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Structure-Function Relationships in Aquaporins

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The identification of members of the aquaporin family as the primary water channels of cell membranes has been followed up by an intense effort to determine how these channels work. Specifically, investigators have sought to learn why these channels are selective for water and how they exclude proton trafficking. Molecular-dynamics studies using elegant, extremely detailed computer models based on accurate crystallographic maps of the channels show the basis for the selectivity of the channel. Channel size, the location of hydrophobic amino-acid side chains, and specific interactions of water dipoles with a charged residue near the most constricted point of the channel indicate that water molecules travel in single file through the center of the channel, and that the orientation of water molecules is manipulated to prevent the formation of a water wire spanning the channel. Finally, the number of water molecules calculated to be aligned in single file in the channel constriction fits predictions based on classic studies of the osmotic permeability: diffusive permeability ratios in water-permeable membranes.
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The limiting plasma membrane is a fundamental requisite of a living cell. It defines the boundary of inside and outside; keeps the genetic material, structural proteins, metabolic machinery, organelles, metabolites, and other substances that allow life to continue inside the cell; and regulates the entry of small and large molecules. The ability of cells to regulate volume and internal osmolarity requires, in many instances, that the limiting membrane of cells restrict the passage of water and salts. This is particularly true when the cell in question resides in an environment that is hypotonic relative to the interior of the cell. In this instance, if water permeability is not restricted, the cell will swell in response to the entry of water down its concentration gradient until the cell ruptures. A classic example of a cell rendered safe in a hypotonic environment by impermeability to water is the oocyte of the clawed frog, *Xenopus laevis*. Amphibians obligatorily reproduce in water, in most cases in water that is very hypotonic relative to the interior of their oocytes. Because of this, any significant amount of water permeability in the oocyte membrane would be lethal to the oocyte, and also to the species. In high contrast, the mammalian erythrocyte is extremely permeable to water but its cell membrane still is highly selective with regard to the entry and exit of specific solutes. When erythrocytes are placed in solutions that show an osmolarity that differs from their internal osmolarity, the erythrocytes behave as osmometers and rapidly swell or shrink in solutions of low or high osmolarity, respectively. The result of exposing erythrocytes to significantly hypotonic solutions is hemolysis.

Epithelia that demarcate compartments of grossly different composition or osmolarity must significantly regulate the permeation of water and other substances. Keratinized epithelia generally are impermeable to most solutes and solvents. Frog skin resists the permeation of external water but transports salt efficiently. Vastly different qualities are required of epithelia whose function includes the absorption of fluid at high flux rates. The small intestine and the renal proximal tubule are examples of epithelia in which salts, organic molecules, and water are absorbed at high rates per area of epithelium with high selectivity with regard to what is absorbed. The volume absorption is an active, energy-driven process, and not simply a leak that permits passage of bulk solution. Volume moves in one direction with a relatively small amount of back leakage. Finally, the adaptability of membranes such as the toad bladder and the mammalian collecting duct show that water permeability may be regulated on demand. Both of these membranes may appear to be nearly totally impermeable to water at baseline, but readily

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allow osmotically driven water reabsorption when stimulated with antidiuretic hormone.

The most basic model of a cell membrane is the simple lipid bilayer. Biophysicists have examined the water permeability of the artificial black lipid bilayer in great detail in an attempt to explain the water permeability of the cell membrane.[1,2] Although water can permeate a black lipid bilayer by diffusion, the permeability attributable to this mechanism is quantitatively insufficient to meet the needs of most cells of transporting epithelia. The diffusive permeability of a lipid membrane may be altered by changing the lipids of which it is composed, but the diffusive permeability of a lipid membrane fails to effectively model a significantly permeable biological membrane. Furthermore, pure lipid membranes show very little osmotic water permeability when the membranes are placed between solutions of differing composition. For simple lipid membranes, theory states that the permeability of water through the membrane by diffusion and the permeability of water through the membrane in response to pressure gradients should be equal.[1,2] In biological membranes these permeabilities tend to differ.

A comparison of the diffusive water permeability of a membrane or epithelium to water, measured by the flux of radioactively labeled water through a membrane in the absence of osmotic gradients across the membrane or epithelium, and the osmotic water permeability, determined by the movement of labeled or unlabeled water in response to transmembrane or transepithelial gradients, can suggest the mechanism by which water transits the membrane or epithelium.[1,2] The greater the ratio of osmotic permeability (Pf) to diffusional permeability (Pd), the more likely that water passes through channels in the membrane. Although lipid membranes are hydrophobic by nature, water molecules diffuse through them, albeit slowly. This slow motion is not affected by osmotic gradients across the membrane. Holes are required for osmotic pressure to be effective. Furthermore, earlier researchers effectively argued that the activation energy required for water permeation, the presence of the phenomenon of solvent drag, and the inhibition of water flux in some membranes by mercury all suggest the presence of a pore, which was most likely a protein associated with plasma membranes.[1-4]

Because of the inability of diffusion through lipid bilayers to explain experimentally observed water permeability and the other considerations mentioned earlier, water channels were hypothesized for decades, but the complexity of transport across the epithelia available for study made the search for this pore extremely difficult. Those epithelia with the greatest permeability and transport also had the property of leakiness. In addition, the renal proximal tubule, the small intestine, and other high-volume transport epithelia show behavior that suggested that both paracellular and transcellular paths for volume absorption were likely to be present.

Strong early evidence for a specific cell membrane water channel came from studies using the toad bladder, which had low osmotic water permeability (Pf) but greater diffusive water permeability (Pd) in the baseline state and developed a much more significant osmotic permeability on exposure to vasopressin (4.5). The ratio of Pf to Pd suggested that a channel was present; the change that occurred in response to the hormone vasopressin indicated that it was highly likely that more channels were added in response to the hormone or that existing channels enlarged. Freeze-fracture–etched electron micrographs of the apical membrane of the toad bladder granular cells showed the presence of small, regularly arranged particles in that membrane; after the addition of vasopressin the percentage of the membrane displaying these particles and the total number of particles in the membrane increased markedly.[4,5] The increase in permeability and the increase in particles significantly corresponded. This would be the result if the particles added to the membrane were indeed water channels.

