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Minireview

Aquaporin water channels: molecular mechanisms for human diseases[1]

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Abstract **Although water is the major component of all biological fluids, the molecular pathways for water transport across cell membranes eluded identification until the discovery of the aquaporin family of water channels. The atomic structure of mammalian AQP1 illustrates how this family of proteins is freely permeated by water but not protons (hydronium ions, H_3O^+). Definition of the subcellular sites of expression predicted their physiological functions and potential clinical disorders. Analysis of several human disease states has confirmed that aquaporins are involved in multiple different illnesses including abnormalities of kidney function, loss of vision, onset of brain edema, starvation, and arsenic toxicity.**
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Key words: Aquaporin; Membrane water channel; Structure and function; Human disease

1. Background

Water is the major component of all human cells and tissues, and the same is true for all other vertebrates, invertebrates, unicellular organisms, and plants. The plasma membrane is the major barrier to the movement of water between cells, but identification of the molecular pathways by which water is absorbed and released from cells remained unknown until long after most classes of membrane transport proteins. This is surprising, since the phenomenon of cell membrane water permeability had been debated for decades by physiologists and biophysicists. It was agreed by most scientists that water passes through biological membranes by simple diffusion through the lipid bilayer.

Based upon indirect observations, a small number of scientists argued that specialized water-selective pores are necessary to explain the high water permeability of red blood cells and renal tubules [1]. Moreover, the water permeability of these tissues could be reversibly inhibited by mercuric ions [2], and the activation energy was similar to diffusion of water in bulk solution, ~5 kcal/mol. In addition, specialized tissues such as mammalian collecting duct or amphibian bladder were known to exhibit fluctuations in water transport regulated by the antidiuretic hormone, vasopressin. Nevertheless, water channel proponents were unable to convince skeptics, since all attempts to isolate or clone molecular water channel proteins had failed, and no mechanism explained the passage of water (H_2O) without passage of protons (H_3O^+, hydronium ions).

2. Discovery of aquaporins

The discovery of a 28 kDa integral membrane protein in red cells and renal tubules [3] ended the controversies about the possible existence of molecular water channels. The protein now known as AQP1 was first purified from red cell membranes and found to exist as a tetramer with intracellular N- and C-termini – an organization similar to several ion channel proteins [4]. The primary sequence of the cDNA revealed two tandem repeats each containing three bilayer-spanning α-helices [5]. The loops connecting the second and third transmembrane segments in each repeat contained several highly conserved residues and the signature motif, asparagine-proline-alanine (NPA). The genetics database at that time included a few homologous proteins from a curious assortment of sources including bovine lens, *Drosophila* brain, bacteria, and plants, but their physiological functions were largely unknown, and the possibility of water transport had apparently not been considered.

Based upon its distribution in highly water-permeable tissues and the existence of sequence-related proteins in plants, the possibility that the 28 kDa protein may confer water permeability was tested by expression in *Xenopus laevis* oocytes which normally have low water permeability. When transferred to hypoosmotic buffer, the cRNA-injected oocytes were found to have markedly increased water permeability, causing them to swell and burst [6]. The water permeability was reversibly inhibited by Hg^{2+}, exhibited low activation energy, and was not accompanied by membrane currents. In addition, the purified red cell 28 kDa protein was reconstituted into lipid vesicles which exhibited the same properties. The unit water permeability was found to be extremely high, $\sim 3\times10^9$ water molecules per subunit per second, while transport of other solutes or ions (including protons) was negligible [7,8]. Notably, the studies in oocytes and reconstituted membranes both confirmed that simple membranes exhibit finite water permeability, but the water permeability of membranes containing AQP1 are up to 100 times greater. Both systems demonstrated that the movement of water represents facilitated diffusion with the driving force determined by osmotic gradients. Thus, both sides in the longstanding debate over transport of water through membranes by simple diffusion or

