

Crystal structure of human aquaporin 4 at 1.8 Å and its mechanism of conductance

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Aquaporin (AQP) 4 is the predominant water channel in the mammalian brain, abundantly expressed in the blood–brain and brain–cerebrospinal fluid interfaces of glial cells. Its function in cerebral water balance has implications in neuropathological disorders, including brain edema, stroke, and head injuries. The 1.8-Å crystal structure reveals the molecular basis for the water selectivity of the channel. Unlike the case in the structures of water-selective AQPs AqpZ and AQP1, the asparagines of the 2 Asn-Pro-Ala motifs do not hydrogen bond to the same water molecule; instead, they bond to 2 different water molecules in the center of the channel. Molecular dynamics simulations were performed to ask how this observation bears on the proposed mechanisms for how AQPs remain totally insulating to any proton conductance while maintaining a single file of hydrogen bonded water molecules throughout the channel.

brain edema | inhibitor discovery | NPA motif

The aquaporin (AQP) family includes both AQPs that conduct water, but not glycerol, and aquaglyceroporins that mediate diffusion of water, glycerol, and certain other small molecules in their neutral form across biological membranes. In humans, 13 different AQPs (AQP0–12) provide for transport in different tissues, each of which has broad clinical importance (1, 2). Besides AQP4, AQP1 and AQP9 are also expressed in the brain (3); AQP1 is expressed in the epithelial cells of the choroid plexus, and has a role in cerebrospinal fluid production, whereas AQP4 is localized to the endfeet of astrocytes in contact with the blood vessels of the blood–brain barrier and in astrocytic processes in contact with synapses. From its tissue-specific concentrated localization in closely packed tetragonal arrays, and the improved response to water intoxication or stroke in AQP4^{-/-} knockout mouse, it is thought that AQP4 is primarily responsible for cerebral water homeostasis (4). AQP4 also may be involved in buffering altered potassium ion concentration after neuronal activity due to its codistribution with KIR4.1 potassium channels in synapses (5). AQP9 is an aquaglyceroporin also found in astrocytes, and with suggested roles in glycerol and monocarboxylate diffusion and energy metabolism in catecholaminergic neurons.

The secretion and absorption of cerebrospinal fluid is precisely controlled, because the brain is encased within the rigid cranium; thus, any increase in intracranial pressure caused by edema can lead to compression of brain tissues resulting in neurological disorders and cell death. Because of its role, inhibitors of AQP4 are sought, although so far with conflicting results. Certain quaternary ammonium compounds (6), antiepileptic drugs (7, 8), and serotonin receptor agonists (9) have been reported to inhibit AQP4 water transport in oocytes with IC₅₀ values down to the low micromolar range. However, Yang et al. (10) report no inhibition up to 100 μM using different assay methods. Therefore, the atomic resolution structure of human AQP4 provides the means of discovering and validating therapeutic agents that might diminish damage from stroke, tumor-associated edema, epilepsy, traumatic head injury, and other CNS disorders associated with brain water imbalance.

AQP4 is the primary target in the autoimmune disease neuro-myelitis optica (NMO). Primarily affecting the optic nerves and

spinal cord, AQP4-specific autoantibodies (NMO-IgG) activate the complement-mediated inflammatory demyelination and necrosis (11). Knowing the structure around the epitopes for the NMO-IgG can facilitate discovery of agents that may compete for, or alter the site without triggering the complement cascade.

On a structural level, AQP4 is unique among AQPs that it exists in 2 isoforms owing to the use of 2 different translation initiation sites at methionine M1, or at M23. The M1 and M23 isoforms have very different effects on array formation with the shorter isoform favoring larger arrays mediated by 2 symmetric interactions between Arg-108 of each molecule and Tyr-250 of another molecule in the neighboring tetramer (12, 13). The C-terminal 3 amino acids, –SSV, serve as the ligand of a PDZ binding partner, α-syntrophin, which is a component of the dystrophin protein complex that links AQP4 to the actin cytoskeleton (14). Such bridged connection between AQP4 and the actin cytoskeleton allows AQP4 to be anchored at the endfeet of astrocytes such that transgenic mice deficient in α-syntrophin completely lack such polarized expression in astrocytes (15).

