 Sample preparation for the FTIR and stopped-flow studies of the crosslinked hAQP1

Reconstituted hAQP1 was crosslinked with 5 µl of 2.3% freshly prepared solution of glutaraldehyde for 2 to 5 minutes at 37°C. The reaction was terminated upon addition of 10 µL of 1 M Tris-HCl, pH 8.0. Samples were spinning down using 300,000×g centrifugation to remove non-bound crosslinker. Mercury chloride was then added to the crosslinked sample and it was incubated for 30 minutes prior to spinning down using 300,000×g centrifugation. Pellets were then resuspended in MilliQ water and dried on a CaF₂ window (Harrick). All the cross-linking experiments were repeated three times and reproducibility of each experiment was confirmed by obtaining similar results.
5.5.6 Sample preparation for the stopped-flow measurement of hAQP1 inhibited with the novel blockers

For this set of the experiments, the control liposomes and proteoliposomes were diluted to 0.2 mg lipid/mL in the liposome buffer (20 mM Tris-HCl, pH 7.0, 50 mM NaCl). Stopped flow measurements were done using the same sucrose concentration and protocols as described above. All the stopped-flow experiments were repeated three times and in each measurement 15 traces were recorded and the reproducibility of each result was confirmed by getting similar results. The absolute amplitudes in the stopped-flow results were reproducibly 7 to 8 V.

5.6 Assay of the protein functionality in the presence of different mercury concentrations

To study inhibitory effects of mercury chloride, we performed stopped flow experiments with hAQP1 proteoliposomes inhibited with three different mercury chloride concentrations. Figure 5-4 presents stopped flow results of inhibited and intact hAQP1 proteoliposomes as well as protein-free phosphatidylcholine (PC) liposomes.
Fig 5-4. Stopped flow experiments following shrinkage of PC liposomes (purple) and proteoliposomes (dark blue) upon hypertonic shock. hAQP1 was reconstituted in PC liposomes (lipid/protein ratio of 10 w/w). Mercury chloride at different molar ratios of Hg/AQP (1, 10 and 30 - red, green and light blue, respectively) was added to proteoliposomes and kinetics of the light scattering changes of samples were measured in the stopped flow apparatus.

The kinetics of water efflux from protein-free liposomes and proteoliposomes could be fitted by single and double exponential functions, respectively, to obtain apparent time constants t (s). Stopped flow results show that we can see inhibition of hAQP1 starting from Hg/AQP molar ratio of 1. In the case of proteoliposomes inhibited with HgCl₂/Protein at molar ratios of 1, 10 and 30, respectively (absolute concentration of 0.83 µM, 18.7 µM and 60 µM HgCl₂ respectively), the curves could be fitted with a
single exponential as expected. This is similar to what we observed previously when we performed the functionality assay of the hAQP1 using stopped-flow kinetic experiments (Chapter 3, Figure 3-4). Equations 5.3 and 5.4 show single and double exponential functions respectively:

\[ F(x) = F_1 e^{-\frac{t}{\tau}} + F_0 \]  \hspace{1cm} (5.3)

\[ F(x) = F_1 e^{-\frac{t}{\tau_1}} + F_2 e^{-\frac{t}{\tau_2}} + F_0 \]  \hspace{1cm} (5.4)

Where \( F_1 \) and \( F_2 \) are the amplitudes of the scattering components, which are negative, and \( F_0 \) is light scattering at \( t = \infty \), while \( \tau_1 \) and \( \tau_2 \) are fitted time constants.

Table 5-2 shows fitted time constants for each sample.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Fitted time constant, (s)</th>
<th>Amplitude</th>
<th>Error of fitted time constants</th>
</tr>
</thead>
<tbody>
<tr>
<td>hAQP1 Proteoliposomes</td>
<td>0.01253</td>
<td>89%</td>
<td>0.00008</td>
</tr>
<tr>
<td></td>
<td>0.1404</td>
<td>11%</td>
<td>0.0031</td>
</tr>
<tr>
<td>Proteoliposomes – 0.83 μM HgCl₂ - (HgCl₂/AQP molar ratio 1)</td>
<td>0.05248</td>
<td></td>
<td>0.004</td>
</tr>
<tr>
<td>Proteoliposomes – 18.7 μM HgCl₂ - (HgCl₂/AQP molar ratio 10)</td>
<td>0.1039</td>
<td></td>
<td>0.00043</td>
</tr>
<tr>
<td>Proteoliposomes – 60 μM HgCl₂ - (HgCl₂/AQP molar ratio 30)</td>
<td>0.1174</td>
<td></td>
<td>0.00053</td>
</tr>
<tr>
<td>PC Liposomes</td>
<td>0.1263</td>
<td></td>
<td>0.00132</td>
</tr>
</tbody>
</table>

Table 5-2. Stopped flow time constants of osmotically induced shrinkage of reconstituted hAQP1 (lipid/protein ratio of 10 w/w), PC liposomes, and proteoliposomes inhibited with mercury chloride with different concentrations added after reconstitution. The mixture was incubated half an hour prior to the measurements. These experiments were conducted three times.
One important fact was observed during sample preparation and inhibition of hAQP1 with mercury chloride. Upon the incubation of hAQP1 proteoliposomes (reconstituted either in PC alone or PC/PS 9:1 mixture) with mercury chloride with an Hg/hAQP1 molar ratio of 10 or 30 (with absolute concentrations value of 18.7 μM and 60 μM HgCl₂ respectively), we noticed that a white sediment appeared at the bottom of the tube after 30 minutes (Figure 5-5). Such precipitate was not seen after incubation of the proteoliposomes with molar ratios of mercury-to-protein lower than 10.

![Fig 5-5. White sediment precipitates after addition of 60 μM HgCl₂ to the hAQP1 reconstituted in PC lipids. Lipid/protein ratio 10 w/w. Incubation time 30 minutes.](image)

The white sediment was also seen in the UCN labeled NMR sample prepared at much higher protein/lipid ratio. In that case, after we removed the sample from the rotor, we saw a white precipitate that looked different from the non-inhibited sample. The concentration of mercury chloride was 80 μM (Hg/Protein molar ratio of 160) and this was when we saw the broadening of peaks and loss of resolution.

The nature of this precipitate and associated changes in the proteoliposomes have been investigated with FTIR spectroscopy as described below in a separate section. As obvious from the stopped flow results, hAQP1 proteoliposomes demonstrated much faster shrinking compared to the control liposomes (similar to the earlier results presented
in the Figure 3-4 in Chapter 3). The reason for faster shrinkage of the proteoliposomes must be the presence of the functional reconstituted hAQP1 that allows water molecules to pass with higher rate than simple diffusion across the membrane. Such a biphasic character for hAQP1 proteoliposomes was shown before and the slow kinetic phase was interpreted as corresponding to the protein-free population [12].

The hAQP1 proteoliposomes inhibited with 0.83 μM HgCl₂ showed a lower time constant of shrinking compared to those at 18.7 μM and 60 μM HgCl₂. This indicates that the rate of vesicles shrinkage is still relatively high and thus some hAQP1 molecules are still active in the membrane. The HgCl₂/AQP molar ratio is 1 for 0.83 μM mercury concentrations. Based on the molar ratio of mercury to protein, the number of mercuric ions per Cys residue of hAQP1 may not be sufficient to inhibit the function of the protein completely (even though two of the four Cys are buried and one is located on the cytoplasmic side, Figure 5-1). Also, orientation of hAQP1 in the membrane is another fact that might make the accessibility of mercury ion to Cys that are oriented towards the inside of liposomes harder. There are two possible orientations of reconstituted hAQP1 molecule in the liposome. One orientation is where Cys 189 is facing the inside of the liposome and one where it points outside so that mercury ion does not need to pass through the membrane to reach this Cys residue.

The hAQP1 proteoliposomes inhibited with 18.7 μM and 60 μM HgCl₂ (HgCl₂/Protein molar ratio of 10 and 30 respectively) have higher time constants of shrinkage than proteoliposomes that were inhibited with 0.83 μM HgCl₂, and are similar to the protein-free liposomes. Increasing the time constant of vesicles shrinkage is likely to be related to more complete blockage of the hAQP1 channel upon binding of mercury.
to Cys189 residue. Based on the molar ratio of mercury to protein that is equal to 10 for 18.7 μM HgCl₂, the availability of mercury ion increases sufficiently and thus all hAQP1 molecules could be inhibited. The molar ratio of mercury to protein is even higher for 60 μM HgCl₂ and is equal to 30, so that mercury ions should easily inhibit hAQP1 reconstituted in the membrane. Nevertheless, one cannot rely solely on the measured rates of shrinkage to understand the inhibition, in view of the formation of the white precipitate described above and without knowing whether mercury affects the size of liposomes and proteoliposomes (see equations 5.1 and 5.2), which we explored using DLS as described below.