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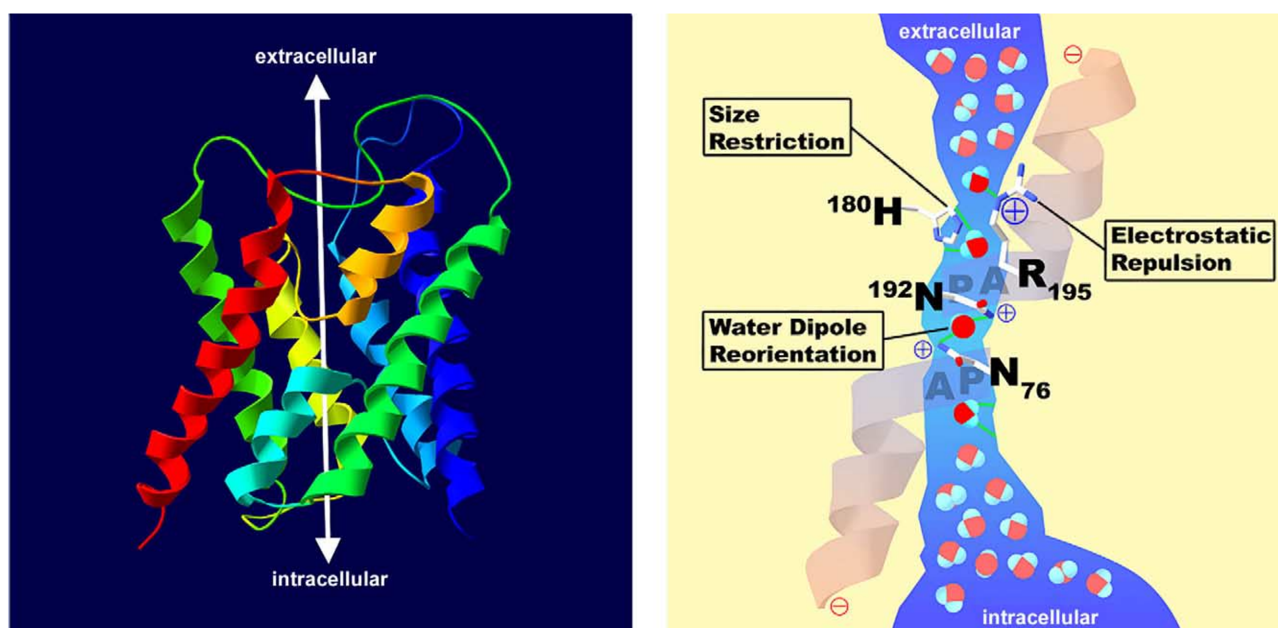

Fig. 1. Structure of AQP1 subunit and schematic of water transport. Left panel: Ribbon model of AQP1 monomer showing six tilted bilayer-spanning domains and two pore-forming loops with short transmembrane α-helices entering the membrane from the extracellular surface (orange) and intracellular surface (cyan). Right panel: Schematic diagram representing channel pore in same orientation. Flow of water from extracellular to intracellular chambers occurs through the narrow column. Proton conduction (hydronium ion, H_3O^+) is prevented at the narrowest segment 8 Å above the channel center by size restriction and electrostatic repulsion (H180 and R195). A second barrier to proton conduction exists at the center of the channel where partial positive dipoles contributed by the short pore-lining α-helices and the two highly conserved asparagines (N76 and N192) in the signature NPA motif cause a transient dipole reorientation of an isolated water molecule. Reproduced with permission from [59].

by hypothetical water channels were partially correct. AQP1 is now widely known as the first recognized molecular water channel, and an out-pouring of research followed from many laboratories.

3. The hourglass form and water transport function

Under different physiologic stimuli, water may be taken up or released by cells, so the molecular architecture for a water-selective transporter must explain bi-directionality. The mercury sensitivity of water transport indicates the existence of a free sulfhydryl within the water channel protein that is blocked by reaction with an Hg^{2+} ion and restored by treatment with reducing agents. The primary amino acid sequence of AQP1 contains four cysteines, but only one (C189) confers mercury sensitivity, suggesting that it may reside within the aqueous pore [9]. The predicted topology of the two tandem repeats first indicated that two NPA-containing loops in an AQP1 subunit reside on the opposite surfaces of the lipid bilayer [4]. Site-directed mutagenesis studies further refined the topology [10], leading to the 'hourglass model', a pseudo two-fold symmetrical structure with the six bilayer-spanning α-helices surrounding the aqueous pore formed from the two NPA-containing loops that enter the bilayer from the opposite surfaces and overlap at the junction of the two NPA motifs [11].