Aquaporin

The story of the isolation and identification of the first recognized aquaporin channel by Peter Agre, for which he was awarded the 2003 Nobel Prize in chemistry, is now well known. Agre et al,[6] Smith and Agre,[7] and Preston and Agre[8] isolated a 28-kd protein in the course of studies intent on purifying and characterizing Rhesus (Rh) factor polypeptides in human erythrocytes. The 28-kd protein was found in great quantity, was glycosylated variably, which explained its electrophoretic mobility, and it was found to exist in its native state as a tetramer. The protein clearly was important; it was the most abundant protein present in erythrocyte membranes. The size of the protein monomer was highly significant; contemporary radiation inactivation experiments had predicted that a hypothetical water channel in the renal proximal tubule likely would have a molecular weight of approximately 30 kd.[9] When the 28-kd protein was sequenced and its primary amino acid structure was considered, it was noted to resemble a group of proteins known as integral membrane proteins, and it ultimately was suggested that it might be a channel protein.[8] Investigation of the possibility that the protein, now named CHIP28, could be a water channel arose from the observation of Dr. John C. Parker, as related in Dr Agre's Nobel lecture, that red blood cells, similar to proximal tubules, had high water permeability. The key experiment was the expression of the newly isolated *CHIP28* gene in the Xenopus oocyte. Normally, these oocytes are tolerant of hypotonic environments because they are impermeable to water. After expression of the CHIP28 protein in the oocyte, however, oocytes exposed to hypotonic solutions swelled and eventually burst.[10,11] These results indicate that the protein that was expressed by the oocyte was in fact a water channel.

With the recognition that CHIP28 was a functional water channel, there was an explosion of studies in search of related proteins in a wide variety of cells and epithelia that showed significant water permeability. Comparison of the gene sequence with already identified protein in a number of databanks indicated that CHIP28, now renamed *aquaporin 1* (*AQP1*), was related to a large group of proteins found in all organisms with membranes.

The Aquaporin Protein Family

Glycerol permeability is the principal characteristic of a large group of proteins related to aquaporins, the glycerol facilitator–like proteins (GlpF).[12] These and other channel proteins are found broadly in the membranes of bacteria, yeast, and animals. Together with the nodulin-like proteins found in plants, the plant plasma membrane integral proteins, and the animal, plant, yeast and slime mold AQP proteins, they comprise a large family of proteins that primarily are divisible into water and glycerol-permeable channels. Two large clusters thus are defined, a GlpF cluster, and an aquaporin (AQP) cluster. The structural basis for the selectivity of the groups is currently a major question in biology.

The members of the aquaporin family of membrane channel proteins seen in human beings have a variety of functions. At this time at least 12 putative aquaporins have been identified. AQP0 is the primary protein component of the lens of the eye. It initially was called *major intrinsic protein* (MIP) when it was found originally in the bovine lens.[13] Although this protein does not seem to function as a channel in the eye, it has been observed to increase the water permeability of Xenopus oocytes when expressed in that system.[14] AQP1, formerly CHIP28, is the most abundant protein in erythrocyte membranes and it has been shown to be the apical membrane water pore in the renal proximal tubule.[15-17] In addition to the kidney, AQP1 also is found in endothelial cells, the choroid plexus of the brain, the ciliary epithelium and corneal endothelium of the eye, and in intestinal lacteal glands. AQP2 is the name given to the antidiuretic hormone-inducible apical membrane water channel seen in the renal collecting duct and its analogs. It has not been found elsewhere.[18-20] AQP3 is a water channel localized in the basolateral membrane of renal collecting duct cells. It also is found in skin, airway epithelia including large airways, the conjunctiva of the eye, and the urinary bladder.[21,22] Unlike AQP1 and AQP2, AQP3 is not an entirely water-specific channel. AQP3 shows permeability to glycerol and water; this is also the case for AQP7, AQP9, and AQP10.

The basolateral water permeability of the renal cortical collecting duct also occurs via a second channel, AQP4, which appears to be a classic water channel.[23] AQP4 also is found in glial cells, glandular epithelia, the lungs, the retina, skeletal muscle, and the stomach. AQP5 is water specific and is found in glandular epithelia, the cornea, alveoli, and along the gastrointestinal tract.[24]

The only human AQP yet shown to convey any ionic permeability is AQP6, which has been localized to the apical membrane of principal cells of the renal collecting duct. At low pH levels, AQP6 functions as a chloride channel in preference to water-permeability properties.[25] The basis for the ionic permeability is an area of intense investigation at the present time. AQP7, as mentioned earlier, is permeable to glycerol and water.[17] It is found in the proximal renal tubule, but probably has a more important role in adipose cells, where it may affect fat metabolism.[25]

AQP8 functions as a water channel at various sites throughout the gastrointestinal tract, in the testes, and in the heart.[26] AQP9 channels glycerol and water in the liver, testis, the brain, and in white blood cells.[27] AQP10 is permeable to a number of small solutes and water and is found in the small intestine. AQP11 is known to be present in the kidney and liver, but its function is undefined. Finally, AQP12 is a channel of unknown permeability found in the pancreas.

Aquaporin Structure

Once AQP1 was identified in terms of function and location, a race began to describe fully and accurately the complete tertiary structure of the molecule and to develop an understanding of the relationship between its structure and its function. Such studies are driven by both the "pure" motivation of the need to understand the most basic principles of transport by the channel and how structure generally determines function, and the "impure" motivation of the race to develop potential clinically applicable inhibitors of the channel. Because aquaporins have critical roles in tubular reabsorption of water, and the permeability of water in the lung, brain, and gastrointestinal tract, they are obvious targets for drug development. Precise knowledge of channel structure and structure-function relationships should, in theory, allow the development of specific water-channel inhibitors. The understanding of the structure-function relationships of water channels has developed rapidly, and it has been aided greatly by massive advancement in computational science in the past decade.