5.7 Dynamic light scattering studies of mercury effects on proteoliposome size

To evaluate the size of proteoliposomes containing hAQP1 inhibited with different concentrations of mercury chloride and protein-free liposomes, we performed DLS measurements. In DLS, the Polydispersity Index (PdI) is used as an indicator of the width of the vesicles size distribution (Chapter 2, Figure 2-11). The PdI formula is as follows:

$$PdI = \left( \frac{\sigma}{d} \right)^2$$

(5.5)

Where σ is the standard deviation and d is mean diameter. In DLS measurement, PdI is calculated through cumulants analysis and is given as a number. Mean diameter is Z-average (Z-average is derived from the cumulants analysis and it is an intensity-
weighted mean diameter). Thus, standard deviation is what is shown as the width of the particle size distribution and is different from spectral width used in other techniques.

Figure 5-6 shows DLS results for hAQP1 proteoliposomes before the mercury treatment.

Fig 5-6. Dynamic light scattering results of hAQP1 reconstituted in PC liposomes (lipid/protein ratio of 10 w/w).

As another control, DLS result of protein-free liposomes is shown in Figure 5-7. They show wider size distribution than hAQP1-reconstituted proteoliposomes, consistent with what was observed previously for other proteins [31]. This suggests that when hAQP1 was inserted into the liposomes, it made vesicles size distribution narrower with the unilamellar lipid formations. In order to see effects of mercuric ion on the size of protein-free liposomes, they were incubated with 60 μM HgCl₂ for 30 minutes (Figure 5-8). We saw no changes occurring to protein-free liposomes upon incubation with high concentrations of mercury chloride.
Fig 5-7. Dynamic light scattering results of 11.1 mg/mL PC liposomes.

Fig 5-8. Dynamic light scattering results of 11.1 mg/mL PC liposomes incubated with 60 μM Hg.

The DLS results on the inhibited proteoliposomes were obtained twice. When we measured DLS right after resuspending white sediments, we saw very broad and asymmetric peaks reflecting the presence of a continuum of aggregates of various sizes, but when we let the sample stay for 1 minute after resuspending sediments, we saw that asymmetric peak changed to two separate peaks with measurable width and diameter for
each peak. Figures 5-9 to 5-11 represent DLS results of proteoliposomes inhibited with three different concentrations of mercury chloride. Table 5-3 shows diameter and width of size distribution from the DLS results.

Fig 5-9. Dynamic light scattering results of hAQP1 reconstituted in PC liposomes inhibited with 0.83 μM Hg (Hg/AQP ratio 1 and lipid/protein ratio of 10 w/w).
Fig 5-10. Dynamic light scattering results of hAQP1 reconstituted in PC liposomes inhibited with 18.7 µM Hg (Hg/AQP ratio 10 and lipid/protein ratio of 10 w/w) a) right after the resuspension of the white sediment and b) 1 minute after resuspending white sediment.
Fig 5-11. Dynamic light scattering results of hAQP1 reconstituted in PC liposomes inhibited with 60 μM Hg (Hg/AQP ratio 30 and lipid/protein ratio of 10 w/w) a) right after the resuspension of the white sediment and b) 1 minute after resuspending white sediment.
Table 5-3. Dynamic light scattering results for PC-reconstituted hAQP1 (lipid/protein ratio of 10 w/w), PC liposomes, and proteoliposomes inhibited with mercury chloride with concentration of 0.83 µM, 18.7 µM and 60 µM (HgCl$_2$/Protein molar ratio of 1, 10 and 30, for the three HgCl$_2$ concentrations respectively) added into the proteoliposomes after reconstitution. The mixtures were incubated for half an hour prior to the measurement.

Based on these results, the diameter of hAQP1 proteoliposomes changes upon increase in the mercury chloride concentration in a complex way. In DLS, the diameter is very sensitive to the presence of aggregates. When mercury chloride was added to the sample, the diameter of the vesicles changed along with the shape of the peaks. Upon increase in the mercury chloride concentration, the size distribution became very
asymmetric, showing secondary peaks (and/or shoulders) suggesting aggregations. At the same time, the main peak shifted towards lower diameter values implying that, along with aggregation, many vesicles became smaller. An additional complicating factor mentioned above is that after the addition of mercury chloride the white precipitate appeared at the bottom of the tube (see above). Thus, we can conclude that the higher mercury chloride concentrations routinely used in the functionality assays may have non-trivial effects on proteoliposome size, and possibly their composition, so that the straightforward interpretation of the shrinkage rate is impossible. We decided to further explore mercury-induced changes of the hAQP1 proteoliposomes by FTIR spectroscopy as described below.

In summary, in our stopped flow results, similar to many previous studies [12], we saw that the activity of hAQP1 in proteoliposomes was inhibited upon the addition of mercury chloride leading to the complete inhibition of hAQP1 at higher concentrations. On the other hand, studies of the size distribution of proteoliposomes by DLS showed the appearance of broad peaks suggesting formation of aggregates and different types of vesicles with increased and decreased sizes. These DLS results, along with the observed formation of the white precipitate, suggested that effects of mercury on hAQP1 are more complex than a simple inhibition of its activity, at least in the higher concentration range, and need more investigation.
5.8 Effects of mercury may depend on the orientation of hAQP1 in the membrane - stopped flow assay to test the orientation effects

The orientation of hAQP1 in the membrane may also play a role in the efficiency of inhibition, and there is evidence that AQPs could be oriented in two directions (N and C terminus toward the cytoplasm or outside) when reconstituted in the lipid membrane [15]. Thus, to test for effects of the orientation of hAQP1 in the membrane, we incubated the protein with 60 µM HgCl₂ before and after reconstitution. Figure 5-12 shows stopped flow results of mercury inhibition before and after reconstitution. We can see that hAQP1 inhibited before and after reconstitution both show slower rate of vesicle shrinkage. Although there is some minor difference in the rates of vesicles shrinkage, the results suggest that inhibition both before and after reconstitution is efficient, at least for the higher mercury concentrations. Nevertheless, in view of the mercury effects on the liposome size (and possibly the composition) described above, interpretation of these results may be more complex.
Fig 5-12. Stopped flow results for hAQP1 reconstituted in PC (lipid/protein ratio 10 w/w) (blue), PC liposomes (purple), proteoliposomes inhibited with hAQP1 with 60 µM HgCl₂ before reconstitution (Hg/AQP molar ratio 30) (green), and after reconstitution (red).

To test whether mercury affects proteoliposome size, which could influence interpretation of the orientational effects, DLS measurements were performed for hAQP1 inhibited with 60 µM HgCl₂ before and after reconstitution. For these measurements, white sediment was discarded and only the supernatant was measured with DLS. The results shown in Figures 5-13 to 5-16 and summarized in table 5-4 present only one symmetric peak, which broadens significantly upon addition of mercury.
Fig 5-13. Dynamic light scattering results of hAQP1 reconstituted in PC liposomes.

Fig 5-14. Dynamic light scattering results of hAQP1 inhibited with 60 μM HgCl₂ (Hg/AQP ratio 30) before reconstitution in PC liposome.
Figure 5-15. Dynamic light scattering results of hAQP1 inhibited with 60 µM HgCl₂ (Hg/AQP ratio 30) after reconstitution in PC liposome.

Fig 5-16. Dynamic light scattering results of 11.1 mg/mL PC liposomes.
Table 5-4. Diameter, width of size distribution, and time constants of osmotically induced shrinking based on DLS and stopped flow results. hAQP1 was reconstituted in PC lipid (lipid/protein ratio 10 w/w). These experiments were conducted three times and the reproducibility of the results was confirmed.

In both cases of the inhibition, width of the size distribution of the inhibited hAQP1 proteoliposomes increased. Comparing these results with the similar series shown in section 5.6, table 5-2, we can see the difference in the fitted time constants for the proteoliposomes. The reason for this is likely to be related to the preparation procedure of
the proteoliposomes, where the amount of the protein molecules that were inserted in the lipid are somewhat different from one preparation to the other. Thus, it is important to have one set of experiments done using the same sample preparation. However, despite some differences in the absolute rates, the trends are the same in both sets, and we have a fast and a slow rate of shrinkage for intact proteoliposomes and slow rates for the inhibited ones.

One important observation is that the parameters of inhibited proteoliposomes are close to those of protein-free liposomes. Also, note that the white sediment was removed before taking DLS measurements and thus we see fewer aggregates in DLS results.