The structure of AQP1 was established by reconstitution of purified protein at high concentrations into synthetic lipid bilayers. The resulting symmetrical arrays of protein are referred to as membrane crystals. Importantly, this approach permitted the functional demonstration that the water permeability was entirely preserved, indicating that the deduced structure represents the physiological state of the protein [12]. The membrane crystals were analyzed by atomic force microscopy as well as electron microscopy. A series of studies by our colleagues and two other groups of investigators led to the same general conclusion supporting the AQP1 monomer hourglass [13–15]. Analysis of membrane crystals by cryoelectron microscopy at ultra-low temperatures yielded the important observation that NPA loops in the center of the membrane are followed by short α-helices that do not span the membrane [16]. An advanced structural model of AQP1 was constructed from a refined dataset at 3.8 Å combined with information derived from mutagenesis studies [17]. An almost simultaneous report by Stroud and colleagues [18] described the atomic structure of the glycerol facilitator (GlpF), a sequence-related protein from *Escherichia coli* solved by X-ray analysis of three-dimensional protein crystals. Modeling the human AQP1 membrane crystallographic information with the GlpF coordinates provided the atomic model for a water channel [19]. The protein is a tetramer formed of four AQP1 monomers, each containing six tilted, bilayer-spanning α-helices surrounding the two NPA-containing loops which enter the membrane from the opposite surfaces and are juxtaposed in the center (Fig. 1, left panel). The atomic structure of bovine AQP1 was independently determined by X-ray analysis [20]. Molecular dynamics simulations of water transport by AQP1 by two different groups [21,22] have led to an advanced understanding of how water can be rapidly transported across membranes while protons (H_3O^+) are repelled.

Columns of water molecules joined by hydrogen bonds are known to permit exceedingly rapid conduction of protons, referred to as the Grotthus effect, analogous to the conduction of electrons through a copper wire [23]. Thus, very specialized