The primary structure of CHIP28 was described initially by Preston and Agre[8] in 1991. The protein initially was cloned from complementary DNA isolated from human fetal liver cells. The protein produced was of an appropriate size for a channel and had a nearly identical amino acid sequence to a prior isolate. Kyle-Doolittle analysis of the primary structure of CHIP28 suggested the resulting construct would have 6 membrane-spanning regions, that both the amino and carboxy termini were located on the cytoplasmic side of the membrane, and that there were 3 (A, C, and E) external loops and 2 (B and D) cytoplasmic loops as shown in Figure 1.[28]

Homology of CHIP28 with the MIP protein family was uncovered by searches of the GenBank DNA database. Several regions of the protein were identical between CHIP 28 and MIP26 (the major intrinsic protein of the bovine lens), but others, including the C terminus, varied considerably. In contrast, the B and E loops had homologous domains with 20% identity in the first and second halves of the molecule. Other members of the MIP family of proteins shared a similar repeat in their primary structures with recurrence of the amino acid sequence asparagine-proline-alanine (NPA) in homologous B and E loops, leading to the speculation that an ancestral gene had undergone duplication, probably before the differences between species from which the proteins were isolated. All the aquaporins appear to contain the NPA repeats and the repeats always occur near the constriction area of the pore in the proposed tertiary structures as indicated in Figure 2.[29] The carboxy and amino terminals appear to represent repeats and further modeling suggested that each end of the molecule functions as a hemipore and that the overall

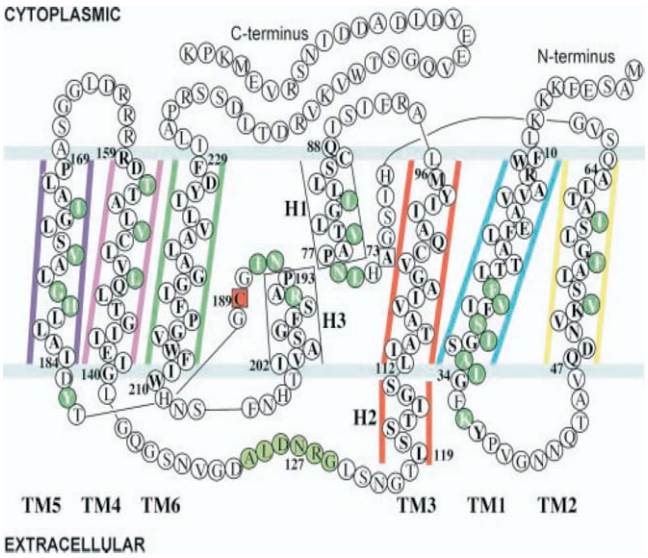

Figure 1 Primary and secondary structure of AQP-1. The primary sequence of AQP1 is shown as present in the cell membrane, as well as indicating the 6 membrane-spanning helices, 2 cytoplasmic loops, and 3 extracellular loops. Portions of 1 extracellular loop and 1 intracellular loop are shown to fold back into the membrane and form what is effectively a seventh membrane-spanning region (center). Amino acids in membrane-spanning regions that have side chains lining the channel or pointing into the channel are colored green. The red square indicates the mercury-sensitive site (C189). The green amino acids in the extracellular loop at the bottom center line the entrance to the water pathway. Reprinted with permission from Ren et al.[28] Copyright 2001 National Academy of Sciences USA.

molecule assumes an hourglass shape with the B and E loops each penetrating the membrane and interacting to form the actual water-selective pore.

An hourglass model of the AQP1 molecule was proposed as early as 1994 based on sequence analysis, hydropathy plotting, and functional analysis.[30] By 1997 the 3-dimensional structure of AQP1 had been determined to the level of 0.6 nm resolution.[31,32] The current generally accepted picture of the AQP1 molecule is hourglass shaped with the 6 membrane-spanning regions forming the overall transmembrane pore. The 6 transmembrane helices are tilted within the membrane as shown in Figure 3.[33] In Figure 2, the membrane-spanning regions are linked by the loops labeled A through E and, most importantly, the B and E loops, rather than sticking out into the cytoplasm and extracellular space, respectively, fold back into the funnel shape of the overall channel and the 2 NPA repeat regions of these loops are located near the center of the pore. The infolded portions of the B and E loops together form a seventh membrane-spanning helix with each of the loops penetrating halfway down the pore.[33,34] The overall impression is that the 6 typical membrane-spanning helical domains defined by classic hydropathy mapping serve to create an hourglass-shaped scaffold that supports the much less substantial but critically more functional half helix loops draped inside each funnel-shaped half of the hourglass. The NPA sequence sites of the B and E loops are brought in juxtaposition, creating an area with 2 nearby negatively charged regions located at the center of the hourglass.

Unique Characteristics of Aquaporin Permeability

When the CHIP28 (AQP1) is expressed in Xenopus oocytes that normally are water impermeable, the subsequent expo-