At this point, there may be several explanations on why inhibitions before and after reconstitution of hAQP1 are equivalent. First, one can conclude that mercury can pass through the membrane bilayer and reach the Cys189 residue that is oriented toward the inside of the liposomes in one of the orientations. Second, the same result could be obtained if hAQP1 is oriented uniformly right-side-out, and Cys189 is always accessible. Finally, considering the dramatic effects of mercury on proteoliposomes size and formation of the white precipitate, our results can be possibly explained by those effects, not directly related to binding to Cys189 of hAQP1.

5.9 Investigation of mercury effects on hAQP1 proteoliposomes with Fourier Transform Infrared spectroscopy (FTIR)

To understand biochemical nature of mercury-induced changes in the proteoliposome morphology as well as the nature of the associated white precipitate, we
used FTIR spectroscopy. FTIR can detect if the protein to lipid ratio of proteoliposomes after inhibition with mercury chloride changes and also reports on the secondary structure of hAQP1 and its possible changes upon interaction with mercury. Figure 5-17 shows FTIR absorbance spectra of hAQP1 inhibited before and after reconstitution.

Fig 5-17. FTIR absorbance spectra of a dry film of hAQP1 reconstituted in PC liposomes (blue spectrum) with lipid/protein ratio of 10 (w/w), hAQP1 inhibited with 60 μM HgCl₂ (Hg/AQP molar ratio of 30) before reconstitution (purple spectrum) and after reconstitution (orange spectrum). Spectra were normalized based on their lipid esters band amplitude. Each spectrum was baseline corrected through the simple linear corrections.

Based on the Amide I band position (located at 1650 – 1655 cm⁻¹), we confirmed the nativity of our hAQP1 sample as judged from its predominantly α-helical conformation. Surprisingly, when we inhibited hAQP1 with mercury either before or
after reconstitution, we could see amide I and II peaks of hAQP1 disappear from the FTIR absorbance spectra, showing a dramatic decrease of protein to lipid ratio. This suggests that mercury precipitates the hAQP1 protein before reconstitution or interferes with lipid reconstitution in some other way. Even more surprisingly, the results also suggest that high concentrations of mercury can destabilize and remove hAQP1 from the proteoliposome membrane after the reconstitution. Accordingly, this suggests that the observed white precipitate could be hAQP1, as verified below.

To find the concentration dependence of hAQP1 precipitation FTIR experiments were performed for proteoliposomes that were inhibited by different concentrations of mercury chloride. These samples were all inhibited after reconstitution of hAQP1 protein in PC liposomes (Figure 5-18).
Fig 5-18. FTIR absorbance spectra of a dry film of hAQP1 reconstituted in PC liposomes (blue) with lipid/protein ratio of 10 (w/w), mercury chloride at different Hg/AQP molar ratios - 1 (green), 10 (orange) and 30 (red) - was added to proteoliposomes after reconstitution. Spectra were normalized based on their lipid esters band amplitude. Each spectrum was baseline corrected through the simple linear corrections.

Based on these FTIR results, we can confirm that significant changes occur in amide I band for the hAQP1 proteoliposomes that were inhibited even at low Hg/AQP molar ratio of 1 (0.83 μM HgCl₂). As the concentration of the mercury chloride increases, we see the amide I peak disappear. At both Hg/AQP molar ratio of 10 and 30 (18.7 and 60 μM HgCl₂), we see almost no amide I peak. This correlates with and could be related to the observation of white sediment at these molar ratios.
Based on the above experiments, the protein nature of the white sediment is suggested. Thus, we needed to verify if the white precipitate contains hAQP1 and whether it is in its native state or underwent some conformational changes. A dry film of the white precipitate was made in order to measure its FTIR absorbance spectrum (Figure 5-19).

![FTIR absorbance spectrum](image)

Fig 5-19. FTIR absorbance spectrum of a dry film of the white precipitate formed upon addition of 60 μM HgCl₂ to the reconstituted hAQP1 in PC lipid with protein-to-lipid ratio of 1/10 (w/w). The resulting spectrum suggests much lower lipid content with protein to lipid ratio of ~1/2.

What could be unambiguously gleaned from the FTIR spectrum of the white sediment is that this is a spectrum of a protein with very small amount of bound lipids, as obvious from the amplitude ratio of the lipid esters band to Amide I band that is equal to
0.29. For comparison, the amplitude ratio of the lipid esters band to Amide I of the native proteoliposomes shown in Figure 5-18 is equal to 2.5.

An important feature of the IR spectrum of the white sediment is the presence of a shoulder at 1575 cm\(^{-1}\), which implies some unusual, possibly \(\beta\)-turn, secondary structure in the protein. On the other hand, comparing Amide I peak of the white sediment precipitate with the normal hAQP1 Amide I peak, there was no difference either in its width or in its position, which suggests that the precipitated hAQP1 is still mainly alpha-helical.

The white sediment sample was also tested by SDS-PAGE (the results are shown in the next section, Figure 5-20 lane 5 from left). The SDS-PAGE results confirm that the white precipitate is hAQP1 protein that shows some oligomers and has bands at \(\sim\)85 kDa for trimers, \(\sim\)63 kDa for dimers, and \(\sim\)30 kDa for monomers.

In summary, we can certainly conclude that the white sediment is hAQP1 that precipitates and leaves the lipid bilayer when Hg/AQP molar ratio is at 10 or higher (18.7 \(\mu\)M HgCl\(_2\) concentration). Thus, higher molar ratios and concentrations of mercury destabilize the membrane-bound conformation of hAQP1 via some unknown mechanism, presumably different from the simple binding to Cys189 at low mercury concentrations. As hAQP1 exists as a tetramer, and the tetramers are known to form 2D crystalline domains [32], we decided to investigate whether mercury-induced protein destabilization may be also related to the effects on its oligomeric state.
5.10 Crosslinking experiments to probe changes of hAQP1 oligomerization

Chemical crosslinking offers a direct method of identifying stable interactions between monomers of oligomeric proteins. Crosslinking technique involves the formation of covalent bonds between two (or more) amino acid residues of different monomers, with specificity based on the cross linker that is being used. Most often, specific bifunctional reagents that contain reactive end groups are used. In our research, we used glutaraldehyde \( \text{CH}_2(\text{CH}_2\text{CHO})_2 \) as an inter-subunit crosslinker for maintenance of stable quaternary structure. Glutaraldehyde crosslinker is a very well-known lysine-reactive reagent used when maintenance of the structural rigidity of the protein is important [29]. Mercury-induced changes of quaternary structure of hAQP1 could be analyzed by comparison of the extent of crosslinking in the case of mercury-bound versus native hAQP1. Additionally, effects of mercury added to the crosslinked hAQP1 samples could give extra information on their interaction.

5.10.1 SDS-PAGE of the proteins crosslinked with and without mercury

We used SDS-PAGE in combination with crosslinking to study mercury-induced changes in quaternary structure of hAQP1. The idea of using treatment with glutaraldehyde is to induce inter-subunit crosslinking, which will be manifested as the presence of dimers, trimers and tetramers on the stained gel if isolated tetramers are present in proteoliposomes. On the other hand, only a monomeric band will be visible if
tetramers are completely destroyed by mercury. Finally, if hAQP1 exists as a 2D crystal of tetramers, as shown below (Figure 5-33), only very high molecular weight products will be visible due to inter-tetramer links. SDS-PAGE results of crosslinking of hAQP1 proteoliposomes with glutaraldehyde are shown in Figure 5-20.
Fig 5-20. SDS-PAGE results of hAQP1 crosslinked before and after mercury inhibition, SYPRO Ruby dye was used for staining. From left to right: (Marker) – All blue marker, (Cross ++) - hAQP1 crosslinked after inhibition of proteoliposomes with 18.7 μM Hg (Hg/AQP molar ratio 10), (Cross +++ - same with 60 μM Hg (Hg/AQP molar ratio 30), (Cross -) - hAQP1 crosslinked without inhibition, (Sed++) - white precipitate formed after addition of 60 μM Hg (Hg/AQP molar ratio 30), (Cross -) - crosslinked hAQP1 without inhibition, repeated, (Cross +) - hAQP1 crosslinked after inhibition with 0.83 μM Hg (Hg/AQP molar ratio 1), (Sed+) - white precipitate formed after addition of 18.7 μM Hg (Hg/AQP molar ratio 10), (Cross -) - crosslinked hAQP1 without inhibition, repeated, (Marker) - marker (Molecular weight of marker is shown in the left in kDa unit).
The SDS-PAGE results (Figure 5-20) show the existence of trimer, dimer and monomer bands for hAQP1 crosslinked after inhibition with mercury at Hg/AQP molar ratios of 10 and 30 (18.7 μM and 60 μM HgCl₂ concentration, respectively), but not at lower concentration. Trimers appeared at 85 kDa, dimers at 63 kDa and monomers at 30 kDa.

