Crystal Structure of AqpZ Tetramer Reveals Two Distinct Arg-189 Conformations Associated with Water Permeation through the Narrowest Constriction of the Water-conducting Channel*

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AqpZ is a homotetramer of four water-conducting channels that facilitate rapid water movements across the plasma membrane of *Escherichia coli*. Here we report a 3.2 Å crystal structure of the tetrameric AqpZ (tAqpZ). All channel-lining residues in the four monomeric channels are found oriented in nearly identical positions with one marked exception at the narrowest channel constriction, where the side chain of a highly conserved Arg-189 adopts two distinct conformational orientations. In one of the four monomers, the guanidino group of Arg-189 points toward the periplasmic vestibule, opening up the constriction to accommodate the binding of a water molecule through a tridentate H-bond. In the other three monomers, the Arg-189 guanidino group bends over to form an H-bond with carbonyl oxygen of the Thr-183, thus occluding the channel. Therefore, the tAqpZ structure reveals two distinct Arg-189 conformations associated with water permeation through the channel constrictions. Alternation between the two Arg-189 conformations disrupts continuous flow of water, thus regulating the open probability of the water pore. Further, the difference in Arg-189 displacements is correlated with a strong electron density found between the first transmembrane helices of two open channels, suggesting that the observed Arg-189 conformations are stabilized by asymmetrical subunit interactions in tAqpZ.

Fluid balance is fundamental to osmoregulation of a large variety of cells. Rapid transmembrane fluxes of water or glycerol are facilitated by a family of membrane channels called aquaporins or aquaglyceroporins (AQPs), depending on their water/glycerol permeability (1, 2). Structures of several AQPs have been determined, including those of human AQP1 (3), *Escherichia coli* GlpF (4), bovine AQP1 (5), and *E. coli* AqpZ (6) as well as sheep and bovine AQP0 (7, 8). All known AQP structures share a common molecular architecture containing four monomeric subunits assembled into a homotetrameric structure. Each monomer is characterized by an hourglass-like structure with an internal quasi-2-fold symmetry, corresponding to an intragenic gene duplication of AQP protein sequences (9). The functional unit of AQPs is a monomer that embraces a central water/glycerol-conducting channel surrounded by six transmembrane helices and two half-spanning helices joined end-to-end in the middle of the membrane by interlocking interactions of two highly conserved NPA tripeptide sequences. The channel lumen surface is strongly amphipathic, with oxygens and nitrogens lined up on one side and carbons on the opposite side (4). Molecular dynamic simulations of AQPs have suggested that water molecules can coordinate each of the hydrogen-bonding groups to form a continuous single file that spans the entire length of the water-conducting channel (10, 11). The rate of permeation is limited by two significant energetic barriers in the channel, corresponding to two channel constriction regions: one is located at the narrowest point at the channel entrance on the periplasmic side and the other at the NPA region about the quasi-2-fold axis. The narrowest constriction has been identified as the selective filter of the channel, which is formed by the side chains of Arg-189, Phe-43, and His-174 in the water-conducting channel AqpZ (6) or Arg-206, Phe-200, Trp-48 in the glycerol-conducting channel GlpF (4). These side chain differences render a narrower and more hydrophilic selective filter in AqpZ, accounting for its selectivity for water over glycerol (6). Recent structural solutions of two AQP0 proteins have shown that the AQP0 water pore is much more constricted compared with those of AqpZ, AQP1, and GlpF (7, 8). The narrowest region of the AQP0 channel is formed by an additional channel constriction in the cytoplasmic half of the pore, suggesting that additional structural components may contribute to limiting water permeation through the AQP0 channels, the rate of which is ~40 times lower than that of AQP1 at neutral pH (12).