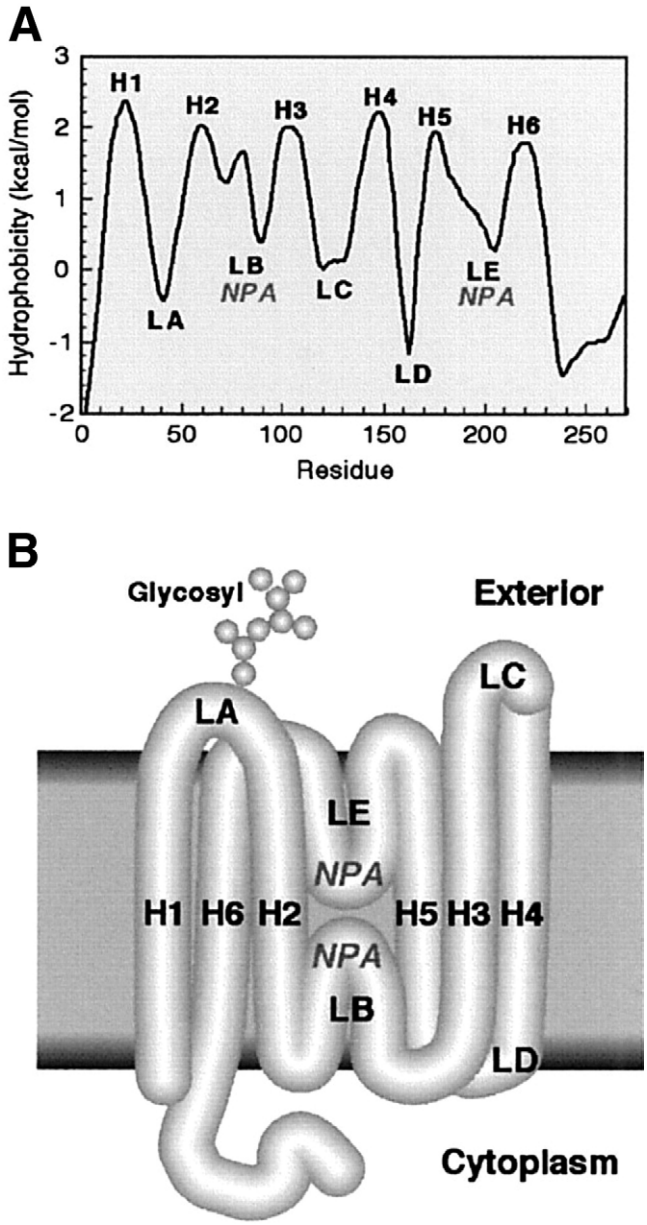

Figure 2 General structure of AQP family proteins, an average of 46 AQP proteins are shown. (A) The mean hydrophobicity plot used to determine the membrane-spanning regions (H1-H6) and the various loops (LA-LE). The position of the NPA sites are those for AQP1. (B) The hourglass model, indicating both the creation of a membrane-spanning region by LB and LE, and the presence of the NPA repeats in proximity at the middle of this structure. Reprinted with permission from Heyman et al.[29] Copyright 1999, Int. Union Physiol. Sci./Am. Physiol. Soc.

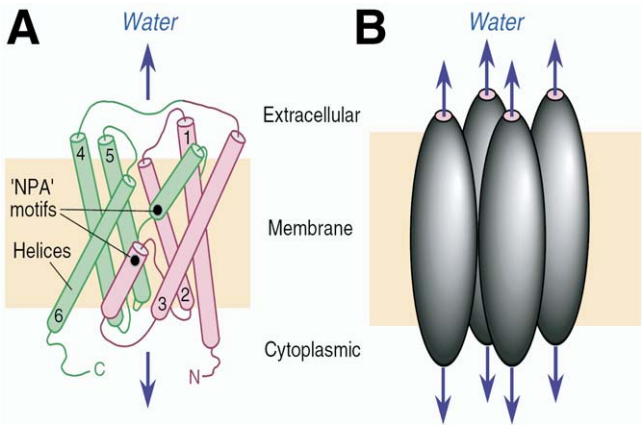

Figure 3 Structure of AQP1, monomer, and tetramer. (A) Tilting of the membrane-spanning region to form an hourglass shape with the NPA motifs in the center of the water path indicated by the arrow. (B) Molecule as found in tetrameric form, indicating that the water flow occurs through the monomers, but not the center of the tetramer. Reprinted with permission from Verkman.[33] Copyright 2005, Company of Biologists Ltd.

sure of the eggs to hypotonic buffer results in their rapid enlargement and rupture.[8,9] This unmistakable result is compatible only with the insertion of new water channels into the cell membrane. However, the experiments also showed that there was no change in membrane current in the oocytes expressing the channel, indicating that ionic permeability was not increased by the addition of the channel.[10] Further studies indicated that AQP1 reconstitution into the membrane also did not increase the permeability of the oocyte membrane to small solutes or protons, which indicates that the pore was remarkably specific for water.[35]

The protein structure of membrane channels determines their permeability.[36] Channels can restrict the passage of substances through the mechanism of size restriction and the alignment of areas of hydrophobicity or hydrophilicity. In addition, ions may be prevented from transiting a pore by the particular arrangement of charge. Even if a channel has a pore size large enough for the hydrated ion to pass, it may be prevented from doing so by electrical repulsion caused by the arrangement of charged amino acid side chains in the channel. Conversely, the arrangement of amino acids in the channel may enhance passage of an ion by electrostatic attraction. The behavior of the AQP1 channel suggested that either a strong size or charge barrier was present. The hourglass shape of the molecule suggests the possibility of size restriction. The NPA repeats near the area of greatest size constriction present a possible site where charge may be a significant factor.

The permeation of the channel by gasses including O_2, CO_2, and NO would appear to be theoretically possible given the size of the molecules.[37] Nakhoul et al[38] expressed AQP1 in Xenopus oocytes and measured an increase in the carbon dioxide permeability of the cells. In fact it might be hypothesized that the purpose of the massive quantities of the AQP1 channel in the membrane of the erythrocyte may be to provide the necessary permeability to CO_2 required for respiratory gas exchange. However, studies performed to investigate the role of AQP1 in erythrocytes have found evidence to the contrary. Yang et al[39] examined the carbon dioxide permeability of red blood cells and lung in normal and AQP1 null mice, but found no difference in permeability between the animals with and without the water channel. The possibility remains, however, that erythrocytes in the null mice derive carbon dioxide permeability by other redundant mechanisms. Colton null erythrocytes do have an effective water permeability provided by other aquaporins than AQP1.

Proton Impermeability—The Problem

In particular, it is the absence of proton permeability that is both interesting and biologically important. Physiologically, it seems necessary that a water channel at the site of urinary acidification be relatively impermeable to protons, otherwise back diffusion of secreted acid would occur through the pore. The observed properties of renal transport indicate that portions of the nephron are both water permeable and proton tight. Massive amounts of filtrate are reabsorbed along the course of the proximal tubule and proton secretion decreases the pH level by 0.7 units. More significantly, vasopressin-stimulated water reabsorption in the collecting duct does not prevent the generation of 3.0 pH unit gradients as water transport occurs. These behaviors are not compatible with a water channel that has significant proton permeability, either in the proximal tubule (AQP1) or the collecting duct (AQP2).

