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Minireview

Atomic structure of a glycerol channel and implications for substrate permeation in aqua(glycero)porins

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Abstract The structure of a glycerol channel from *Escherichia coli* at 2.2 Å resolution serves as a basis for the understanding of selective transmembrane substrate permeation. In the course of permeation, glycerol molecules diffuse through a tripathic channel with their alkyl backbone wedged against a hydrophobic corner, such that OH groups become acceptors and donors of hydrogen bonds at the same time. The structure of the channel explains the preferential permeability for linear carbohydrates and absolute exclusion of ions and charged solutes. Its gene-duplicated sequence has a structural counterpart in a pseudo two-fold symmetry within the monomeric channel protein. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Aquaporin; Aquaglyceroporin; Glycerol; Channel; Membrane permeation

1. Introduction

The glycerol channel from *Escherichia coli* (GlpF) is a member of the aquaporin family (AQPs) that facilitates the passive and selective permeation of water and small uncharged organic molecules across biological membranes [1–3]. Sequence comparisons reveal that the water channel subfamilies, as well as the glycerol channel subfamilies, diverged from each other in ancient times [4]. In all organisms, the role of AQPs is facilitation of rapid membrane permeation by water and specific nutrient molecules. The AQPs are keys to the establishment and maintenance of organism-wide and tissue-specific homeostasis, and are thus of paramount importance to the proper growth and function of multi-celled organisms. AQPs also serve to maintain specialized tissue-specific non-homeostatic conditions such as the counter-current exchange system of the mammalian kidney, the production of cerebrospinal fluid in the central nervous system, and fluid exchange in a variety of secretory glands, erythrocytes, and in specialized tissues such as the ocular lens [5–11].

The AQP family arose by tandem gene duplication [12] where the N-terminal segment (residues 6–108) has ~20% conservation with the C-terminal segment (residues 144–254) [13]. Both segments contain the highly conserved -Asn-Pro-Ala- signature sequence (-NPA-). The human genome contains ten AQP genes (AQP0–AQP9) [14], of which AQP3 [7], AQP7 [15] and AQP9 [16] are known to conduct glycerol.

GlpF conducts its primary substrate glycerol and is stereo- and enantioselective for the homologous series of linear polyalcohols called alditols [17,18]. GlpF can also conduct water though with ~five-fold less efficient than the water channel AqpZ [19]. Like most AQPs, GlpF is highly insulating for all cations and anions, including hydroxide and hydronium ions, and thus preserves the electrochemical potential across the cell membrane [20].

2. Structure determination

We initially described the atomic structure of GlpF determined at 2.2 Å resolution by X-ray crystallography [18]. Iterative refinement and model adjustment yielded a crystallographic *R* factor of 19.7% (*R*free of 22.2%). The structural coordinates are available from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (accession code 1FX8).

3. Results and discussion

3.1. The fold of the monomer channel

We expect that most aspects of the folding of the AQPs are conserved. Thus, GlpF provides a good template for understanding the subtle differences between AQPs in the context of a conserved framework. GlpF is a tetrameric complex consisting of four identical channels arranged around a four-fold symmetry axis normal to the membrane plane. Surrounding each monomer channel are six and two half membranes spanning α-helices arranged in a right-handed supertwisted fashion (Fig. 1A,B). The two homologous segments representing the genetic duplication are related by a quasi two-fold axis that passes through the central plane of the bilayer (Fig. 1A) to almost intersect the four-fold axis of the tetramer (Fig. 1B). Both the fold and several of the channel lining features of the two segments are related to each other via a pseudo two-fold symmetry axis (Fig. 1B,C).

The N-terminal segment (residues 6–108) forms one side of the channel and begins with two membrane spanning helical segments, M1 (residues 6–34) and M2 (40–60). These are followed by a key stretch of seven amino acids (61–67) that

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Abbreviations: GlpF, the glycerol channel from *Escherichia coli*; AQPs, aquaporins