The emerging evidence suggests that AQP channels are not constitutive conduits of water or glycerol. The crystal structures of AQP1, GlpF, and AqpZ all showed that their narrowest constrictions (selective filters) are open to water/glycerol binding, whereas the electron diffraction structure of the sheep AQP0 revealed a closed water pore. Intriguingly, the x-ray structure of bovine AQP0 exhibited a water-filled channel that contained constriction regions narrower than would be required for the passage of a water molecule. Thermal fluctuations of AQP0 side chain rotamers appeared to cause dilation of channel constrictions to larger sizes sufficient for a water molecule to pass; thus, the bovine AQP0 channel was thought to be dynamically open to water passage. This finding raised a question as to how the side chain dynamics play a role in controlling the AQP channel opening or closing. Molecular dynamic simulations of AqpZ revealed thermal fluctuations of the Arg-189 side chain between two distinct conformational states, resulting in an open or closed channel conformation (13). The thermodynamic gradient is expected to be quite shallow for this thermally activated conformational equilibrium. It is not clear whether the simulated Arg-189 thermal fluctuations could lead to steady states of channel...
opening and closing in milliseconds to hundreds of milliseconds, a time scale commonly observed in ion channel kinetic studies (14, 15).

In the present study, we determined the AqpZ tetrameric structure in the space group of P4₁2₂ with two tetrath- asymmetrical unit; thus, the four monomeric structures were solved independently without crystallographic averaging. Intriguingly, the tAqpZ structure revealed two distinct conformations among the four monomeric channels. One of the four channels was found in a fully open state, whereas the remaining three were closed by the side chain displacement of Arg-189 that occluded the selective filter. The tAqpZ structure therefore provides structural evidence for the presence of two distinct channel conformations during water permeation. A unique crystal packing contact between two open channels is implicated in a role of trapping the Arg-189 side chain. The tAqpZ structure therefore provides structural evidence for the presence of two distinct channel conformations during water permeation. A unique crystal packing contact between two open channels is implicated in a role of trapping the Arg-189 side chain. Further, Arg-189 is the only channel-lining residue in tAqpZ that shows a significant side chain displacement. This finding highlights the localization of the channel conformational dynamics to the narrowest constriction region.

**EXPERIMENTAL PROCEDURES**

**Expression, Purification, and Crystallization**—As described previously (16), His-AqpZ was overexpressed in BL21 (DE3)pLysS cells and purified by one-step metal affinity chromatography. The N-terminal His tag was then removed by thrombin digestion, resulting in AqpZ that was further purified by size-exclusion high performance liquid chromatography and concentrated to ~10 mg/ml prior to crystallization screens. The best AqpZ crystals were obtained in hanging drops by a 1:1 ratio of protein/precipitant solution and concentrated to ~20 mg/ml. The crystals belonged to space group P4₁2₂ with two tetramers/asymmetrical unit; thus, the four monomeric structures were solved independently without crystallographic averaging. Intriguingly, the tAqpZ structure revealed two distinct conformations among the four monomeric channels. One of the four channels was found in a fully open state, whereas the remaining three were closed by the side chain displacement of Arg-189 that occluded the selective filter. The tAqpZ structure therefore provides structural evidence for the presence of two distinct channel conformations during water permeation. A unique crystal packing contact between two open channels is implicated in a role of trapping the Arg-189 side chain. Further, Arg-189 is the only channel-lining residue in tAqpZ that shows a significant side chain displacement. This finding highlights the localization of the channel conformational dynamics to the narrowest constriction region.

**RESULTS**

**Determination of the tAqpZ Structure**—Diffraction data to 3.2 Å were collected under cryoconditions. AqpZ crystals belonged to space group P4₁2₂ (a = b = 119.152, c = 380.394) with two tetrath-asymmetrical unit, related by a 2-fold NCS axis perpendicular to the crystallographic c-axis. The structure was solved by molecular replacement and initially refined to an R cryst of 19.1% and R free of 36.9% without any NCS restraint. Applying gradual weighted restraints between two tetraths led to minimization of tetrath-tetrath root mean square differences (r.m.s.d.) to 0.01 Å, accompanied by narrowing of the R cryst-R free difference. Thus, a strict 2-fold NCS constraint was adopted to further refine the structural model, thereby reducing the R cryst of 23.1% and R free of 25.0%. At this stage of refinement, several strong electron densities were evident on the surface of the membrane-spanning domain. The locations and appearances of these electron densities were indicative of ordered detergents and lipids. The numerical values of R cryst and R free were further improved to 22.5 and 24.7%, respectively, by modeling these detergent/lipid densities.

The strongest water electron densities were observed on the tetramer peripheral surface as well as in the center of the AqpZ tetrath (Fig. 1A). The strongest water electron densities were observed within four monomeric channels, A, B, C, and D (Fig. 1B).