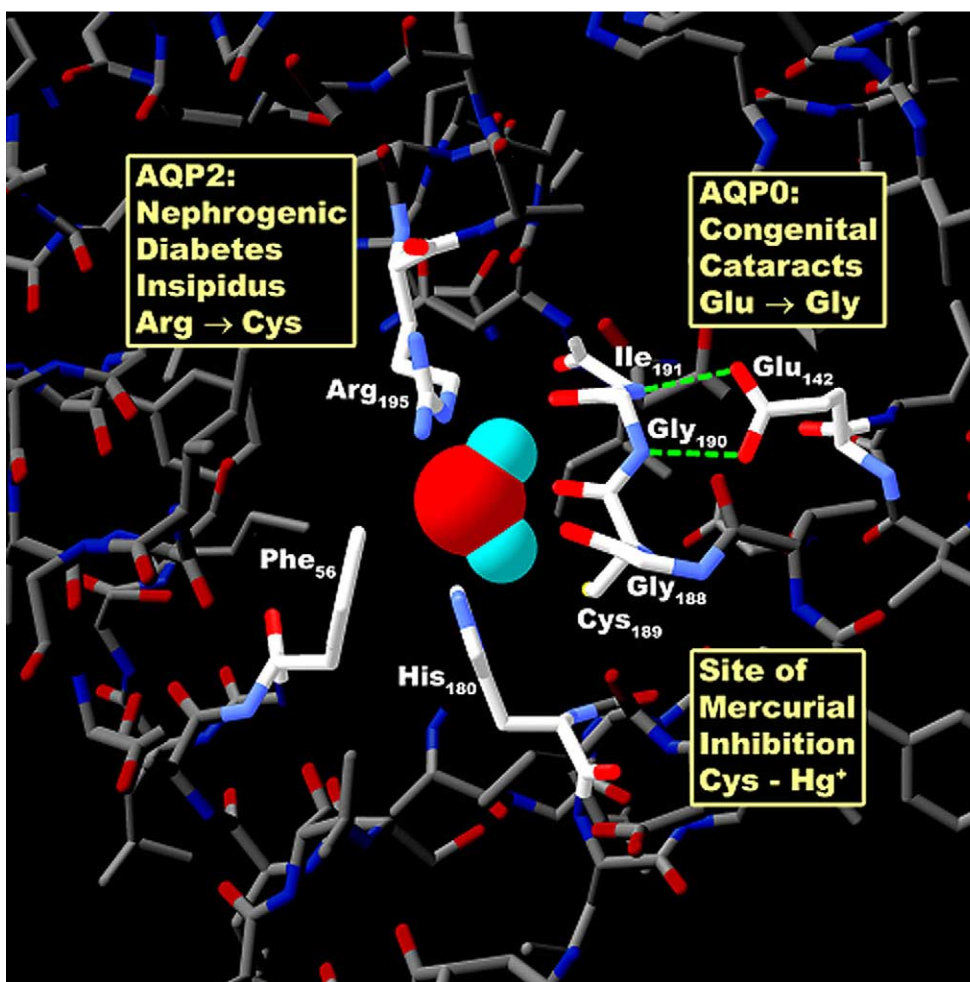

Fig. 2. Atomic model of AQP1 subunit in horizontal cross-section. A single water molecule at the narrowest segment of the channel surrounded by functionally important residues (F56, R195, H180, and C189). Hydrogen bonding occurs between carbonyl oxygens on the peptide backbone (G188, C189, G190, and I191) at the other surface of the pore. The site of mercurial inhibition (C189) and clinically important residues in corresponding residues in other aquaporins (AQP2 and AQP0) are denoted. Reproduced with permission from [59].

molecular architecture is needed to permit the passage of water but not protons. Hydrogen bonding causes water to be a liquid and permits rapid exchange of protons between adjacent water molecules in bulk solution. The hourglass structure of AQP1 has cone-shaped extracellular and intracellular vestibules that are filled with water. Separating the vestibules is a narrow channel 20 Å in length through which the water molecules must pass in single file without hydrogen bonding to each other.

The narrowest segment of the channel occurs ∼8 Å above the channel center where entry of molecules is controlled by size restriction and electrostatic repulsion (shown in longitudinal section in Fig. 1, right panel). At this level, the 2.8 Å channel diameter is similar to the van der Waals diameter of a single water molecule (shown in cross-section in Fig. 2). Most of the residues lining the 20 Å channel are hydrophobic and are contributed by the transmembrane α-helices, and at this level, one wall of the pore is formed by a phenylalanine (F56) contributed by the second transmembrane α-helix. The adjacent surface of the pore is the side chain of the arginine (R195) that follows the second NPA motif (Fig. 1, right panel, and Fig. 2). This arginine bears a fixed positive charge and is conserved among aquaporin homologs from nearly all forms of life. A histidine (H180) faces the pore from the opposite wall of the channel. This histidine is conserved in all water-transporting members of the aquaporin gene family. At neutral pH, H180 is predicted to bear a partial positive charge. Thus, R195 and H180 provide a size restriction, for molecules larger than water, and fixed positive charges to repel protons and other cations. In addition, a string of four carbonyl oxygens of the peptide backbone (G188, C189, G190, and I191) line the pore, serving as energetically favorable substitutes for hydrogen bonding (Fig. 2).

The mercury sensitivity of AQP1 is well explained by location of the specific residue (C189) at the narrowest segment of the channel at the same level as H180 and R195 (Fig. 2). The orientation of the sulfhydryl side chain at a channel-lining position explains why the residue is blocked by Hg^{2+} ions. Cysteines are present at the corresponding position in several other members of the aquaporin family (AQP2, AQP5, AQP6, and AQP9). Prior to the introduction of modern loop diuretics, patients with refractory fluid overload were treated with calomel (mercurous chloride oxidizes to mercuric chloride) which delivers profound renal diuresis. Interestingly, inhibition of AQP1 is very specific for Hg^{2+} and a few organomercurials such as PCMB, whereas larger sulfhydryl agents,

such as *N*-ethylmaleimide, presumably fail to inhibit due to size constraints at this site.

Halfway down the 20 Å channel, the two signature NPA motifs are juxtaposed by van der Waals associations with each other in a pseudo two-fold axis of symmetry (Fig. 1, right panel). The dipoles caused by the two short α-helices facing each other result in partial positive charges surrounding the conserved asparagines. An isolated water molecule is believed to transiently form partial hydrogen bonds with both asparagines, thus undergoing a temporary dipole reorientation of the water molecule. After further descent through the channel, an isolated water molecule then interacts with a second string of carbonyl oxygens (L75, H74, A73, G72) on the peptide backbone as they exit the channel into the cytoplasmic vestibule. The presence of relatively few sites where water can interact with the walls of the pore contributes to the rapid speed for the water movement.