AQP4 is not sensitive to inhibition by mercury (16), because it does not have the reactive cysteine residue in the lumen of the channel corresponding to Cys-191 in AQP1 (17). AQP4 conductance is reduced >50% by phosphorylation mediated by protein kinase C at Ser-180 (18, 19), and increased ≈40% by protein kinase G activity at Ser-111 (20). The gating mechanism by phosphorylation events may be similar to that of the spinach AQP SoPIP₂;1 (21, 22).

To delineate the cellular organization, immune related properties, and the prospects for inhibitor development, we determined the 1.8-Å crystal structure of human AQP4 from heterologously expressed protein. To date, there are only 3 crystal structures reported for mammalian AQPs, 2 purified from naturally rich sources, AQP1 in red blood cells, AQP0 from the eye lens (23, 24), and human AQP5, from protein heterologously expressed in *Pichia pastoris* (25).

Results

Overall Architecture. Crystals of the M1 isoform full-length AQP4 diffracted to ≈8 Å in space group I4. Trypsinization improved the resolution. Both full-length and trypsinized protein (hAQP4) have water conductance in reconstituted proteoliposomes (Fig. S1a). The hAQP4 crystallized in space group P4₂1₂, although packing interactions between the tetramers are completely different from in the electron diffraction structure of rAQP4 (13). The rAQP4 structure was determined to 3.2 × 3.6 Å with no water or glycerol

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The authors declare no conflict of interest.

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.rcsb.org (PDB ID code 3GD8).

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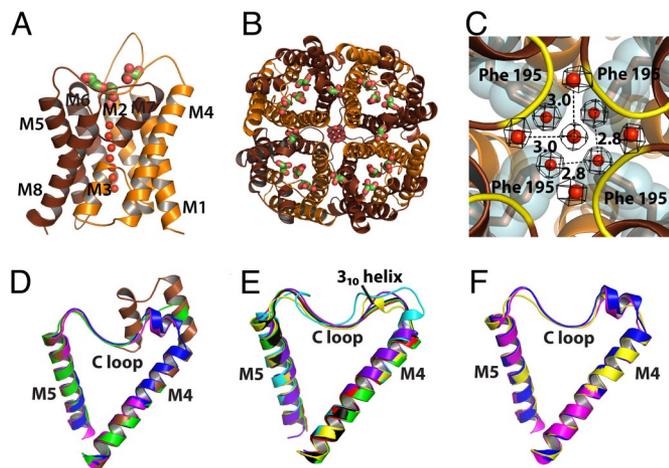


Fig. 1. General features. (A and B) Monomer and tetramer views of hAQP4 in diagram representation. Brown and orange colors represent the N- and C-terminal pseudo 2-fold related portions. Water molecules are represented as red spheres, and glycerol molecules are shown as green sticks. (A) The side view of the monomer. Helices are labeled M1 to M8. (B) The tetramer viewed from the extracellular side down the crystallographic 4-fold symmetry axis. (C) The network of water molecules found at the intracellular side of the central pore. The central pore is at the crystallographic 4-fold symmetry axis, and is formed by the tetramer. The 2Fo-Fc density of the water molecules is shown in black, contoured at 1.2 σ . The backbone amides of Ser-188 and Gly-189 are colored yellow in diagram representation. Phe-195 is shown as brown stick and cyan surface. (D–F) Diagram representation of the C loop of all of the AQP X-ray structures solved to date. (D) *E. coli* GlpF (brown), archeal AqpM (magenta), spinach AQP SoPIP2;1 (blue), and PfAQP (green). (E) Rat AQP4 (yellow), human AQP4 (black), human AQP5 (red), *E. coli* AqpZ (cyan), bovine AQP0 (green), and bovine AQP1 (purple). (F) Comparison of the 3_{10} helix of rat AQP4 (yellow) with the 2-turn helix of AqpM (monomer) and spinach AQP (blue). All structural renderings were made with PyMOL (<http://www.pymol.org>).