The positioning of two homotetramers in the asymmetric unit showed 5’ skewing of the tetrath quasi-4-fold axes around the crystallographic c-axis. However, applying additional tetrath 4-fold NCS constraints did not improve structural refinement, suggesting that there are genuine structural differences among the four AqpZ monomers. Alignment of Cα traces yielded a r.m.s.d. of 0.25–0.29 Å among four monomers, with differences mainly visible in the extracellular loops where different crystal contacts were made between AqpZ monomers. Slightly higher r.m.s.d. differences (0.27–0.43 Å) were obtained when different crystal contacts were made between AqpZ monomers. Similar structural differences (r.m.s.d. = 0.44 Å) were also noted between two monomers in the P4 AqpZ crystals (6).

**Structure of the Water-conducting Channel**—The monomeric structure of AqpZ displays six transmembrane helices and two half membrane-spanning helices in a right-handed helical bundle that revolves around a pseudo-2-fold symmetry axis in the membrane plane as described previously (6). The AqpZ channel consists of a periplasmic and a cytoplasmic vestibule, both conical in shape and connected by a narrow amphipathic pore ~25 Å in length. The hydrophilic face provides two patches of solvent-accessible carboxyl oxygens, each con-

**TABLE 1**

| Data collection and refinement statistics | 1.0 |
|-----------------------------------------|-----|
| Resolution (Å)                          | 50.0–3.2 |
| Reflections total/unique                | 129,681/46,497 |
| Completeness                            | 99.6 (97.0) |
| Multiplicity                            | 28.2 (2.4) |
| Rmerge (%)                              | 12.2 (34.6) |
| Rfactor (%)                             | 9.8 (3.3) |
| Space group                             | P4₁2₂ |
| Unit cell dimension (Å)                 | a = b = 119.067, c = 380.23 |
| R cryst/R free (%)                      | 20.1/23.9 |
| R.m.s.d. bonds (Å)                      | 0.008 |
| R.m.s.d. angle (degree)                 | 1.3 |
| Reflections in working/test set         | 43,046/21,434 |
| Nonhydrogen protein atoms               | 6,576 |
| Nonhydrogen heteroatoms                 | 132 |
| Waters                                  | 270 |
| Average B-factor, all atoms/Wilson plot (Å²) | 35.9/43.9 |
Structure of the AqpZ Tetramer

Different Water Binding in AqpZ Channels—Clusters of discreet electron densities were observed in each of the AqpZ monomer channels (Fig. 1B). The strongest electron densities were located in the periplasmic half of the central amphipathic pore between the selective filter and the NPA junction, resulting in unambiguous assignments of several water molecules. Unexpectedly, the number of water molecules and their locations are different among the four monomer channels. In the selective filter region, there are two water molecules (WA1, WA2) in monomer A, one (WB1) in monomer B, and no water observed in monomers C and D (Fig. 2, A--D). Locations of WA1 and WA2 do not overlap with WB1. WA1 is above the selective filter, where it is hydrogen bonded to the NH of the Arg-189 guanidino group (O-N distance of 2.7 Å). WA2 snugly nests within the selective filter, forming a tridentate H-bond with the NE2 of the His-174 imidazole group (O-N 2.6 Å), NE of Arg-189 guanidino group (O-N 2.6 Å), and Thr-183 main chain carboxyl oxygen (O-O 2.9 Å). WB1 is below the selective filter coordinated by the Thr-183 carbonyl oxygen (O-O 3.1 Å) and the Arg-189 NH1 (O-N 2.7 Å). In the NPA region, there are two water molecules (WA3 and WA4) in monomer A, three in monomer B (WB2, WB3, WB4), two in monomer C (WC1, WC2), and one in monomer D (WD1). These water molecules occupy the same general positions. From NPA junction to the cytoplasmic vestibule, the innermost waters (WA3, WB2, WC1) are present in monomers A, B, and C, forming a coordinating H-bond with either ND2 of Asn-186 or Asn-63 (O-N distances, 2.9–3.0 Å). The middle waters (WA4, WB3, WC2, WD1) are invariantly present in all four monomers and bind to His-61 carbonyl oxygen (O-O distances, 2.6–2.9 Å). The outermost water (WB4) is only seen in monomer B with an H-bond with the carbonyl oxygen of Gly-60 (O-O distance, 2.9 Å). The orientations of water molecules in all four monomers are consistent with a bipolar ordering with their oxygen atoms facing the center of the channel as described previously (11).