These predictions were verified and further delineated by molecular dynamics simulations [21,22]. Interestingly, the orientation of a water molecule changes as it passes through the channel. The oxygen faces down when the water molecule enters from the extracellular side. The water molecule flips when interacting with both asparagines of the NPA motifs, and moves further down the channel with the oxygen facing upwards (Fig. 1, right panel). This description is written from the perspective of water entering a cell through an aquaporin. The same molecular events will occur in the reverse order as water is released from a cell through an aquaporin. This reverse flow is equivalently important. In many physiological processes water may enter cells or be released from cells. In the case of polarized epithelia, water enters at one surface of the cell and is released from the other surface.

4. The aquaporin family of membrane channels

After AQP1 was demonstrated to be a molecular water channel, a flurry of homology cloning efforts by multiple laboratories led to the identification of sequence-related proteins in nearly all forms of life. Eleven mammalian aquaporins have now been identified and at least partially characterized (Fig. 3). These conform to two subsets of proteins – those selectively permeated by water (classic aquaporins) and those permeated by water plus glycerol (aquaglyceroporins). This is an oversimplification, since AQP0 and AQP6 have been found to exhibit biophysical functions different from water or glycerol transport. *E. coli* has one of each (AqpZ and GlpF). The atomic structure of the aquaglyceroporin GlpF has been solved [18], and transport of glycerol through the channel has been simulated by molecular dynamics [22]. The detailed mechanism by which glycerol is transported by aquaglyceroporins will be presented in the minireview in this issue by R. Stroud.

In many cases, the sites of expression predict physiological and pathological roles. The mammalian homologs are expressed in specific tissues, but it should be noted that aquaporins are not expressed in all cell types. For example, no aquaporin has been identified in neurons. Moreover, the developmental expression of aquaporins is often complex. For example, AQP1 is expressed in rat renal tubules only after birth but not before [24]. In contrast, AQP3 and AQP7 are expressed in immature dendritic cells but not mature cells [25]. In cells expressing more than one aquaporin, the different homologs usually reside in different membranes. For example, in salivary glands, AQP3 is present in basolateral membranes where water is taken up from the interstitium, and AQP5 resides in the apical membrane where water is released during salivation [26].

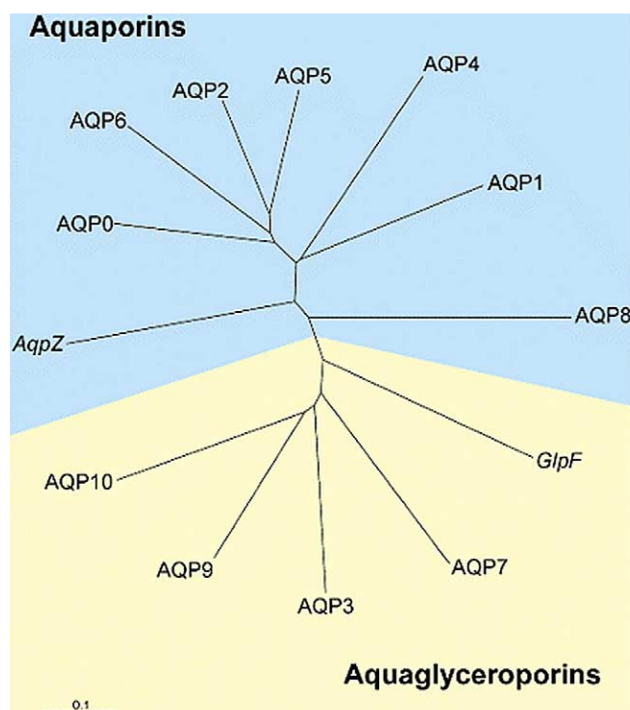

Fig. 3. Human aquaporin gene family and *E. coli* homologs. Subsets correspond to classic aquaporins and aquaglyceroporins. Represented (blue) are cDNA sequences encoding homologs with demonstrated high water permeability (AQP1, AQP2, AQP4, AQP5 and the *E. coli* AqpZ) and closely related sequences with other potential functions (AQP0 and AQP6). Also represented (yellow) are aquaglyceroporins with glycerol and water permeability (AQP3, AQP7, AQP9, AQP10 and the *E. coli* GlpF). Reproduced with permission from [60].