The absence of ionic permeability of a transmembrane pore may have several causes. The pore may be too small to permit passage of the ion in the hydrated or unhydrated state, and charges within the pore can prevent the passage of ions as a result of electrostatic repulsion. These strategies are insufficient, however, to explain fully the lack of proton permeability, which is a much more interesting phenomenon. Protons are chemically unique in that they may behave not only as hydrated or unhydrated ions, but also as quantum objects. Protons are mobile in aqueous systems in 2 ways, first as hydronium ions (H_3O^+) that move through the solution in a random walk as any other cation and, second, as the unhydrated proton species that moves along linearly arrayed water ice via a tunneling mechanism. The mechanism is analogous to the movement of electrons in a conductive metal. The major requirement for the extreme mobility of protons in water is that water dipoles form an unbroken chain for the distance between the 2 points. Bulk water, even when at room temperature, is organized, or ice-like, to a very high degree. In the de Grotthuss[40] mechanism protons rapidly move along water molecules by rearrangement of hydrogen bonds. When a proton is placed adjacent to the end of a line of hydrogen-bonded water molecules, it reacts strongly with the oxygen in water, resulting in the formation of H_3O^+, but because the molecule it has just reacted with shares hydrogen bonds with the next, a rearrangement of charge can, effectively, instantly transfer the excess proton to the next water molecule in line in a process that can be repeated along the

line of water molecules as fast as water dipoles can oscillate. The result is that the proton appears to diffuse a significant distance with relativistic speed, even though the originally added proton does not itself move, just as an electron added to the end of a metal wire results in the near instant delivery of an electron at the other end of the wire. The particular electron does not move, but an electric current results, and free electrons are available at the end of the wire. In an even more wire-like situation, water in the near vicinity of proteins form structured, ice-like layers and water molecules interacting with polar side groups of the protein may form single-file arrangements that function as wires for proton passage by the hydrogen bond rearrangement mechanism. The addition of a proton to one end of a water wire results in the instantaneous availability of a proton at the other end of the wire.

The Path of Water

To investigate the peculiar properties of the water channel, computer simulations using molecular dynamics protocols were performed in 2001 by Kong and Ma,[41] using 2 available radiograph crystallographic 3-dimensional structures for the molecule. In these modeling studies, the most precise available molecular structure is input together with a high-precision estimate of the distribution of charge along the model, and careful input of the hydrophobicity or hydrophilicity of areas along the channel. Simulated water molecules then are added, and the resulting path by which water may pass through the channel then is calculated. When the simulations were run, both 3-dimensional structures gave data that indicated that the area near the NPA repeats constricted water flow through the molecule, and that the size of the constriction fluctuated over the 10-nanosecond period of simulation. Figure 4 is a stereoscopic crossed-eyes image showing the areas where water molecules most commonly are located within the computer model of the molecule.

In addition, Kong and Ma[41] also found that the innermost and outermost areas of the channel appeared to be lined with enough hydrophobic residues to ensure that few interactions occurred between water and the wall of the channel. Water thus is funneled smoothly into the constricted center of the channel. In contrast, water flow near the NPA region appeared to occur in single file, but it also was suggested that fluctuation of the size of the channel could break any continuous single file of water and thus prevent the creation of a transmembrane water wire. In addition, the study showed that the Asn residues at 76 and 192 were critical to water permeability because replacement of these moieties in the simulated molecule by hydrophobic residues eliminated water permeability.

In 2001, deGroot and Grubmuller[34] used a real-time molecular dynamics simulation to compare the movement of water through 2 related but significantly different pores, AQP1 and the *Escherichia coli* GlpF. These proteins differ substantially in selectivity. GlpF permits the rapid diffusion of glycerol through the *E coli* membrane but the channel is less permeable to water than AQP1. The channel proteins also differ with regard to their structure. GlpF actually has a larger pore radius than AQP1 in its crystallographic measurements, but water travels through the GlpF pore at a slower rate when transit is measured. deGroot and Grubmuller[34] modeled each of the pores in 3 dimensions, simulating the full tetrameric structure of the molecule as each is believed to exist in the membrane, including a lipid bilayer model in their calculations. They then simulated how water molecules would penetrate the pore from inside and outside the simulated lipid membrane. The elements that went into these calculations included precise mathematic models that accounted for the complete steric structure of the AQP1 and GlpF monomers arranged as tetramers, the distribution of charge on the amino acid residues, the effects of hydrophobicity and hydrophilicity, and the potential motion of the molecule. The simulation also models the diffusive motion of water, and the interactions between water dipoles and the amino acid residues of the channel. When all these structural factors were taken into account and simulation was run, the model predicted that in any 10-nanosecond period, 205 water molecules would visit the 4 pores of the tetramer, and 16 water molecules would transit the entire pore, 4 per pore, with half the transits in each direction. No water molecule crossed the membrane via the cavity created at the center of the tetramer by the packing of the monomers.

The model created by deGroot and Grubmuller[34] allows a calculation of the path of water through the molecule. These calculations indicated several critical interactions between water and specific residues along the path through the pore. Proceed-

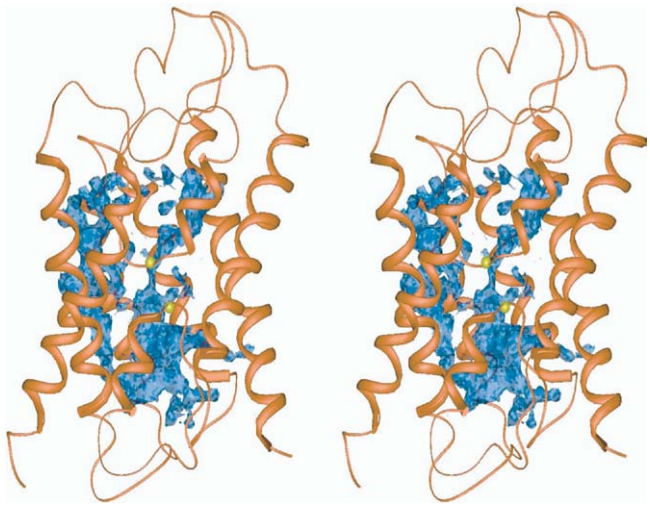

Figure 4 Location of water in the AQP1 water channel. The figure is a stereoscopic image of the ribbon structure map of AQP1, indicating the sites inside the channel where water is found most frequently. It was produced by a molecular-dynamics simulation based on the structure of the molecule, the location of hydrophilic and hydrophobic residues, and the distribution of charge, and predicts where water will be located most commonly during a 10-nanosecond period of simulation. The small yellow spheres are the Asn-76 and Asn-192 residues of the NPA repeats. The figure shows the main water pathway travels by the NPA repeats where it appears to go through a narrow constriction. Reprinted with permission from Kong and Ma.[41] Copyright 2001 National Academy of Sciences USA.