On the other hand, for hAQP1 crosslinked without inhibition, there is only one high molecular weight band (or group of bands) that appeared on top of the lane, probably representing crosslinking of multiple hAQP1 molecules in the lattice of tetramers (Figure 5-20). This is consistent with the earlier work showing formation of hAQP1 tetramer 2D-crystalline arrays in proteoliposomes [32] and is confirmed by our SAXS data (see below).

The existence of monomeric, dimeric, and trimeric bands with the molecular weight equal to monomer, dimer or trimer structure of the hAQP1 in the samples crosslinked after the mercury treatment suggests that mercury destroys the lattice of hAQP1 tetramers, and possibly the tetramers themselves, at least partially. However, the oligomers that were seen for the white precipitate suggest that the main effect of mercury is on the lattice rather than on the tetramers, unless the re-formation of hAQP1 oligomers is promoted in SDS.
5.10.2 Stopped-flow experiments on the crosslinked samples with and without mercury

As the SDS-PAGE results presented above suggest that mercury can interfere with the oligomeric state of the protein by destroying the lattice of tetramers and possibly the tetramers themselves, we decided to stabilize the oligomeric state of hAQP1 by crosslinking and apply mercury afterwards. Please note that the order of applying the crosslinker and mercury chloride is opposite to that in the previous section, where crosslinking was used as an analytical tool to reveal the supramolecular structure. Here, we were hoping that crosslinker-induced stabilization of the lattice will prevent precipitation of hAQP1 out of the membrane and reveal conformational changes of individual monomers of hAQP1. Thus, we repeated the functionality assays on the crosslinked hAQP1 proteoliposomes with and without mercury using the stopped flow apparatus.

To do this, hAQP1 samples containing 50 μg to 100 μg of protein were reconstituted in PC lipid, and crosslinked with 5 μl of 2.3% freshly prepared solution of glutaraldehyde for 2 to 5 minutes at 37°C. The reaction was terminated upon addition of 10 μl of 1 M Tris-HCl, pH 8.0. Non-bound glutaraldehyde was washed away by centrifugation at 300,000×g. Next, crosslinked hAQP1 proteoliposomes were incubated for 30 minutes with 0.83, 18.7 and 60 μM mercury chloride and used for stopped flow measurements as before. Crosslinked and inhibited proteoliposomes were submitted to a 225 mOsm/kg inwardly directed sucrose gradient at 20 °C.
It appears that the glutaraldehyde crosslinking has stabilized hAQP1 in the membrane as expected, as formation of the white precipitate at high mercury concentrations was no longer observed. At the same time, the crosslinking has not affected properties of the channel, as the rate of vesicles shrinking was very similar in crosslinked and non-crosslinked samples. The most interesting stopped-flow results we saw were in the case of incubation of hAQP1 proteoliposomes with higher concentrations of mercury chloride, which produced a very fast rate of vesicles shrinkage, at the highest mercury concentration exceeding that in the control. Figures 5-21, 5-22, and 5-23 represent stopped flow results of crosslinked and inhibited hAQP1 as well as hAQP1 proteoliposomes and a control sample that is crosslinked and not inhibited.
Fig 5-21. Stopped flow results of PC liposomes (purple) and proteoliposomes (dark blue) shrinking upon hypertonic shock. hAQP1 was reconstituted in PC liposomes (lipid/protein ratio of 10 w/w). Reconstituted hAQP1 was crosslinked with glutaraldehyde and then inhibited by mercury with Hg/AQP molar ratio of 1 (0.83 μM HgCl₂) (orange). Control sample is hAQP1 reconstituted and crosslinked with glutaraldehyde (black curve). hAQP1 inhibited by mercury with Hg/AQP molar ratio of 1 that was not crosslinked is shown in red.
Fig 5-22. Stopped flow results of PC liposomes (purple) and proteoliposomes (dark blue) shrinking upon hypertonic shock. hAQP1 was reconstituted in PC liposomes (lipid/protein ratio of 10 w/w). Reconstituted hAQP1 was crosslinked with glutaraldehyde and then inhibited by mercury with Hg/AQP molar ratio of 10 (18.7 μM HgCl₂) (orange). Control sample is hAQP1 reconstituted and crosslinked with glutaraldehyde (black curve). hAQP1 inhibited by mercury with Hg/AQP molar ratio of 1 that was not crosslinked is shown in red.
Fig 5-23. Stopped flow results of PC liposomes (purple) and proteoliposomes (dark blue) shrinking upon hypertonic shock. hAQP1 was reconstituted in PC liposomes (lipid/protein ratio of 10 w/w). Reconstituted hAQP1 was crosslinked with glutaraldehyde and then inhibited by mercury with Hg/AQP molar ratio of 30 (60 μM HgCl₂) (orange). Control sample is hAQP1 reconstituted and crosslinked with glutaraldehyde (black curve). hAQP1 inhibited by mercury with Hg/AQP molar ratio of 1 that was not crosslinked is shown in red.

Table 5-5 represents fitted time constants of shrinking of crosslinked hAQP1 proteoliposomes with and without mercury and the appropriate non-crosslinked controls. The mercury concentration dependence of shrinking kinetics in the crosslinked sample is dramatically different from that observed for non-crosslinked samples presented above. As discussed above (and shown again in Figures 5-21 to 5-23 for the sake of
comparison), for non-crosslinked samples one observes slower proteoliposome shrinking with increased mercury concentration, which approached the rate of protein-free liposomes at higher concentrations. We showed that the latter may be associated with hAQP1 leaving the membrane, while at low mercury concentration the inhibition is presumably related to the classic mechanism of the pore blockage at Cys189.

In contrast, in the crosslinked samples we observed a complex kinetics, where at lower mercury concentration we see some inhibition which coexists with fast phases of shrinking, while at the highest concentration the shrinking becomes very fast, faster than in the mercury-free sample. We offer a tentative logical explanation to that phenomenon based on the fact that hAQP1 no longer leaves the membrane after being crosslinked. We propose that the slow phase of shrinking observed at low and medium concentration of mercury represents the classic mechanism of the pore blockage at Cys189. On the other hand, accelerated shrinking observed at the highest mercury concentration is a result of mercury-induced conformational change of the monomer, which makes hAQP1 more leaky. It may be the same conformational change which would result in the destruction of the oligomeric state and precipitation of hAQP1 out of the membrane if the samples were not stabilized by the crosslinking.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Time constant, (s)</th>
<th>Amplitude</th>
<th>Error of fitted time constants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crosslinked Proteoliposomes</td>
<td>0.01727, 0.1151</td>
<td>97%, 3%</td>
<td>0.0001, 0.0042</td>
</tr>
<tr>
<td>Crosslinked hAQP1 with Hg/AQP molar ratio of 30</td>
<td>0.005028, 0.0584</td>
<td>91%, 9%</td>
<td>0.000088, 0.0015</td>
</tr>
<tr>
<td>Crosslinked hAQP1 with Hg/AQP molar ratio of 10</td>
<td>0.01974, 0.1589</td>
<td>72%, 28%</td>
<td>0.000084, 0.001</td>
</tr>
<tr>
<td>Crosslinked hAQP1 with Hg/AQP molar ratio of 1</td>
<td>0.01857, 0.1365</td>
<td>82%, 18%</td>
<td>0.000081, 0.0016</td>
</tr>
<tr>
<td>Proteoliposomes</td>
<td>0.01253, 0.1404</td>
<td>98%, 2%</td>
<td>0.00008, 0.00312</td>
</tr>
<tr>
<td>PC liposomes</td>
<td>0.1263</td>
<td></td>
<td>0.0013</td>
</tr>
</tbody>
</table>

Table 5-5. Fitted time constants and amplitudes obtained from stopped flow results of crosslinked hAQP1 inhibited by mercury with Hg/AQP molar ratio of 1, 10 and 30 μM. Results for non-crosslinked hAQP1 and protein-free liposomes are also shown as controls. PC lipid was used to reconstitute hAQP1 with lipid to protein ratio of 10 w/w.
All the experiments described above were repeated three times and the reproducibility of the results was confirmed.