Two Distinct Arg-189 Conformational Orientations in the Channel-selective Filters—Channel-lining hydrogen bond donor and acceptor groups in the four monomer channels are approximately held in the same positions, with one marked exception occurring in the selective filter where the side chain of Arg-189 was found in two distinct conformational orientations. Omit maps on Arg-189 (before inclusion of waters) show a difference in the placement of the guanidino group of Arg-189 in the monomer A as compared with those in monomers B, C, and D, which are essentially identical (Fig. 1C). In monomer A, the Arg-189 guanidino group is orientated parallel to the length of the channel, creating an opening in the selective filter for accommodation of a bound water molecule, WA2 (Fig. 2A). This upward Arg-189 orientation is approximately identical to those of Arg-189 equivalents observed in all of the AQP x-ray structures determined thus far. In monomers B, C, and D, the Arg-189 guanidino group bends over toward the carbonyl oxygen of Thr-183 to form an NH1-O hydrogen bond and closes up the selective filter (Fig. 2, B--D). To validate two different fits of the Arg-189 side chain, we switched Arg-189 side chains between monomers A and B-D, removed water molecules from the model, and applied energy minimization and simulated annealing. Arg-189 side chain orientations in both monomers A and B--D returned to their original conformations. Furthermore, we replaced the Arg-189 side chains in monomer A with several randomly selected rotamers and found that these different rotamers converged on a single upward orientation by energy minimization and annealing in the absence of water molecules. Likewise, Arg-189 rotamers in monomers B and C converged on the same downward orientation regardless of the initial rotomeric conformations. B-factors for atoms in Arg-189 were in the range of 21–30 for monomer A, 23–26 for monomer B, 34–38 for monomer C, and 36–41 for monomer D. The

tributed by four adjacent main chain carbonyls of Asn-182, Thr-183, Ser-184, and Val-185 from the periplasmic side and those of quasi-2-fold related Gly-59, Gly-60, His-61, and Phe-62 from the cytoplasmic side. Between these main chain carbonyls is the highly constrained and interlocked NPA conjunction centered about the quasi-2-fold axis where Asn-186 and Asn-63 project ND2 groups of their side chains to the channel. This complete set of pore-lining carbonyl oxygens and asparagine amines are arranged on the hydrophilic face to form a contiguous chain of hydrogen bond acceptors and donors for coordination of water molecules, thus establishing a water-conducting pathway. On the opposing hydrophobic face of the channel, an abundance of valines, phenylalanines, and isoleucines provides steric limits to shape the size of the pore. The channel contains two highly conserved regions, the selective filter and the NPA region. Located ~10 Å away from the NPA junction on the periplasmic side is the selectivity filter formed by the side chains of His-174, Arg-189, and the carbonyl of Thr-183 on the hydrophilic face and the aromatic ring of Phe-43 on the opposite hydrophobic face (Fig. 2).
overall B-factor values of Arg-189 in monomers C and D were similar to the average AqpZ B-factor (35.9), whereas binding of water molecules to monomers A and B reduced the Arg-189 B-factors to below the average. Therefore, these analyses supported the two different fits of the Arg-189 guanidino group. The observed side chain displacement was unique to Arg-189, because side chain orientations of all other channel-lining residues were nearly identical. Further, we only observed two distinct Arg-189 side chain conformations among four monomer channels. This observation was in agreement with molecular dynamic simulations of AqpZ showing that Arg-189 side chain could only adopt one of two distinct conformations, as opposed to many randomly rotameric conformations (13).