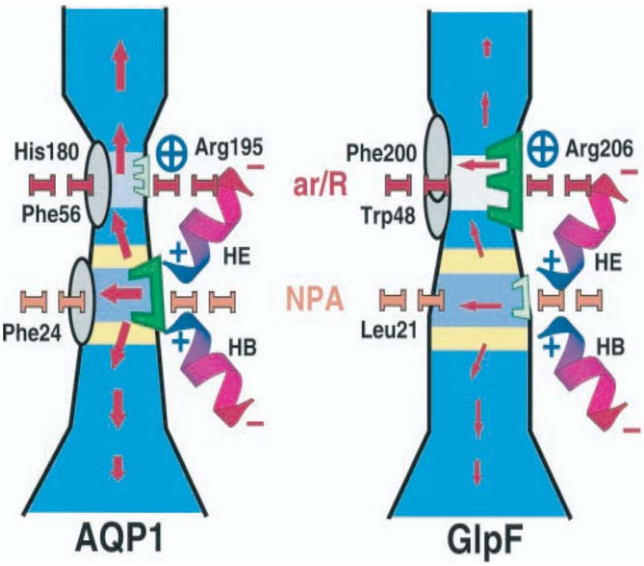

Figure 5 Summary of water permeation through AQP1 and GlpF. The diagrams show the relative size of the critical portions of the pores including the area of most constriction. An ar/R region involving arginine is shown at the narrowest portion of both pores. This is shown with a red bar and indicates the upper filter of the pore. It is tighter in AQP1. The shade of blue in the channel indicates the predicted strength of water-water H bonds, and shows that these are weakest in the region of arginine because of interactions with this amino acid residue. This area thus is predicted to be a proton filter because it interferes with the formation of water wires. Despite the larger size of the constriction area, GlpF exerts greater effect on water interactions. In GlpF, there are strong interactions between water and arginine, and repulsion of water by tryptophan and phenylalanine, greatly reducing the ability of water to pass by the area in green, and providing selectivity for glycerol in preference to water. The orange bar shows a second filter area at the site of the conserved NPA regions. This area is predicted to restrict permeability by size. There is a strong predicted interaction between water and the channel protein at arginine here, indicated in green. The yellow areas of both diagrams indicate hydrophobic regions that slow water entry. Reprinted with permission from deGroot and Grubmuller.[34] Copyright 2001 AAAS.

ing from the cytoplasmic side of the pore, the opening was reported to be relatively wide with water interacting weakly with Gly, Ala, and His residues in the outer funnel. The model predicted a simultaneous interaction between water and the 2 arginine side chains of the NPA regions of the B and E loops on one side and Phe, Val, and Ile on the other side of the pore. Once past this region, transiting water molecules interact with a Ile, Gly, and Cys grouping, and then with a site comprised of Phe, His, and a positively charged Arg moiety. At this point, strong hydrogen bonds were predicted to form between water and the His and Arg sites. After passage here, some minor interactions occurred and the water molecule eventually exited the pore. Figure 5 shows the primary issues just discussed.

The key findings of this modeling exercise were the identification of the strong water-arginine interaction at the NPA sites in the middle of the pore, and the broader observation that an extended chain of water molecules never appeared to develop through the pore. The strong arginine site was prevented to prevent the passage of any positively charged ions. The lack of a continuous water chain through the channel eliminated the possibility of a water wire mechanism of proton permeation. Notably, the arginine site was present in, and served to limit proton permeability of, both AQP1 and GlpF. Other members of the family (aquaglyceroporins) similarly have located Arg residues and thus would be expected to share in the property of proton impermeability.

A final conclusion of the model was that there appeared to be specific differences between AQP-1 and GlpF that can explain the selectivity of AQP1 for water over glycerol. These included the partial occlusion of the pore by a Phe residue in AQP1 that serves to increase interactions of water with the NPA site and increases water permeability while reducing permeation of larger molecules such as glycerol. A Leu residue at the same site in the GlpF channel is less intrusive on the channel pathway and more readily allows the passage of glycerol while serving to decrease the velocity of water permeation. The NPA region in the GlpF molecule further serves as a filter by apparently narrowing after passage of glycerol molecules, reducing the pore available for water passage. The overall conclusions of the model are that the NPA regions of these 2 channels and other water channel molecules of this family are size selective, and that arginine residues nearby the constriction area serve to reduce ion, and particularly proton, permeation.

Further examination of related members of the aquaglyceroprotein family provided additional insight into the mechanisms of selectivity. The 3-dimensional structures of AQP1 and GlpF are believed to be nearly superimposable, and have at their heart oxygen ladders that steer water to the center portions of the pore by means of hydrogen bonding.[42] These ladders are broken by the amide groups on the asparagines in the 2 NPA groups. Available measurements at the time of the study indicate the presence of a 0.28 nm constriction near the arginine region of the central selectivity area in orthodox aquaporins, whereas aquaglyceroporins have a significantly larger central pore area of 0.35 nm. Beitz et al[43] isolated an aquaglyceroporin from *Plasmodium* (*P*) *falciparum* that showed high permeability to both water and glycerol. Despite the presence of amino acids said to be typical for glycerol-specific pores based on the effects of mutations in related molecules at these sites, the pore is water permeable. Beitz et al[43] performed multiple mutations at sites along the molecule and found that glutamate (E125) at a site near a regularly conserved arginine (R196) in the mouth of the pore was critical. Substitution of serine at this site eliminated water permeability, presumably by electrostatic effects.

Yet more detail regarding the glycerol permeability of GlpF was generated by Fu et al[44] with computer modeling techniques in 2002, making use of a 0.22 nm map of the protein that included glycerol molecules in place along the channel (Fig 6). In the model, glycerol molecules lined up in single file with a selection site for size located in the middle of the typical hourglass configuration, and enhancement of this size