molecules observed. The X-ray structure of hAQP4 at 1.8-Å resolution shows water molecules throughout the channel, 5 glycerol, and 1 octyl glucoside molecule. Each monomer, surrounded by 6 and 2 half-length alpha-helices (M1 to M8), tetramerizes along the crystallographic 4-fold *c* axis (Fig. 1A and B) (26).

Central 4-Fold Axis. The physiological 4-fold axis insulates against all solutes and water. On the cytoplasmic side, a 4-fold arrangement of water molecules is stabilized by the backbone amides of Ser-188 and Gly-189 (Fig. 1C). Throughout ≈ 21 Å of the midmembrane section, Phe-195, Leu-191, and Leu-75, repeated 4 times, create a hydrophobic block. This observation contrasts with the 4-fold axis in *Plasmodium falciparum* aquaglyceroporin, PfAQP, where the region is blocked by 4 aliphatic chains of phospholipids or fatty acids (27), and the human AQP5 where a single lipid molecule is found (25).

Rat AQP4 and Human AQP4. Although hAQP4 and the electron diffraction structure of rAQP4 in lipid bilayers crystallize in the same space group (P4₂2₁), their crystal lattice contacts lie on different surfaces of the protein. The hAQP4 3D crystal contains head-to-head contacts only, because tetramers within the horizontal plane are too far apart ($a = 82.1$ Å) to make contact with each other (Fig. S2a). The rAQP4 2D crystal lattice has tetramers closer together ($a = 69.0$ Å), and contains both in-plane and between-plane contacts of the latticed tetramers. Based on the molecular contacts in the crystal, the interaction between the short 3_{10} helices in the C loop was proposed to be a possible mechanism for AQP4-mediated cell–cell adhesion (Fig. S2b) (13). Although the sequences of the C loop are the same in hAQP4 and rAQP4 (Fig. S3), hAQP4 does not adopt the short 3_{10} helix in this region (Fig. S4).

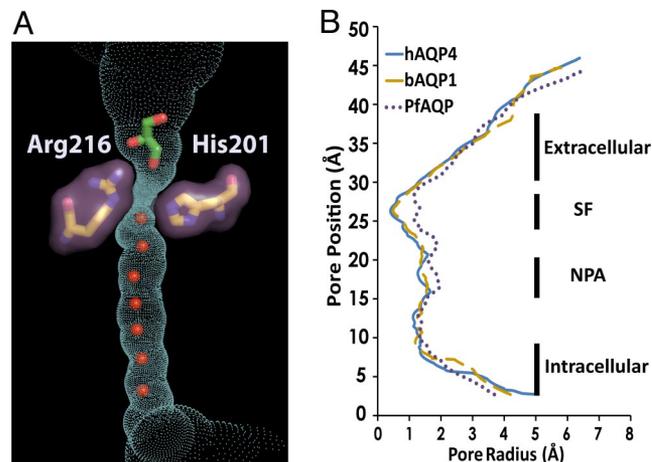


Fig. 2. The conducting pore. The trace of the pore inner surface is shown in cyan. The selectivity filter residues, Arg-216 and His-201, are shown as sticks with surfaces in purple. The glycerol molecule is shown as green stick, and the water molecules in the channel are shown as red spheres. (B) Plot of the channel radius versus position along the pore for human AQP4, bovine AQP1 (bAQP1), and the *P. falciparum* AQP (PfAQP). Regions of the channel are labeled as extracellular vestibule, the selectivity filter (SF), the NPA motif, and the intracellular vestibule. The pore inner surface and its dimension are calculated using Hole 2.0 (51).