5.10.3 Dynamic Light Scattering (DLS) results for the crosslinked samples

For additional verification of the prevention of mercury-induced morphology changes to proteoliposomes by the crosslinking, we measured diameter and width of size distribution of crosslinked inhibited hAQP1 proteoliposomes through dynamic light scattering. In these DLS results (Table 5-6 and Figures 5-24 to 5-26) we observed narrower width of vesicle size distributions after incubation of samples with mercury chloride, which is exact opposite of the trend, observed for non-crosslinked inhibited hAQP1, where the peaks became broader. The narrower width of size distribution becomes more pronounced at higher mercury concentrations and is consistent with the absence of the white precipitate and stabilization of hAQP1 in the membrane. The exact cause for the increase in uniformity upon treatment with mercury is not clear at this point, but may be related to the conformational change in hAQP1 manifested by its increased leakiness (see above).
<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean Diameter (nm)</th>
<th>Standard deviation or Width (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control sample – non crosslinked hAQP1</td>
<td>80.12</td>
<td>27.46</td>
</tr>
<tr>
<td>Crosslinked hAQP1 inhibited by mercury with Hg/AQP molar ratio of 1</td>
<td>90.06</td>
<td>14.55</td>
</tr>
<tr>
<td>Crosslinked hAQP1 inhibited by mercury with Hg/AQP molar ratio of 10</td>
<td>82.45</td>
<td>10.17</td>
</tr>
<tr>
<td>Crosslinked hAQP1 inhibited by mercury with Hg/AQP molar ratio of 30</td>
<td>68.09</td>
<td>7.27</td>
</tr>
</tbody>
</table>

Table 5-6. Dynamic light scattering results for crosslinked reconstituted hAQP1 (lipid/protein ratio of 10 w/w). Crosslinked samples were incubated with mercury with Hg/AQP molar ratio of 1, 10 and 30 for half an hour prior to measurement.
Fig 5-24. Dynamic light scattering results for crosslinked hAQP1 reconstituted in PC liposomes inhibited with mercury with Hg/AQP molar ratio of 1 (lipid/protein ratio of 10 w/w).

Fig 5-25. Dynamic light scattering results for crosslinked hAQP1 reconstituted in PC liposomes inhibited with Hg/AQP molar ratio of 10 (lipid/protein ratio of 10 w/w).
Fig 5-26. Dynamic light scattering results for crosslinked hAQP1 reconstituted in PC liposomes inhibited with Hg/AQP molar ratio of 30 (lipid/protein ratio of 10 w/w).

The stopped flow and DLS results showed that through using crosslinking, effects of mercury chloride on disruption of hAQP1 lattice and tetramers could be prevented. While the lattice of tetramers is stabilized in its membrane-bound state by crosslinking, the conformational changes of monomers could still occur and be observed through the accelerated shrinking. To verify these hypotheses, we employed two structural methods, FTIR and SAXS, as described below.

5.10.4 FTIR studies of secondary structure and conformational changes of crosslinked inhibited hAQP1

To get further structural insights and to test the hypotheses suggested above FTIR was used to analyze the crosslinked inhibited hAQP1 samples. FTIR absorbance spectra of crosslinked inhibited hAQP1 versus non-crosslinked inhibited hAQP1 are shown in Figures 5-27, 5-28 and 5-29.
As expected from the lack of mercury-induced white precipitate (proven to be hAQP1) in the crosslinked proteoliposomes, we saw the presence of amide I peak in these samples, even for the highest molar ratio of 30 (60 μM HgCl₂). As mentioned above, amide I peak has disappeared in non-crosslinked inhibited samples due to protein precipitation. These results confirm the presence of hAQP1 protein in the mercury-treated lipid bilayer as a result of stabilizing effects of crosslinking on hAQP1. An additional interesting observation is a shift of the Amide I band to lower wavenumbers (from 1662 to 1649 cm⁻¹) upon mercury treatment, suggesting a decrease in the alpha-helical content. This is consistent with our conclusions from the stopped flow experiments on the mercury-induced conformational changes in the monomers present even in the crosslinker-stabilized lattice of tetramers.
Fig 5-27. FTIR absorbance spectra of a dry film of reconstituted hAQP1 in PC liposomes (blue) with lipid/protein ratio of 10 (w/w), crosslinked hAQP1 inhibited by mercury with Hg/AQP molar ratio of 1 (orange) and non-crosslinked hAQP1 inhibited by mercury with Hg/AQP molar ratio of 1 (red). Spectra were normalized based on their lipid esters peak and baseline corrected by subtracting a linear function.
Fig 5-28. FTIR absorbance spectra of a dry film of reconstituted hAQP1 in PC liposomes (blue) with lipid/protein ratio of 10 (w/w), crosslinked hAQP1 inhibited by mercury with Hg/AQP molar ratio of 10 (orange) and non-crosslinked hAQP1 inhibited by mercury with Hg/AQP molar ratio of 10 (red). Spectra were normalized based on their lipid esters peak and baseline corrected by subtracting a linear function.
Fig 5-29. FTIR absorbance spectra of a dry film of reconstituted hAQP1 in PC liposomes (blue) with lipid/protein ratio of 10 (w/w), crosslinked hAQP1 inhibited by mercury with Hg/AQP molar ratio of 30 (orange) and non-crosslinked hAQP1 inhibited by mercury with Hg/AQP molar ratio of 30 (red). Spectra were normalized based on their lipid esters peak and baseline corrected by subtracting a linear function.

5.11 Small-angle X-ray scattering

To confirm the proposed effects of mercury on hAQP1 crystallinity, we used small-angle X-ray scattering (SAXS), which can report on the periodical structures in the sample via Bragg peaks.

SDS-PAGE of the crosslinked untreated samples along with earlier EM work [33] suggested the existence of a lattice of tetramers in our proteoliposomes, which was analyzed first. Indeed, for the untreated hAQP1 proteoliposomes (lipid to protein 10 w/w)
lattice formation was observed in the sample (Figure 5-30). There are three peaks in this spectrum with a position 0.081, 0.115, and 0.162 Å⁻¹, respectively. This could be interpreted as a tetragonal lattice with lattice parameter around 7.8 nm. In accordance with our hypothesis, this periodic structure was eliminated by the treatment with 60 μM mercury chloride (Figures 5-30 and 5-31).
Fig 5-30. SAXS results for untreated hAQP1 proteoliposomes (lipid to protein 10 (w/w), reconstitution in PC lipid, black). There are three peaks in this spectrum with a position 0.081, 0.115 and 0.162 Å⁻¹, respectively. This can be interpreted as a tetragonal lattice with lattice parameter around 7.8 nm. The periodicity disappears after the mercury treatment Hg/AQP molar ratio of 30 (60 µM, blue).
For hAQP1 inhibited at Hg/AQP molar ratio of 1 (0.83 μM HgCl$_2$) and cross-linked inhibited sample at Hg/AQP molar ratio of 30 (60 μM HgCl$_2$), the scattering plots looked very similar to those of the non-inhibited hAQP1 sample. They have three peaks and could be considered as having a tetragonal lattice structure as well (Figure 5-31). This is consistent with the lack of the white precipitate at low mercury concentration and in the crosslinked samples, as well as with SDS-PAGE results at low mercury concentration. Thus, it appears that both of these samples preserve the lattice of tetramers, consistent with our hypothesis.

![SAXS results for hAQP1 inhibited at Hg/AQP molar ratio of 1 and 30 (0.83 μM, blue, 60 μM HgCl$_2$, red) and cross-linked hAQP1 inhibited at Hg/AQP molar ratio of 30 (60 μM HgCl$_2$, black) (lipid to protein ratio 10 w/w, reconstituted in PC lipid).](image-url)

Fig 5-31. SAXS results for hAQP1 inhibited at Hg/AQP molar ratio of 1 and 30 (0.83 μM, blue, 60 μM HgCl$_2$, red) and cross-linked hAQP1 inhibited at Hg/AQP molar ratio of 30 (60 μM HgCl$_2$, black) (lipid to protein ratio 10 w/w, reconstituted in PC lipid).
We also measured SAXS of the white precipitate that was formed after incubation of hAQP1 with mercury at Hg/AQP molar ratio of 30 (Figure 5-32), to see if any crystallinity was preserved in the precipitated hAQP1. In the plot, there were two relatively sharp harmonics (marked by solid arrows) indicative of lipid membrane stacking with a lamellar periodicity of 3.8 nm; there is another sharp scattering (marked by broken arrow) with a lamellar periodicity of 4.3 nm. Nevertheless, no peaks corresponding to in-membrane lattice were observed, leading to the conclusion that precipitated hAQP1 is not crystalline, in agreement with the idea of lattice disruption by mercury.
Fig 5-32. SAXS results for white precipitate formed after incubation of reconstituted hAQP1 with mercury at Hg/AQP molar ratio of 30 (60 μM HgCl₂).

To test if the same kind of mercury-induced lattice disruption occurs at higher protein to lipid ratio used for our ssNMR experiments we measured SAXS for this sample as well and this result is shown in Figure 4-1, chapter 4. The result shows clear lattice formation of hAQP1 reconstituted in the PC/PS liposomes with the protein to lipid ratio of 2 w/w.

As expected from the mercury-induced deterioration of the ssNMR spectral quality, this periodic structure was eliminated by mercury as obvious from SAXS results.
for hAQP1 reconstituted in PC/PS lipid with the protein to lipid ratio of 2 w/w inhibited with 60 μM HgCl₂ (Figure 5-33).