5. Molecular mechanisms for human diseases

Aquaporins have been identified in numerous tissues, and the importance of these proteins in several disease states is becoming clear. In some instances, the structure of the proteins provides detailed understanding into the molecular mechanisms for protein dysfunction. In other instances, the subcellular localization of the protein provides obvious clues. Examples of each are illustrated below.

AQP1 is extremely abundant in both apical and basolateral membranes of renal proximal tubules and in capillary endothelium [27]. A major role of the kidney is to concentrate glomerular filtrate (∼180 l/day) to the final urine (∼1 l/day). AQP1 in the renal proximal tubule and descending thin limb of Henle has been proposed to contribute to the counter-current mechanism for renal concentration. Exceedingly rare humans lack the Co blood group antigen, a specific extracellular domain of AQP1 protein [28]. Nonsense mutations have been identified in two AQP1-null individuals. In the third, a structurally severe missense mutation was identified in which a leucine has replaced the proline that normally caps the end of the first transmembrane α-helix, reducing protein expres-

sion by 99% [29]. In the unstressed state, the AQP1-null individuals were not clinically affected. However, in a carefully supervised hospital-based study, AQP1-null individuals could only concentrate their urine to ~450 mOsm after fluid deprivation for 24 h [30]. This is a significant defect, since normal individuals can concentrate their urine to >1000 mOsm and do so every night when deprived of fluid during sleep. Capillary water permeability was measured in a second study. The AQP1-null individuals exhibited a markedly decreased fluid release from blood vessels, a process needed for the function of sweat glands and other water-transporting epithelia [31].

The final stages of urinary concentration occur in the renal collecting ducts. In the diuretic state, AQP2 resides in intracellular vesicles in principal cells where the location prevents reabsorption of water from the glomerular filtrate [32,33]. This is well recognized in our everyday lives, since intake of excessive fluid or inhibition of vasopressin release from the central nervous system by alcohol or caffeine causes our kidneys to secrete large volumes of dilute urine. Individuals with mutations in the gene encoding AQP2 suffer from a severe form of nephrogenic diabetes insipidus (NDI) and release up to 20 l of urine per day [34]. Several of the individuals with recessively inherited NDI were found to be homozygotes or compound heterozygotes for substitutions in pore-lining residues (one example is shown in Fig. 2). Secondary reductions in AQP2 expression have also been identified in other polyuric states, including enuresis. In normal individuals, release of vasopressin causes the AQP2 proteins to become exocytosed into the apical plasma membrane, permitting water reabsorption from filtrate. If too much AQP2 protein is expressed, water reabsorption may become excessive, and this is believed to contribute to common fluid overload states found in patients with congestive heart failure or pregnancy (see review [35]).

The mechanisms by which kidneys control urine acidification are less well defined but are likely to involve AQP6. Unlike other aquaporins, AQP6 exhibits minimal water permeability and resides alongside H^+ ATPase in intracellular vesicles in acid-secreting α-intercalated cells of renal collecting duct [36,37]. Very surprisingly, this member of the family behaves as a pH-regulated anion channel with greatest selectivity for nitrate [38,39]. Specific variations in the AQP6 structural model explain the selectivity by the presence of hydroxyl-bearing residues (Y34 and T63) that are predicted to coordinate a nitrate anion within the pore. Our current hypothesis is that AQP6 may serve as a negative regulator for intracellular populations of H^+ ATPase, an enzyme known to be inhibited by nitric oxide.

Aquaporins are also found in many non-renal tissues. AQP0 (also known as major intrinsic protein) is expressed in ocular lens fiber cells [40]. Lenses are avascular and anuclear. To maintain transparency, fiber cells must remain viable throughout life. Lens is not a tissue with high water permeability. AQP0 exhibits low water permeability [41], and other studies predict that AQP0 serves as a cell-to-cell adhesion molecule [42]. Inherited defects in the gene encoding AQP0 have been identified in two large kindreds with dominantly inherited cataracts affecting small children [43]. Interestingly, the mutations occur in highly conserved residues believed to be critical to the protein structure [44]. The highly conserved glutamate in the fourth transmembrane α-helix (E134) forms hydrogen bonds with amide moieties in the peptide backbone needed for appropriate orientation of the carbonyl oxygens lining the pore (Fig. 2). All affected members of the E134G family suffer from lamellar opacities corresponding to the perimeter of the lens at birth. A highly conserved threonine at position 138 normally resides adjacent to E134 in the α-helix. The substitution T138R will form an ion pair with E134, thereby perturbing the architecture of the aqueous pore. Unlike the E134G family, all members of the family with the T138R mutation suffer from polymorphic opacities throughout the lens due to deposition of protein throughout childhood and adult life. Identification of major perturbations to AQP0 structure in families with severe, congenital cataracts suggests that more subtle polymorphisms in AQP0 may be a risk factor contributing to precipitation of cataracts that are much more commonly found in older individuals.