Different Crystal Packing Interactions in the Membrane-spanning Domain—To understand how the Arg-189 in monomer A could be stabilized to a conformation differing from those in monomers B, C, and D, we examined the difference in crystal packing contacts between monomer A versus B, C, and D. As shown in Fig. 3A, there are three different types of intersubunit contacts in the xy-plane: A-A and B-B contacts at the interface of two AqpZ tetramers within an asymmetric unit and a C-D contact between two asymmetric units. The tetramer-tetramer interface is formed by an angular association (≈5° in the xy-plane) of two monomer A proteins; thus, monomer A is more extensively involved in the tetramer-tetramer contact. Monomer B only partially contributes to the tetramer interface, whereas monomer C and D are completely disconnected from the tetramer interface. The buried surface areas at the A-A, B-B, and C-D interfaces are 1087.8, 885.3, and 500.8 Å², respectively. A close examination of the A-A contact revealed a strong electron density that connected two aromatic rings of Phe-10 in the first transmembrane helices (Fig. 3A). The elongated appearance of the electron density was suggestive of a lipidic nature, but we were not able to make a molecular assignment at the current resolution. The contact point at Phe-10 is close to the highly conserved Glu-8, which forms two hydrogen bonds with main chain amines to orient four successive main chain carbonyls of Gly-59, Gly-60, His-61, and Phe-62 on the channel lumen surface. The carbonyl of Phe-62 forms another highly conserved hydrogen bond with ND2 of Asn-186 as a part of the interlocking hydrogen bond network that holds the two NPA signature sequences together. The OD1 of Asn-186 forms yet another highly conserved hydrogen bond with the main chain amine of Arg-189. Thus, Arg-189 is connected to Glu-8 through a chain of highly conserved hydrogen bond interactions (Fig. 3B). The geometry of this chain of hydrogen bonds remains almost identical among the four subunits, suggesting the rigidity of these hydrogen bonds, mainly attributed to the constrained NPA-NPA interlocking interaction. It appears that the side chain of Arg-189 is the weakest link at the end of this rigid chain of hydrogen bonds, which may allow the impact of crystal packing contact at Phe-10 to be delivered effectively to Arg-189. Apparently, this unique lateral crystal packing contact between two monomers A is correlated with the open conformation of this subunit. Crystal stacking contacts in the z direction are also different among four subunits. However, there is no obvious correlation to implicate how differences in vertical stacking contacts may contribute to stabilization of the Arg-189 conformations.

FIGURE 2. Water binding in the selective filters of monomers A, B, C, and D as indicated (viewed from the membrane plan, periplasmic side on the top). Note that the guanidino group of Arg-189 in monomer A adopts a different conformational orientation than those in monomers B, C, and D.
DISCUSSION

All the known AQP crystal structures thus far have been determined with crystallographic averaging of monomeric structures due to crystal packing symmetries. The tAqpZ structure represents a unique tetrameric solution that allows the observation of two distinct Arg-189 side chain orientations in the channel-selective filter. This observation is partially consistent with molecular dynamic calculations that also suggested the presence of two distinct conformations. Nevertheless, the Arg-189 side chain in molecular dynamic simulations appeared to be unusually dynamic, in rapid equilibrium between two distinct conformations driven only by thermal fluctuations. In contrast, the B-factors of the Arg-189 side chains in the tAqpZ structure were similar or lower.
than the average protein B-factor, showing no sign of excessive flexibility. Further, the omit maps on Arg-189 exhibited distinct side chain features, indicative of two orientations of the Arg-189 guanidino group even at a limited resolution. Refinement of Arg-189 conformations consistently converged to one of the two distinct orientations, regardless of the initial rotameric conformations. These results suggest that the observation of two distinct Arg-189 conformations in tAqpZ crystals is valid. It is not clear, however, whether the two Arg-189 conformations correspond to open and closed states of an AqpZ channel or whether they represent two dynamic conformations found during water permeation through the narrowest constriction of the channel. Without kinetic measurement of possible opening and closing of AqpZ channels by single channel recording, the crystal structure of tAqpZ only provides clues as to the transient nature of the Arg-189 conformation. The structural difference between the “open” and “closed” conformation is mainly localized to the Arg-189 side chain orientations. This lack of a more global protein conformational change suggests a marginal free energy barrier separating the two Arg-189 conformational states. Thus, the observed two Arg-189 conformations are likely interconvertible by thermal fluctuation. The thermodynamic equilibrium between two Arg-189 conformations is anticipated to regulate the open probability of the water pore, whereas the shallow thermodynamic gradient between the two Arg-189 conformations may be inadequate to stabilize the AqpZ channel into either one of these two states without additional stabilizing free energy input.