Fig 5-33. SAXS results for hAQP1 reconstituted in the PC/PS liposome (protein to lipid ratio of 2 w/w) inhibited with 60 μM HgCl₂.
To summarize, SAXS results fully confirmed the data of other experiments and ensuing hypotheses regarding the disruption of crystallinity of hAQP1 by mercury and its elimination by crosslinking.

5.12 Functional Assays of hAQP1 with novel non-mercurial blockers

The discovered problems with mercury-induced protein precipitation and large scale conformational changes prompted us to explore other hAQP1 blockers. Based on the studies conducted by Seeliger et al. [34], three novel hAQP1 blockers were identified. These three blockers could inhibit water flux of hAQP1 in *Xenopus laevis* oocytes when applied at micromolar concentrations [34]. It was proposed that these blockers can bind to Lys 36 residue of hAQP1 that is located on the extracellular side close to the arginine/aromatic selectivity filter [34]. The compounds’ names are 3, 3′-(1,3-phenylene) bisacrylic acid (Compound 1), disodium 3-methyl-4-(2-quinolinylmethylene)-2-pentenedioate (compound 2) and N-(1,3-benzodioxol-5-ylmethyl)-N′-2,1,3-benzothiadiazol-5-ylthiourea (compound 3) are the names of the novel compounds introduced as hAQP1 blockers [34]. Chemical structures of these compounds are shown in Figure 5-34.
Fig 5-34. Structures of three novel blockers of hAQP1: a) 3, 3’-(1,3-phenylene) bisacrylic acid (Compound 1), b) disodium 3-methyl-4-(2-quinolinylmethylene)-2-pentenedioate (compound 2), c) N-(1,3-benzodioxol-5-ylmethyl)-N’-2,1,3-benzothiadiazol-5-ylthiourea (compound 3).

Half-maximal inhibitory concentrations (IC$_{50}$) for these compounds were measured for hAQP1 expressed in *Xenopus laevis* oocytes, based on the percentage of water blockage in osmotic swelling experiments [34].
Compound 1’s half-maximal inhibitory concentration (IC$_{50}$) was measured to be 8.1±0.8 µM [34]. Compound 2’s half-maximal inhibitory concentration (IC$_{50}$) was measured to be 17.0±0.5 µM and for compound 3 half-maximal inhibitory concentration (IC$_{50}$) was equal to 17.5±0.5 µM [34]. It should be noted that the results of assays for various hAQP1 inhibitors are not always consistent for oocytes, red blood cells, and proteoliposomes [7, 35–39].

5.12.1 Compound 1

Given all this information, we decided to test these three compounds in vitro via the stopped flow functionality assays of hAQP1 reconstituted in PC/PS liposomes. A control experiment was carried out in parallel with liposomes free of protein. Different concentrations of compound 1 (4, 8, 32 and 64 µM) were used to test the inhibitory effects of this novel compound on reconstituted hAQP1. Stopped flow results of the inhibited hAQP1 with compound 1 are shown in Figure 5-35.
Fig 5-35. Stopped flow results for PC/PS liposomes (dark blue) and proteoliposomes (red) shrinking upon hypertonic shock. hAQP1 reconstituted in PC/PS liposomes (lipid/protein ratio of 10 w/w) was incubated for 30 minutes with 4, 8, 32 and 64 μM concentration of compound 1 (green, purple, light blue and orange).

The kinetics of shrinking suggest efficient inhibition by compound 1 even at the lowest concentration tested. The kinetics of water efflux for the native proteoliposomes and proteoliposomes inhibited with 4, 8 μM and 32 μM of compound 1 could be fitted with a double exponential function showing fast and slow phase. For the proteoliposomes treated with 64 μM of compound 1, the kinetics could be fitted by a single exponential function and only showed a slow phase of vesicles shrinkage (Table 5-7).
<table>
<thead>
<tr>
<th>Sample</th>
<th>Fitted time constant (s)</th>
<th>Amplitude</th>
<th>Error of fitted time constants</th>
</tr>
</thead>
<tbody>
<tr>
<td>hAQP1 proteoliposomes</td>
<td>0.008358</td>
<td>96%</td>
<td>0.000086</td>
</tr>
<tr>
<td></td>
<td>0.05762</td>
<td>4%</td>
<td>0.0026</td>
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<tr>
<td>hAQP1 inhibited by 4 µM compound 1</td>
<td>0.1062</td>
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<td>hAQP1 inhibited by 8 µM compound 1</td>
<td>0.1025</td>
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<td>hAQP1 inhibited by 32 µM compound 1</td>
<td>0.1143</td>
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<td>0.000523</td>
</tr>
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<td>hAQP1 inhibited by 64 µM compound 1</td>
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<tr>
<td>PC/PS liposomes</td>
<td>0.162</td>
<td></td>
<td>0.00105</td>
</tr>
</tbody>
</table>

Table 5-7. Fitted time constants and amplitudes for shrinking of hAQP1 PC/PS liposomes (lipid/protein ratio of 10 w/w) and proteoliposomes inhibited with 4, 8, 32 and 64 µM concentration of compound 1.
Being mindful of the effects of mercury on the proteoliposome morphology described above, dynamic light scattering measurements were done to measure the vesicle size distribution in the presence of compound 1 (Table 5-8).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean Diameter (nm)</th>
<th>Standard deviation or Width (nm)</th>
</tr>
</thead>
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<td>hAQP1 proteoliposomes</td>
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<tr>
<td>hAQP1 inhibited with 4 μM compound 1</td>
<td>79.6</td>
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<td>75.91</td>
<td>15.82</td>
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<td>hAQP1 inhibited with 32 μM compound 1</td>
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<tr>
<td>PC/PS liposomes</td>
<td>80.61</td>
<td>31.68</td>
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</table>

Table 5-8. DLS results for hAQP1 reconstituted in PC/PS liposomes (lipid/protein ratio of 10 w/w) and proteoliposomes inhibited with 4, 8, 32 and 64 μM concentration of compound 1.
Figures 5-36 to 5-41 show DLS results for hAQP1 proteoliposomes inhibited with compound 1.

Fig 5-36. Dynamic light scattering results for hAQP1 reconstituted in PC/PS liposomes (lipid/protein ratio of 10 w/w).

Fig 5-37. Dynamic light scattering results for hAQP1 reconstituted in PC/PS liposomes inhibited with 4 μM compound 1 (lipid/protein ratio of 10 w/w).
Fig 5-38. Dynamic light scattering results for hAQPI reconstituted in PC/PS liposomes inhibited with 8 μM compound 1 (lipid/protein ratio of 10 w/w).

Fig 5-39. Dynamic light scattering results for hAQPI reconstituted in PC/PS liposomes inhibited with 32 μM compound 1 (lipid/protein ratio of 10 w/w).
Fig 5-40. Dynamic light scattering results of hAQP1 reconstituted in PC/PS liposomes inhibited with 64 μM compound 1 (lipid/protein ratio of 10 w/w).

Fig 5-41. Dynamic light scattering results for PC/PS liposomes.

We can clearly see an increase in the width of size distribution and diameter of the vesicles as the concentration of compound 1 increases. Despite a similar pattern of increase in the width of size distribution and diameter of vesicles that we saw in the case of mercury chloride, we did not see any white precipitate as a result of incubation of hAQP1 proteoliposomes with this compound. Also, the size distribution of vesicles shows a more uniform and symmetric shape. Nevertheless, these changes in morphology
are negligible at the lowest concentration of the compound used (4 µM), which at the same time shows the full inhibition of hAQP1. It should be concluded that compound 1 is a good candidate for being a specific inhibitor of hAQP1, free of the problems observed for mercury if used at moderate concentrations.

5.12.2. Compound 2:

Next, we tested compound 2, using stopped flow measurements as described above. Different concentrations of compound 1 (18, 36, 72, and 114 µM) were used to test the inhibitory effects of this novel compound on reconstituted hAQP1. The concentrations of compound 2 were higher because the reported half-maximal inhibitory concentration of compound 2 in oocytes is higher than for compound 1. Stopped flow results of this experiment are shown in Figure 5-42.
Fig 5-42. Stopped flow results for PC/PS liposomes (dark blue) and proteoliposomes (red) shrinking upon hypertonic shock. hAQP1 reconstituted in PC/PS liposomes (lipid/protein ratio of 10 w/w) was incubated with 18, 36, 72, and 114 μM concentration of compound 2 (green, light blue, orange and purple).