AQP4 is expressed as two isoforms corresponding to alternative transcription initiation at M1 or M23 [45]. Unlike most aquaporins, AQP4 is not inhibited by mercurials, due to the presence of an alanine at the pore-lining site occupied by a cysteine in AQP1 and other homologs (Fig. 3). In brain, AQP4 is highly concentrated at the perivascular membrane of astroglial cells where the protein is believed to regulate movement of water between brain parenchyma and vascular space [46]. At this site, AQP4 exists in highly symmetric square arrays [47] determined by the relative concentrations of M1 and M23 [48]. AQP4 is restricted to the perivascular membrane by dystrophin-associated proteins [49]. Interestingly, targeted disruption of the gene encoding AQP4 or mislocalization of the mouse AQP4 protein were found to be protective for animals stressed by hyponatremic acute brain edema or ischemic brain injury [50–52]. Unlike other organs and tissues, the brain is encased within the rigid cranium and has limited room to accommodate swelling without irreversible damage to neurons. The survival and recovery for many human patients sustaining traumatic head injury or stroke depends on prevention or rapid reduction of brain edema, indicating that pharmacological manipulation of AQP4 may offer potentially important therapeutic opportunities.

The mammalian aquaglyceroporins (AQP3, AQP7, and AQP9) are permeated by water plus glycerol, and their sequences are closer to bacterial GlpF (Fig. 3). AQP3-null humans have been identified, and red cell glycerol transport is markedly diminished [53]. AQP7 (also referred to as AQP-ap) is expressed in adipocytes where the protein releases glycerol generated by metabolic degradation of triglycerides [54]. Glycerol is taken up by liver through AQP9 where it is converted to glucose [55]. Expression of AQP9 is markedly increased in liver by starvation and in uncontrolled diabetes mellitus [56]. Utilization of glycerol becomes extremely important during prolonged fasting and starvation, and an individual homozygous for a missense mutation in the fourth transmembrane α-helix (G264V) exhibited greatly diminished glycerol release during exercise [57]. Although not structurally similar to glycerol, $As(OH)_3$ is predominantly uncharged at pH 7, and both AQP7 and AQP9 were found to transport arsenite [58]. This may be of relevance to explaining the problem of hepatic arsenic toxicity, since in many areas of eastern India and Bangladesh, tube wells were installed to protect the population against cholera which contaminates surface water. Alarmingly, the well water has recently been discovered to contain high concentrations of arsenic. The World Health Organization estimates that more than 100 million individuals

are now consuming toxic levels of arsenic daily, and an epidemic of hepatocellular carcinoma is resulting.

6. Implications

It is increasingly clear that aquaporins are important in the defense against a great diversity of environmental stresses. The ability to freely transport water but not protons is essential for plants and bacteria where the membrane potentials are determined by proton gradients. Moreover, this explains how our kidneys prevent us from dying of dehydration by reabsorbing 99% of the water from the glomerular filtrate, while at the same time they protect us from acidosis by secreting and not reabsorbing the protons. The heat wave that struck Europe this past August resulted in the deaths of tens of thousands of individuals who succumbed to heat exhaustion, a process in which the body cannot maintain proper body temperature due to improper hydration and is seriously compounded by defective ability to concentrate urine and diminished capacity to sweat – physiological processes which are both known to be affected in the elderly and in which aquaporins are now known to play essential roles. Structural information about aquaporins has provided insight into a severe form of cataracts, predicting that subtle polymorphisms may be a risk factor, similar to UV exposure, for the more typical age-onset forms of cataract. The ability of the brain to survive swelling within the rigid cranium, the ability to utilize stored fat for energy, and the vulnerability to arsenite hepatotoxicity are all processes made clearer by structural and functional information about aquaporins. Our challenge will be to use this information to benefit the patients and those at risk.

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