The Arg-189 in tAqpZ crystals appeared to be trapped into two distinct stable conformational states by asymmetrical crystal packing contacts. Unlike earlier AQP crystal structures where there was no or little packing interaction involved with the transmembrane domain, the tAqpZ structure reveals lateral tAqpZ-tAqpZ interactions in the transmembrane domains and demonstrates that asymmetrical crystal packing contacts are correlated with differences in Arg-189 side chain displacements. The conformations of four Arg-189 side chains are stabilized differently, whereas orientations of other side chains are essentially identical in four monomer channels. This finding highlights the dynamic nature of the Arg-189 side chain in a rather stable local protein fold. The unique Arg-189 conformation in monomer A is linked to a crystal packing interaction at Phe-10 in the first transmembrane helix. Because the molecular dynamics calculations indicated that the orientations of Arg-189 are in equilibrium between two thermally activated rotameric states, it is anticipated that a modest free energy input may be able to stabilize Arg-189 in one of the two conformations. It is possible that interactions at Phe-10 may allosterically alter the equilibrium of Arg-189 conformations through a chain of highly constrained hydrogen bonds. Nevertheless, free energy differences derived from other asymmetrical crystal packing contacts may also contribute to stabilization of the Arg-189 in different conformations.

Most mechanisms ascribed to ion channels involve reversible transitions among discrete open and closed conformational states. The opening or closing of an ion channel results in discontinuous flow of electric current in an all-or-none fashion down an electrochemical potential gradient across the channel. Thus, channel opening and closing can be followed directly by single channel current measurements. Because water/glycerol permeation through AQP channels is not accessible to electrophysiological studies, high resolution crystal structures may be used to access the opening or closing of the water-conducting pore. A simplistic view of an open channel consists of one or several binding sites arranged in a transmembrane pathway that are accessible from both sides of the membrane at the same time (21). The Arg-189 conformation observed in monomer A allows a water molecule binding to the narrowest constriction of the channel; thus, monomer A is in an open channel conformation. The assessment of the closed channel conformation is not so clear-cut. The static water pore structure in the bovine AQP0 was noted to be too narrow for water permeation, but side chain rotameric movements could sufficiently widen the channel constrictions for water passage (8). Taking thermodynamics into consideration, membrane channels may be represented by a sequence of potential wells separated by activation barriers (22). Water permeation through membrane channels may involve a sequence of thermally activated jumps between sites of low potential energy. Because the activation barriers (channel constrictions) in AQP0 could be surmounted by thermal fluctuations of side chain rotamers, AQP0 could be considered a dynamically open channel. On the other hand, asymmetrical crystal contacts in tAqpZ trap monomers B, C, and D into a thermodynamically stable conformation in which NH1 of Arg-189 bends over to form a strong hydrogen bond (see below) with the main chain carbonyl oxygen of Thr-183 in the channel-selective filter, thus making the narrowest constriction of the channel not available for water binding. The main chain-side chain interaction between Thr-183 and Arg-189 was shown to be stable regardless of the initial Arg-189 rotameric conformations. Molecular dynamic simulations of the presteady state of the AqpZ channel also demonstrated that the closure of the selective filter by Arg-189 could lead to a complete dehydration of the water pore (13). These observations suggest that the Arg-189 conformation in monomers B, C, and D may impose an insurmountable activation barrier for thermally activated water permeation. Thus, monomers B, C, and D are in a closed channel conformation, stabilized in vitro by asymmetrical subunit interactions of crystal packing contacts. By analogy, in vivo AqpZ monomers may interact differently with cellular regulatory proteins, thereby regulating the overall tetramer water conductivity by varying the number of open or closed channels in an AqpZ homotetramer.