The kinetics of water efflux from the native proteoliposomes and inhibited proteoliposomes were analyzed (Table 5-9). The kinetics for proteoliposomes and proteoliposomes inhibited with 18 μM compound 2 could be fitted using double exponential function showing two phases, fast and slow, of vesicles shrinkage (Table 5-9). A clear inhibition of water efflux which saturates at 36 μM can be seen, suggesting that compound 2 is not as efficient as compound 1, which saturated at 4 μM.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Fitted time constant (s)</th>
<th>Amplitude</th>
<th>Error of fitted time constants</th>
</tr>
</thead>
<tbody>
<tr>
<td>hAQP1 proteoliposomes</td>
<td>0.008358</td>
<td>96%</td>
<td>0.000086</td>
</tr>
<tr>
<td></td>
<td>0.05762</td>
<td>4%</td>
<td>0.0026</td>
</tr>
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<td>hAQP1 inhibited with 18 μM Compound 2</td>
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<td>hAQP1 inhibited with 72 μM Compound 2</td>
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<td></td>
<td>0.00038</td>
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<td>hAQP1 inhibited with 114 μM Compound 2</td>
<td>0.1702</td>
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<td>0.00043</td>
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<tr>
<td>PC/PS liposomes</td>
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<td>0.00105</td>
</tr>
</tbody>
</table>

Table 5-9. Fitted time constants and amplitudes for shrinking of hAQP1 PC/PS liposomes (lipid/protein ratio of 10 w/w) and proteoliposomes inhibited with 18, 36, 72 and 114 μM compound 2.
5.12.3 Compound 3:

Finally, stopped flow measurements were performed using different concentrations of compound 3 with the same sucrose concentration and protocol as for the other compounds.

Different concentrations of compound 3 (17, 34 and 68 µM) were used to test the inhibitory effects of this novel compound on the reconstituted hAQP1. Stopped flow results of this experiment are shown in Figure 5-43.
Fig 5-43. Stopped flow results for PC/PS liposomes (dark blue) and proteoliposomes (red) shrinking upon hypertonic shock. hAQP1 reconstituted in PC/PS liposomes (lipid/protein ratio of 10 w/w) was incubated with 17, 34, 68 μM concentration of compound 3 (green, purple, light and orange).

The kinetics of water efflux from native proteoliposomes and inhibited proteoliposomes were analyzed (Table 5-10) and a clear and complete inhibition is observed even at the lowest concentration tested (17 μM). This concentration was chosen to start with because the reported half-maximal inhibitory concentration of compound 3 in oocytes is around 17 μM. This suggests that compound 3 may be somewhat more efficient than compound 2.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Fitted time constant(s)</th>
<th>Amplitude</th>
<th>Error of fitted time constants</th>
</tr>
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<tr>
<td>hAQP1 proteoliposomes</td>
<td>0.008358</td>
<td>96%</td>
<td>0.000086</td>
</tr>
<tr>
<td></td>
<td>0.05762</td>
<td>4%</td>
<td>0.0026</td>
</tr>
<tr>
<td>hAQP1 inhibited with 17 μM Compound 3</td>
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<td>19%</td>
<td>0.00035</td>
</tr>
<tr>
<td></td>
<td>0.163</td>
<td>81%</td>
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<td>hAQP1 inhibited with 34 μM Compound 3</td>
<td>0.1579</td>
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<td>0.2104</td>
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<td>0.00028</td>
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<tr>
<td>PC/PS liposomes</td>
<td>0.162</td>
<td></td>
<td>0.00105</td>
</tr>
</tbody>
</table>

Table 5-10. Fitted time constants and amplitudes for shrinking of hAQP1 PC/PS liposomes (lipid/protein ratio of 10 w/w) and inhibited proteoliposomes with 17, 34, and 68 μM compound 3.

Further slowing of shrinking was observed between 34 μM and 68 μM concentrations of compound 3, where after addition of 68 μM compound 3, the rate of vesicle shrinkage became even slower than for protein-free liposomes. This might be related to effects of high concentration of compound 3 on lipids and ensuing proteoliposome morphology, which should be investigated further.
5.12 Conclusions

Mercury chloride is a traditional blocker of hAQP1 and many other aquaporins, and it is used for functionality assays of these proteins. It was found by Preston et al. that mercury could bind to Cys 189 in hAQP1 and inhibit its function [2]. Previous and existing assays use relatively high mercury chloride concentrations, such as 0.1 mM [10], 0.3 mM [40], 1 mM [12] for proteoliposomes or 3 mM HgCl$_2$ for oocytes [2]. Our results presented above show that such high concentrations of mercury chloride may create artifacts related to protein denaturation and precipitation rather than track the specific interaction of mercury with hAQP1.

While we have not detected any significant effects on hAQP1 structure at lower mercury concentrations, we can conclude that upon addition of higher molar ratios of Hg to AQP, such as 10 and 30 (18.7 µM and 60 µM HgCl$_2$ concentrations), hAQP1 can leave the membrane and precipitate. Effects of mercury chloride at lower concentrations could be still discussed as a covalent binding of mercury to Cys 189 and inhibition of its function. However, at higher molar ratio of Hg/AQP such as 10 or 30, specific inhibition of hAQP1 is not the only effect of mercury.

In view of this, returning to the stopped flow measurements, we conclude that when we have higher concentrations of mercury chloride, we are not specifically measuring the inhibition of hAQP1 but are just looking at protein-free liposomes shrinkage due to their exposure to high sucrose gradient. Accordingly, in our FTIR and SDS-PAGE results we confirmed that the white precipitate is hAQP1. Thus, hAQP1
could fall out of the membrane due to the conformational changes of monomers and ensuing lattice and tetramer distortions induced by higher concentration of mercury, presumably upon its binding to some residues other than Cys189.

In support of our conclusion we found the literature evidence stating that some of the helices in hAQP1 are only marginally hydrophobic and can leave the membrane during the folding process. Based on studies by Virkki et al [41], there is a possibility for helix 3 in hAQP1 to come out of the membrane and then bring the preceding loop back into the membrane. Further, helix 2 is very hydrophilic and it is only hydrophobic in regions closer to helix 3 in hAQP1. Based on the hydrophobicity of the helix 2 in hAQP1, it is concluded that helix 2 along with helix 3 and helix 4 could leave the membrane [41]. Thus, monomers are not very stable in the membrane by themselves and there should be an interaction between them to form a stable tetrameric structure in the membrane.

In another relevant study by Buck et al. [42], it was shown that helix 2 of one monomer could interact with helix 5 of another monomer and this interaction is very important for stable tetramer structures of hAQP1. It was also shown that Asn 49 from helix 2 is involved in hydrogen bonding interaction inside the monomer with Asp185 from helix 5 and this interaction helps in stabilization and proper folding of the AQP1 monomer. On the other hand, Lys 51 could also interact with Asp185 from another AQP1 monomer and help in stabilization of AQP tetramer though this interaction [42]. Upon this interaction, hydrophilic helix 2 stabilizes itself via its interaction with helix 5 [42]. Thus, inter- and intra-monomer contacts between helices are also very vital for stability of hAQP1 monomers [42].

We propose that precipitation of hAQP1 at higher mercury concentration could be
related to disruption of inter- and intra-monomer contacts as a result of binding of mercury to residues other than Cys 189. One possible residue is Asp 185 that is known to interact with Loop A in the same monomer (Asn 49), and Lys 51 in another monomer [42]. For mercury chloride to interact with Asp 185 a higher molar excess than in the case of Cys is likely needed. If the interaction of mercury with Asp 185 disrupts the inter- and intra-monomer interactions, it can destabilize hAQP1 tetramers and cause them to leave the membrane. On the other hand, our SDS-PAGE gel results showed that white sediment contains some oligomers. This result suggests that a whole hAQP1 tetramer could leave the membrane upon the addition of high concentrations of mercury chloride. The idea of mercury-induced lattice destabilization is supported by our results on the crosslinked samples, which demonstrate that stabilization of the hAQP1 lattice of tetramers by glutaraldehyde prevents protein precipitation and preserves 2D crystallinity of the samples as measured by SAXS. Still, stopped-flow and FTIR results on these samples suggested that mercury-induced conformational changes of monomers persisted even in the crosslinked samples.

In summary, an important conclusion from our results is that mercury chloride may not be an appropriate choice for hAQP1 functionality assays and other alternatives should be considered. Novel blockers that were identified by Seeliger et al. [34] seem to be one of the alternatives, and we showed the inhibitory effects of compound 1 as one of these novel blockers. On one hand, the fact of not having any precipitation upon incubation of hAQP1 proteoliposomes with higher concentrations of these three compounds indicates that we might not have the same problem we encountered in the
case of higher mercury chloride concentrations. On the other hand, these compounds may also have some additional effects on lipids which should be investigated further.
References


Chapter 6
Concluding remarks and future goals
6.1 Concluding remarks

This study explored the structure and function of human Aquaporin-1 in lipids. hAQP1 is an important protein that functions as a water transporter in the cell membranes of many tissues in the human body. The overproduction or malfunction of this protein was shown to be associated with many human diseases. In this work, hAQP1 was studied by different biophysical techniques, including structural methods such as solid-state NMR (ssNMR, the main technique in this work), small angle X-ray scattering (SAXS), and Fourier transform infrared spectroscopy (FTIR). The functional assays for water transport activity were done by stopped flow experiments and aided by dynamic light scattering (DLS).