Extracellular Vestibule, Selectivity Filter, and Conducting Pore.

AQP4 is a water-selective channel. Signature to the water-selective channels, His-201 lies directly in the selectivity filter, reducing the channel diameter to ≈ 1.5 Å, sterically excluding the passage of glycerol (Fig. 2). AQP4 was purified and crystallized in the presence of 5% (vol/vol) glycerol (0.7 M), and 3 glycerol molecules are found in the extracellular vestibule, although not in the selectivity filter where the 2 glycerol-conducting AQPs, GlpF, and PfAQP, bind glycerol identically to one another (26, 27) (Fig. S5). In the water-selective rAQP1, the double mutant Phe56Ala and His180Ala (Phe-77 and His-201 in hAQP4) (Fig. S5) allows for the passage of glycerol, showing that steric occlusion is one mechanism for exclusion of larger solutes (28).

The ≈ 25 -Å long conducting pore contains a line of water molecules and no solute molecule. However, the electron density of the water molecules are distributed along the pore with residual positive $F_o - F_c$ density observed in between water positions indicating increased anisotropic distribution along the channel axis, implying low-energy barriers between the water molecules along the direction of the channel (Fig. 3). As in other AQPs, the pathway through the channel is amphipathic. The hydrophobic sides are formed by the side chains of Phe-77, Ile-81, Val-85, Leu-170, Ile-174, and Val-197. The 8 backbone carbonyls of Gly-93, Gly-94, His-95, and Ile-96, from the cytoplasmic side and Gly-209, Ala-210, Ser-211, and Met-212 form the hydrophilic hydrogen bond acceptors for 8 positions of water molecules in transit. This arrangement allows bidirectional conductance of water from either side of membrane.

The asparagines 213 and 97 of the 2 almost totally conserved Asn-Pro-Ala (NPA) motifs form the canonical “fireman’s grip-like” structure in the center of the pore (26), and provide the defining force that orients water as it passes through the midpoint of the channel. However, in hAQP4, each asparagine donates its single, highly oriented hydrogen bond to a separate water molecule (Fig. 3). This arrangement is a key variant, because in 3 other water-selective AQP structures (23, 24, 29), the 2 asparagines donate hydrogen bonds to a single water molecule.

Conductance of hAQP4 in Proteoliposomes. Both full-length hAQP4 and trypsinized hAQP4 were reconstituted into proteoliposomes, and water and glycerol conduction were measured. The proteoli-