The driving force for a closed-to-open conformational transition appears to be electrostatic in nature. As shown in Fig. 4A, the closed state is energetically favored by bridging the Arg-189-Glu-138 charge
pair through the Thr-183 carbonyl oxygen and Ser-184 main chain amine nitrogen. NH1 from the Arg-189 guanidino group forms an H-bond with the Thr-183 main chain carbonyl oxygen, which is negatively charged due to the resonance of the main chain peptide bond under the influence of the Glu-138 carboxylate through a side chain-main chain hydrogen bond to the Ser-184 main chain amine nitrogen (O-N distance 3.0 Å). In the channel open state, the Arg-189 NH1-Thr-183 oxygen H-bond is replaced by two H-bonds involved with two water molecules: WA1 bound to Arg-189 NH2 and WA2 to Thr-183 oxygen. WA2 also bind to Arg-189 NE, thus connecting the Arg-189-Glu-138 charge pair through a string of hydrogen bonds mediated by WA2, the Thr-183 carbonyl oxygen, and Ser-184 main chain amine nitrogen (Fig. 4B). Therefore, the Arg-189 conformational equilibrium between the open and closed state is associated with two alternative hydrogen bond networks between Arg-189 and Glu-138. In both cases, the hydrogen bond networks are stabilized by an indirect Arg-189-Glu-138 interaction. This finding emphasizes the electrostatic contribution of the Glu-138 to the Arg-189 side chain dynamics and provides a mechanistic clue as to the observation of only two distinct Arg-189 conformations, as opposed to many random rotameric states. Intriguingly, as noted earlier, the quasi-2-fold symmetry-related Glu-8 might also contribute to Arg-189 conformational dynamics through a rigid chain of hydrogen bonds mediated by the NPA-NPA interlocking network. Arg-189, Glu-138, and Glu-8 are among the most conserved residues in the AQP protein family. The extremely high level of genetic conservation attests to their functional importance.

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REFERENCES
1. Preston, G. M., and Agre, P. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 11110–11114
2. Preston, G. M., Carroll, T. P., Guggino, W. B., and Agre, P. (1992) Science 256, 385–387
3. Murata, K., Mitsuoka, K., Hirai, T., Walz, T., Agre, P., Heymann, J. B., Engel, A., and Fujiyoshi, Y. (2000) Nature 407, 599–605
4. Fu, D., Libson, A., Miercke, L. J., Weitzman, C., Nollert, P., Krucinski, J., and Stroud, R. M. (2000) Science 290, 481–486
5. Sui, H., Han, B. G., Lee, J. K., Walai, P., and Jap, B. K. (2001) Nature 414, 872–878
6. Savage, D. F., Egea, P. F., Robles-Colmenares, Y., O’Connell, J. D., III, and Stroud, R. M. (2003) PloS Biol. 1, E72
7. Gonen, T., Sliz, P., Kistler, J., Cheng, Y., and Walz, T. (2004) Nature 429, 193–197
8. Harries, W. E., Akhavan, D., Miercke, L. J., Khademi, S., and Stroud, R. M. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 14045–14050
9. Wistow, G. J., Pisano, M. M., and Chepelinsky, A. B. (1991) Trends Biochem. Sci 16, 170–171
10. de Groot, B. L., and Grubmuller, H. (2001) Science 294, 2353–2357
11. Tajkhorshid, E., Nollert, P., Jensen, M. O., Miercke, L. J., O’Connell, J., Stroud, R. M., and Schulten, K. (2002) Science 296, 525–530
12. Chandy, G., Zampighi, G. A., Kreman, M., and Hall, J. E. (1997) J. Membr. Biol. 159, 29–39
13. Wang, Y., Schulten, K., and Tajkhorshid, E. (2005) Structure 13, 1107–1118
14. Katz, B., and Miledi, R. (1972) J. Physiol. 224, 665–699
15. Anderson, C. R., and Stevens, C. F. (1973) J. Physiol. 235, 655–691
16. Daniels, B. V., Jiang, J. S., and Fu, D. (2004) Acta Crystallogr. Sect. D Biol. Crystallogr. 60, 561–563
17. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol., Macromol. Crystallogr. 276, 307–326
18. Tong, L. A., and Rossman, M. G. (1990) Acta Crystallogr. Sect. A 46, 783–792
19. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D Biol. Crystallogr. 54, 905–921
20. Read, R. J. (1986) Acta Crystallogr. Sect. A 42, 140–149
21. Lauger, P. (1980) J. Membr. Biol. 57, 163–178
22. Lauger, P., and Apell, H. J. (1982) Biophys. Chem. 16, 209–221