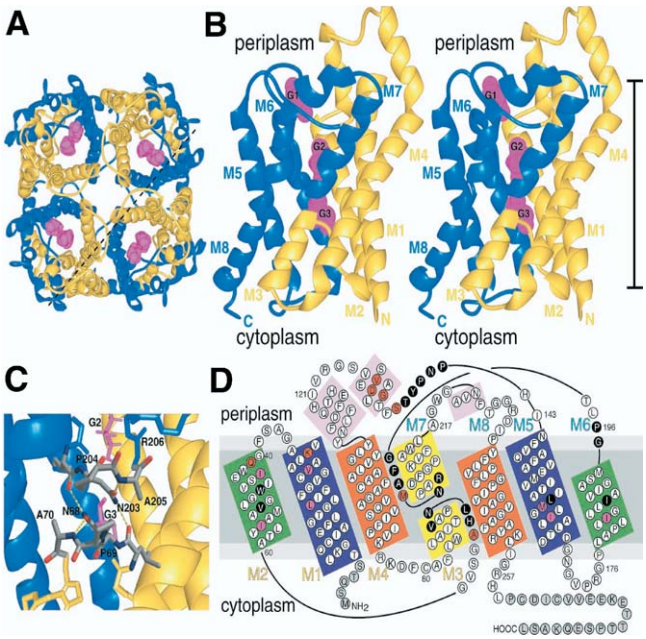

Figure 6 The structure of GlpF. The structure of the *E coli* glycerol channel is shown. (A) Top-down view of the tetramer structure shown in a ribbon representation. A central core exists, but all channel activity occurs through the individual monomer. (B) Crossed-eye stereoscopic view of the GlpF molecule with 3 glycerol molecules in the channel. The side bar indicates the overall thickness of the membrane. (C) Center of the molecule where the NPA motifs meet in the center of the channel and possible hydrogen bonds are shown by dotted lines. (D) Overall primary and secondary structures, with 6 membrane-spanning regions and a seventh membrane-spanning region formed by the interaction of 2 loops, one from each side, with an NPA motif in the center of this region. The figures contain significantly more detail and the reader is referred to the original article for full explanation. Reprinted with permission from Fu et al.[44] Copyright 2000 AAAS.

selectivity resulted from the influence of the NPA groups of infolded M3 and M7 half helices.[44]

The question of proton impermeability of the aquaglyceroporin GlpF of *E coli* was examined again by Tajkhorshid et al[45] in 2002. The crystal structure of this molecule was known at 0.22 nm detail, and the shape of the channel through which glycerol passes had been defined. In addition, the theoretic structure of a mutant with less selectivity for glycerol and greater water permeability also was studied. Molecular dynamic modeling was performed for the occurrence of 7 to 9 water molecules in passage through the available channel. The results of the modeling exercise indicated that water molecules on the 2 sides of the center of the channel always would be oriented oppositely (ie, the oxygen aspect of the boomerang-shaped water dipole faces one direction in the inner side of the molecule and in the opposite direction in the outer side of the molecule). The model thus predicted that a continuous wire could not exist, and the line of water molecules going through the channel always was broken in the middle by the flip in orientation. The structural key to this behavior was the interaction of water molecules and the asparaine molecules in the NPA repeats of the B and E loops. Asn-68 and Asn-203 form hydrogen bonds with each water molecule transiting the center of the channel, breaking any hydrogen bonds with preceding and subsequent water dipoles in their passage through the channel as this occurs.[45] Both the wild-type channel and the more water-soluble GlpF mutant had structures that predicted this behavior, and neither showed appreciable permeability to protons, despite the greater size of the pore in the mutant.

Miloshevsky and Jordan[46] performed a Monte Carlo reaction pathway study of the passage of water molecules through the AQP1 channel in 2004 and predicted from the results the study that single-file water diffusion is required at a site between the NPA locus and a selectivity filter site. Water molecules are prevented from forming a continuous chain from one membrane surface to the other because water passing by the NPA site must bind to these residues by forming 3 hydrogen bonds. This again requires that bonds between this water molecule and the preceding and subsequent water molecules are broken. As in the prior study using the GlpF model, this serves to break the wire and eliminates the possibility that protons can pass through the channel in a quantum mechanical manner.

Most recently, proton and hydroxyl permeation of the *E coli* GlpF channel was examined in a model that included a line of water molecules in the channel in positions relative to protein residues predicted by prior models.[47] With the water in the channel, protons or hydroxyls were added to see if wire-like transit of protons or the indirect transit of hydroxyl ions by progressive shifting of protons from the aligned water would occur. The study confirmed prior predictions regarding proton transfer; the orientation of water in the NPA region would prevent proton transfer along the potential water wire. In the case of the addition of hydroxyl ions, the macrodipoles at the NPA center prevented hydroxyl ions from entering the most critical site of the channel. Jensen et al[47] were able to show that protons and hydronium ions could enter the outer parts of the channel, but could pass through the heart of the channel, and the protons that entered in this manner were expelled again, most likely by a deGrotthuss[40] mechanism.[47]

Channel Structure, Pf, and Pd

A common thread of all the models and calculations just reviewed is that water (and glycerol in the case of GlpF) travels through at least a portion of the channel in a single-file arrangement. Zhu et al[48] examined Pf and Pd in the water channel structures as they are now known to exist. The molecular dynamics techniques were used to determine the flux rate for labeled water in the absence of osmotic gradients to determine diffusive permeability (Pd). A new mathematic model then was generated to determine the flux of water in the presence of hydrostatic gradients or Pf. In this calculation hydrostatic pressure was used rather than osmotic pressure, but the 2 forces are equivalent with regard to generating a water flux through a pore. Three regions of water were modeled with regard to the pore, a middle region in which a unidirectional force theoretically was applied to the molecules, a second region into which the pushed molecules moved, and a third region from which the pushed molecules were taken. The net unidirectional rate at which pushed wa-

ter was driven through the pore was calculated and the resulting permeability was derived. The Pf for the water channel thus derived by Zhu et al[48] in these studies was 7.1×10^{-14} cm^3/sec. The investigators then used the flux of water through the channel from the deGroot and Grubmuller[34] study to provide an estimate of Pd; as discussed earlier, this rate was 16 complete permeations in 10 nanoseconds for the tetrameric channel structure, or a rate of 0.2 unidirectional transitions of the channel per nanosecond. (The 16 permeations per 10 seconds number refers to bidirectional motion; a Pd experiment would measure the motion of labeled water in only 1 direction.) This rate of permeation was calculated to be equivalent to a diffusive permeability of 6.0×10^{-15} cm^3/sec. Thus, the mathematically predicted ratio Pf/Pd for the molecular model of the AQP1 channel is 11.9. In 1996, Mathai et al,[49] using erythrocytes deficient in AQP1 for comparison, estimated the Pf/Pd ration of AQP1 to be 13.2. Classic theory argues that the Pf/Pd ratio is related to the number of steps water makes in crossing a channel or, alternatively, the average number of water molecules present along the length of the channel. The current model of the channel with water in place puts 7 molecules in line (with the break in the center) in the constriction site. Only 8 steps thus are required to push through the area. The remainder of the Pf/Pd ratio therefore is believed to be related to events in the vestibules at either side of the constriction. In a figure that was awarded the 2004 visualization challenge prize by *Science* magazine, Tajkhorshid and Schulten[50] showed the passage of water through AQP1 in a model assembling a full tetramer of the protein, a molecular model of the lipid bilayer, and simulated water on both sides of the membrane and in passage through the channel (Fig 7). The figure includes all aspects of the channel discussed earlier.