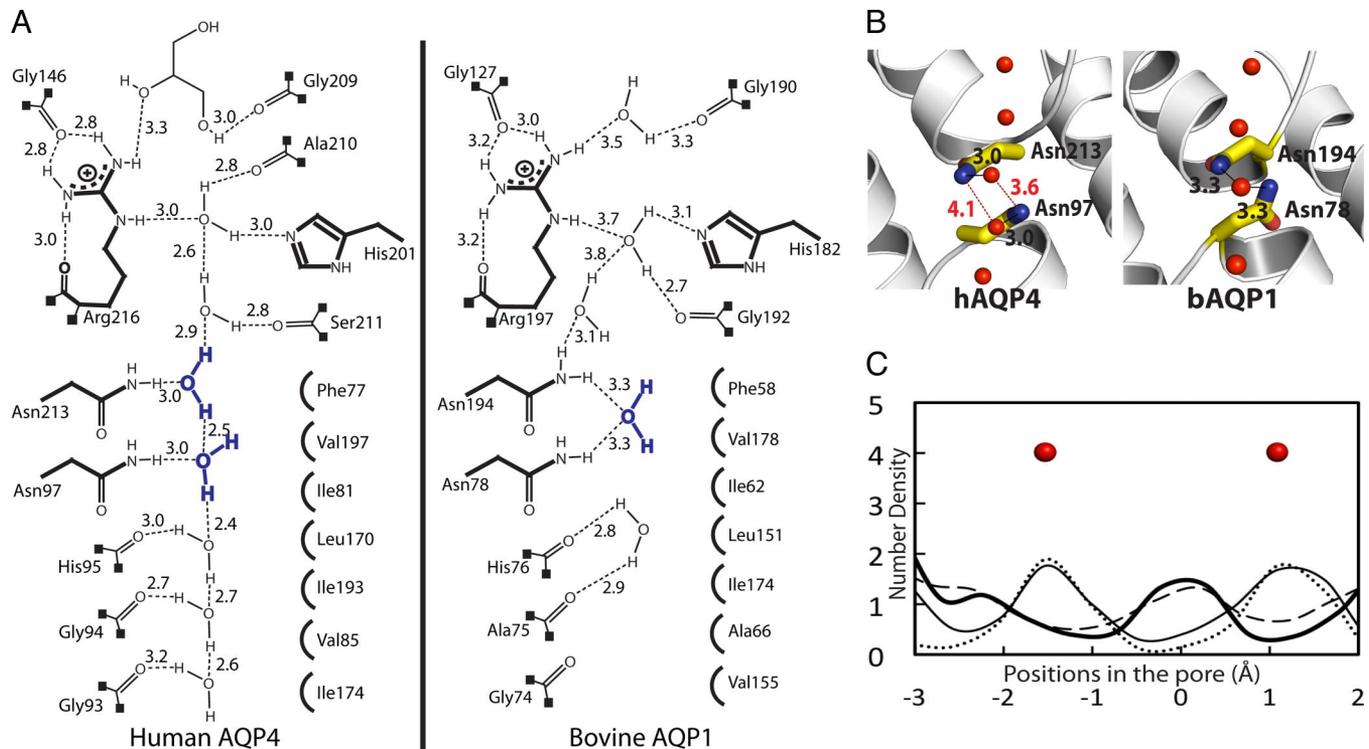


Fig. 5. The NPA motifs. (A) Schematic representation of the hydrogen bonding network through the channels of hAQP4 and bAQP1. The distances are between heavy-atom to heavy-atom. (B) Stick representation of the NPA motifs. Distances that are too long to be a hydrogen bond are colored in red. (C) Plot of the MD simulations of hAQP4 from 4 different experiments. Details are described in Discussion.

in the vestibule could represent “fragment binding sites” for defining inhibitors that would bind from the extracellular side.

Because there are tremendous prospects for drugs that inhibit AQP4, and indeed many inhibitors of AQP4 have been described in the literature to date, we cocrystallized hAQP4 with 5 mM of 3 such compounds, tetraethylammonium (TEA), acetazolamide, and rizatriptan, and determined their cocrystal structures. In oocytes swelling assays, these compounds were reported to be AQP4 inhibitors with IC_{50} in the micromolar range (6–9); however, we have not been able to detect any compound bound in the structures. We then reconstituted purified hAQP4 into liposomes, and measured water conductance in the presence of these compounds. TEA up to 10 mM had no effect on hAQP4 water conductance. Acetazolamide and rizatriptan do inhibit water conductance with an approximate IC_{50} in the low millimolar range (≈ 3 and ≈ 1 mM, respectively; Fig. S1b). We argue that water conductance measurement using purified proteoliposomes is more reliable than in oocytes. In a similar experiment, acetazolamide was also found to inhibit rAQP4 in the mM range, but not hAQP1 (33). These results call into question the previous interpretation of TEA, acetazolamide and rizatriptan as micromolar inhibitors of AQP4.

Arginine 216 Environment Determines Conductance of Water in AQPs.