The first challenge for ssNMR studies of hAQP1 was to obtain a homogeneous isotopically labeled sample in sufficient quantities, which would yield high spectral resolution suitable for the structural studies. We developed a protocol for the production of doubly ($^{13}$C/$^{15}$N) isotopically labeled hAQP1 using methylotrophic yeast Pichia pastoris as an expression system. Previously, the expression of the hAQP1 in P. pastoris was shown to produce exceptionally high yields and we modified and extended this protocol for isotope labeling in shake flasks [1, 2]. The purified hAQP1 was reconstituted in PC/PS lipid and SAXS measurements confirmed the presence of 2D crystals in the sample. The highly ordered 2D crystal hAQP1 sample yielded excellent ssNMR resolution. The obtained one-dimensional and two-dimensional spectra had a very good dispersion with the line widths of ~ 0.5 ppm for carbon and for nitrogen. Through the obtained 2D NMR spectra, many of the amino acids with short side-chains were
identified. Additionally, a large number of amino acids were identified using 3D NMR experiments.

The high level of chemical shift assignments was achieved through the three-dimensional NMR experiments including NCACX, NCOCX and CANCO. By using these experiments we were able to assign resonances for 192 out of 269 amino acids of hAQP1. The secondary structure of hAQP1 in the membrane was derived from the chemical shifts obtained in the ssNMR experiments and compared with the known structures obtained through X-ray and EM techniques [3–7]. While the secondary structure that was derived from the NMR data was mostly consistent with the known structures, there were some differences in the loop regions and in the helical boundaries. These discrepancies may be related to the following facts: the conformational changes of the protein in 3D crystals, the cryo-temperatures that can affect the dynamic motions of the protein, and/or the low resolution of the EM data.

Specifically, the secondary structure and dynamics of hAQP1 obtained from our ssNMR data revealed the presence of some elements of β-structure in loops A and D, and a highly structured nature of the long loop C, which is believed to be functionally important as it may interact with the ar/R filter of the channel. The presence of the cis conformation for the Y37-P38 peptide bond in the loop A of hAQP1 was another new finding from the CSI results of ssNMR experiments.

The ssNMR results also allowed for probing site-specific water accessibility and hydrogen-bonding of the side chain and backbone atoms. As demonstrated by our study, the exchangeable backbone amide or side chain nitrogens that participate in the hydrogen bonding interactions with the water molecules and are not protected by the intraprotein
strong hydrogen bonding can be detected through the site-specific H/D exchange experiments. The H/D exchange results showed that most of the residues located in TM helices are not exchangeable, surprisingly, including some residues in the water channel. Interestingly, we found that Arg195 in the ar/R filter has two exchangeable and one non-exchangeable side-chain nitrogens. This might be related to the involvement of the non-exchangeable side-chain nitrogen of Arg195 in the interaction with loop C residues, either backbone carbonyl of Gly125 of bAQP1 [3] or Asn127 and Asp128 sidechains [4]. The conserved Asn residues in the NPA motif showed no exchangeability of the side chain nitrogen atoms of Asn76 and very slow time-scale exchange for Asn192 residue. These results may be rationalized assuming that the two Asn residues participate in hydrogen bonding interaction with the oxygen atom of the water molecules through their amide protons and that water does not dissociate in the channel, consistent with the lack of proton conductance of hAQP1.

An attempt to study interaction of hAQP1 with mercury by ssNMR faced an unexpected problem, as the spectral quality has dramatically deteriorated, in contrary to what could be expected from the simple pore blocking due to mercury binding by Cys189. The reasons for this behavior were investigated using several alternative techniques.

As expected, the stopped-flow results on the mercury-inhibited hAQP1 showed a slower rate of the vesicles shrinkage, indicating lower or no activity of the protein at the higher concentrations of mercury. At the same time, the DLS results reported on the changes in the size distribution of the hAQP1-bearing vesicles upon the mercury treatment, suggesting strong aggregation. This was in line with the observation of white
sediment appearing during the incubation of hAQP1 proteoliposomes with mercury chloride. FTIR measurements and SDS PAGE confirmed that the precipitate is hAQP1, suggesting that it can leave the lipid membrane due to mercury-induced conformational changes. SAXS measurements confirmed that the 2D crystallinity observed in the intact samples disappears in the inhibited samples. Stabilization of the hAQP1 2D crystal lattice by glutaraldehyde cross-linking (verified by SAXS and SDS-PAGE) lead to elimination of the precipitation of hAQP1 by mercury chloride. At the same time, mercury-induced changes in water conductance, previously obscured by protein precipitation, could be observed. Thus, these results suggest that mercury chloride at higher concentrations destroys the lattice of hAQP1 tetramers and destabilizes its membrane-bound form, providing an alternative mechanism for mercury inhibition of hAQP1.

Finally, we showed that several novel blockers of hAQP1 previously tested in oocytes are effective on the purified protein and do not suffer from the artifacts observed for mercury, which makes them more acceptable as potential therapeutic agents.

6.2 Future directions

6.2.1 NMR studies of hAQP1

One of the important directions for future research is to determine the atomic resolution structure of hAQP1 by ssNMR. The general strategy used in structural analysis of hAQP1 can be divided into following main stages: resonance assignment, identification of secondary structure elements and torsion angles, measurements of the
distance restraints, and, finally calculation of the structure. The process may be complicated by close intermonomer contacts, which will have to be carefully separated from the intramonomer restraints.

The resonance assignments and the secondary structure of hAQP1 that were obtained through our 3D ssNMR experiments have already provided important information that is necessary for tertiary structure determination. The dihedral angle constraints derived from the chemical shift analysis by TALOS+ program is one of the prerequisites in determining the 3D structure of the protein. At the same time, the internuclear distances will have to be measured for determining the tertiary fold of the protein [8]. For this purpose, the dipolar assisted rotational resonance (DARR) and proton-driven spin diffusion (PDSD) techniques can be used in order to obtain information about the interresidue constraints in hAQP1 [9]. But, to gain enough resolution in these measurements the sparsely labeled hAQP1 may be required, which is not a straightforward task, even though its protocol is under development. These experiments will hopefully provide medium and long-range distance restraints, identifying the interhelical contacts of the protein and thus, define its tertiary structure.

6.2.2 Inhibition studies of hAQP1

The destruction of the crystalline lattice of hAQP1 tetramers that occurs upon its inhibition by mercury chloride needs to be further investigated by the biochemical experiments. One aspect of this study should be focused on the point mutations of amino acids that could possibly interact with mercury (i.e., Asp 185, which is involved in the
intermonomer contacts) at higher molar excess. In this kind of experiment, one can probe the mercury resistance in these mutants, being mindful of other effects the mutations could introduce. The time-dependence of the inhibition of hAQP1 by mercury chloride also has to be investigated because of the observed slow time scale of the effects of mercury. Through the stopped-flow kinetic experiments as well as FTIR spectroscopy, one could follow the time-dependency of this inhibition.

Identifying and characterizing novel blockers of hAQP1 is another future direction. Using our resonance assignments, ssNMR can provide information on the binding sites and mechanism of inhibition of these novel blockers of hAQP1. Specifically, ssNMR experiments will allow mapping the interacting atoms through the chemical shift perturbations that will be induced by interaction of the novel blockers with hAQP1. In principle, the structural model of the ligand-bound hAQP1 could also be obtained by measuring specific protein-ligand distances through the dipolar assisted rotational resonance (DARR) and proton-driven spin diffusion (PDSD) techniques and, in the case of large conformational changes, repeating the structure determination routine described above. Finally, the dynamics of the protein-ligand interaction is of great interest as well. The dynamic motions of the protein and how they depend on interaction with drugs can be probed by the measurements of the N-H and C-H couplings and measurements of site-specific relaxation rates [10–12]. As a result, a rational search for better inhibitors for hAQP1 will be enabled. Therefore, through these approaches, different diseases related to the overproduction of hAQP1 may possibly be cured or show decreased symptoms. Thus, these experimental techniques and the results may be useful for pharmaceutical companies that are seeking to find suitable drugs for hAQP1.
Reference


## Appendix 1.1

Chemical shift assignments table for 192 assigned amino acids of hAQP1

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