What is most amazing about these studies and results is the final degree of agreement between theoretic and experimental measurements. The estimates of flux rate and permeability, and the ratios of Pf and Pd derived strictly from precise structural data arising from sequence, radiograph crystallography, and basic principles of charge distribution and hydrophobicity/hydrophilicity, approach those measured in nature. The suggestion is therefore that the model in use is likely to be correct, and that structure determines function.

Mercurial Sensitivity

A final characteristic of water channels to consider is the sensitivity of the channel to mercury. This sensitivity was the probable basis for the activity of merchydrin and other mercurial diuretics that were effective, but toxic, drugs long since abandoned by clinical medicine. Studies using mutational strategies indicated the location of the mercury binding site. A single cysteine residue at position 189 in AQP1 appears to confer the sensitivity of the molecule to mercury in the extracellular fluid.[51] The position of this cysteine is shown as the red residue in Figure 1.[28]

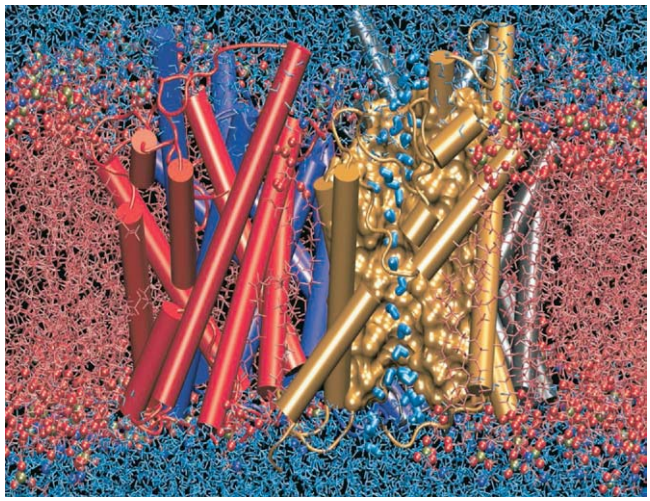

Figure 7 Water permeation via the AQP1 water channel. The figure was produced by Drs. Tajkhorshid and Schulten at the University of Illinois to show the passage of water through AQP1. A full tetramer is shown with monomers of AQP1 shown in red, blue, silver, and gold. The red band is a simulation of the plasma membrane. The blue at top and bottom represents water on both sides of the membrane. The gold monomer is split open to show water molecules (enlarged relative to those shown above and below) en passant, highlighting the constriction of the channel. Of critical importance is the flip in orientation of water dipoles between the top and bottom of the channel because the water molecule in the center interacts with the critical argenine residue. The number of water molecules shown in line approximates the number indicated by Pf/Pd ratios measured in the tissue and calculated from computer modeling.[45] Reprinted with permission from Tajkhorshid and Schulten,[50] and Tajkhorshid et al.[45] Copyright 2004 AAAS.

Structural Lessons From an Odd Member of the Family

Recent research into the structural basis of channel activity has included the investigation of those members of the channel family that show nonclassic behavior. In contrast to other members of the aquaporin family, AQP6 appears to function as an anion channel with a flickering conductivity and only limited water permeability.[17,52] The molecule differs from its closest structural relatives (AQP0, AQP2, and AQP5) at 1 critical area. Although these standard water-permeable, ion-impermeable aquaporins consistently show a glycine residue at position 57, AQP6 shows asparagines at position 60, which is the analogous site in this molecule. Replacement of the Asn-57 with Gly-57 converts AQP6 from an anion channel without water permeability into a water channel without anion channel activity. The obvious question is why does a single amino acid substitution produce such a specific and profound alteration of activity? Although the answer was not shown in this study, the reason Liu et al[52] chose to study the site was that the Gly-57 site was conserved so consistently in other aquaporins. In AQP1 the glycine at position 57, which in the middle of the second transmembrane domain, interacts with another glycine in the fifth transmembrane region. The transmembrane domains appear to be linked and may fix

the overall structure of the molecule by effectively linking transmembrane helices. When the authors performed other mutational substitutions at this site in AQP6 and at the analogous position in AQP1 the molecule that was expressed did not locate to the plasma membrane, indicating that the site is critical to channel activity.[52] Other critical sites for the anion permeability of AQP6 appear to be Thr-63 and Lys-72.[53,54]

Summary

In summary, an astounding amount of information has been accumulated in the past 15 years. In 1990, the water channel was a theoretic necessity, but an unknown quantity. In the time since, the extended family of aquaporins, aquaglyceroporins, GlpFs, plant plasma membrane integral proteins, and nodulin-like proteins has been characterized and its taxonomy outlined. The fundamental, commonly shared structure of the families has been outlined by studies in several principle members, and the shape, size, and restrictions of the pathways for water and glycerol penetration have been outlined in those membranes. When these proposed structures and pathways have been put to the test of careful mathematic modeling of their function, the results suggest behavior that corresponds amazingly well with the empiric data derived from living cells. In addition, the recent modeling data appear to answer the deep physiologic question of why protons do not fall through the hole.

Whether these studies eventually will lead to the development of specific and effective diuretic agents is still a matter of question. Human beings with total absence of functional AQP1 show a phenotype nearly indistinguishable from normal, suggesting that simple inhibition of 1 channel, even if it is the most abundant in the kidney, may not produce an instant cure for the fluid retention seen in heart failure, cirrhosis, or the nephrotic syndrome owing to the presence of a number of back-up permeability mechanisms. The amazing redundancy of physiologic systems may yet defeat this approach to the therapeutic goal in these conditions.

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