Because the glycerol conducting PfAQP conducts water as well as a water channel, whereas GlpF conducts water very poorly, we proposed that the efficiency of water conductance may be proportional to the effective neutralization of the formal positive charge on the arginine side chain by 5 hydrogen bond acceptors, 3 from the protein and 2 from the waters in transit (27). The hAQP4 is a water-specific channel, and similar to all high water conductance AQPs, the $N\epsilon H$, $N\eta^1 H$, $N\eta^2 H$ of Arg-216 in the selectivity filter are all hydrogen bonded to other acceptor oxygens of the protein, leaving 2, one each from $N\epsilon H$ and $N\eta^1 H$ as donors to the waters in transit (Fig. 4). For the water-selective bAQP1, the arginine (Arg-197) environment is very similar to that in hAQP4 (Fig. 4).

However, in the glycerol conducting GlpF, with low water conductance, the selectivity filter arginine (Arg-206) has only one of the $N\eta^1 H$ satisfied (Fig. 4), so it is possible that the extra cost of desolvating the fully charged guanidinium leaves GlpF “holding on” to water molecules in transit. This higher degree of the guanidinium cation buffering by the protein may be the basis for higher efficiency water transport among the AQPs.

Variant Role of the Dual NPA Motifs in Proton Exclusion. The interlocking of 2 almost totally conserved NPA motifs in AQPs provides 2 highly oriented donors from $N\delta H_2$ of the 2 asparagines to the center of the channel. We proposed that they have a key role in insulation against any conduction of protons or ions while supporting a single file of hydrogen bonded water molecules throughout the entire length of the channel (34). In the crystal structures of the water-selective *Escherichia coli* AqpZ and bAQP1, the central water is positioned in between the 2 asparagines of the NPA motifs, and receives 2 hydrogen bonds from the 2 asparagine $N\delta H_2$ s (represented by bAQP1 in Fig. 5A and B). Such orientation of the central water may be a factor in preventing proton conduction through the channel by the highly cooperative hop-and-turn Grotthuss relay mechanism (35, 36), because the central water cannot rotate to accept or exchange its proton (26, 37). However, the hAQP4 crystal structure does not have a water molecule centrally located between the 2 NPA motifs. Instead, each asparagine of the NPA motifs (213 and 97) donates a hydrogen bond to a different water molecule (Fig. 5). This arrangement is also observed in the crystal structures of hAQP5 and the spinach AQP SoPIP2;1. Therefore, in this subclass of AQP structures, these 2 water molecules may each be somewhat freer to rotate in a concerted fashion leaving 1 additional non-hydrogen bonded water hydrogen. These AQPs equally insulate against any proton leakage, suggesting that the 2 models for the central waters may represent intermediates that are very close in free energy.

To see whether this observation constitutes a difference in

cannot comment on the interaction of Ser-180 with the C-terminal domain, we think it is unlikely that the gating of AQP4 at Ser-111 is similar to SoPIP2₁, because the D loop of hAQP4 is 4 residues shorter than in SoPIP2₁, and residues in the N-terminal domain that are involved in binding to the D loop (Asp-28 and Glu-31) are different amino acids in hAQP4 (Fig. S3).

The high resolution structure of the phosphorylated protein, the M1 isoform, and complexes with binding elements of syntrophin may address these issues of larger scale assemblies beyond that of the tetrameric AQP4.

Materials and Methods

Materials and methods for protein expression and purification, crystallization, proteoliposome assay, and MD simulations are described in *SI Materials and Methods*.

For data collection and model building, diffraction data were collected using a wavelength of 1.11 Å at Beamline 8.3.1 at the Advanced Light Source (Lawrence Berkeley National Laboratory). Data were processed by using HKL2000 (43). Molecular replacement was performed with Phaser (44) using the rAQP4 electron

diffraction structure (PDB code: 2D57) (13), as a search model. Subsequent iterative cycles of manual building and retrained refinement were done using Coot (45) and Refmac5 in CCP4 (46). TLS refinement in Refmac5 was applied in the last stage using 19 TLS groups obtained from the TLS analysis server (Table S1 and Table S2) (47, 48). Riding hydrogen atoms were generated during refinement, but not written to the output. Structure was assessed using PROCHECK (49) and MolProbity (50). Data processing and refinement statistics are summarized in Table 1.

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