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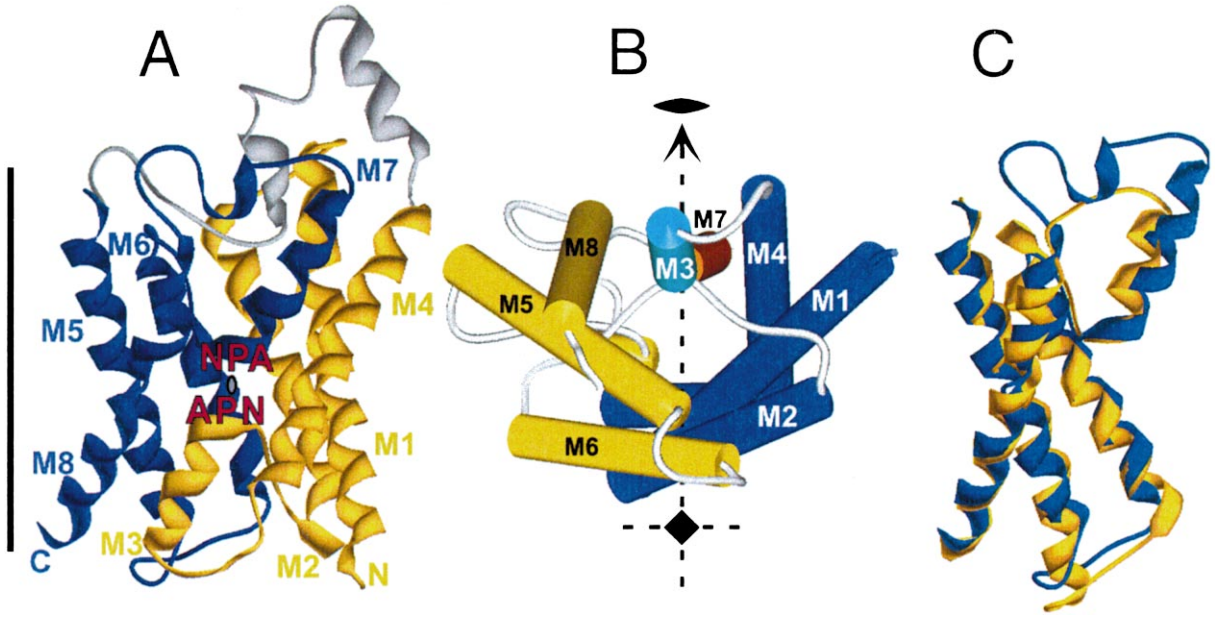


Fig. 1. The AQP fold. A: A monomer of GlpF viewed down the quasi two-fold symmetry axis with the cytoplasmic side down. The two segments are related by a quasi two-fold symmetry axis that passes through the center of the molecule, as indicated, between the positions where the two signature -NPA- sequences (residues 68–71 and 203–205) form the junction between helices M3 and M7. Most of these features are conserved among all AQPs. The two gene-duplicated segments, colored in yellow and blue, are separated by a linker region on the periplasmic side that is quite variable between AQPs (shown in gray). The vertical bar (35 Å) represents the position of the membrane. B: The channel viewed from the cytoplasmic side shows how the two half helices, M3 and M7, form a coaxial bend conjugating at their NPA–NPA junction to form a narrow channel constriction inside the channel. The two segments form a right-handed twisted bundle of essentially seven helical rods, such that the quasi two-fold axis passes between the two half helices, M3 and M7. The location of the four-fold symmetry axis of the tetrameric channel complex and the pseudo two-fold symmetry axis are indicated. C: Superposition of the N-terminal (yellow, 6–108) onto the C-terminal segment (blue, 144–254). The periplasmic linker region is excluded. The RMSD of the (340) main-chain atoms between their positions is 1.32 Å, emphasizing the closely homologous structure formed by each.

project into the center of the channel as an extended polypeptide chain and orient three successive carbonyls into the cytoplasmic vestibule where they act as hydrogen bond acceptors. This chain initiates M3 (68–78), the first of two short α-helices that begin with a -NPA- sequence near the center of the bilayer and return to the cytoplasmic side. This third transmembrane helix M4 (85–108) is packed between M3 and M1 (Fig. 1A).

The C-terminal segment (144–254) reiterates a similar transmembrane topology, but beginning from the periplasmic side rather than from the cytoplasmic side. Two more α-helices, M5 (144–168) and M6 (178–194), are followed by a second extended chain incursion into the transbilayer region from the outside. It also contributes three successive carbonyls as hydrogen bond acceptors into the vestibule, but on the periplasmic side, and initiates the second half-spanning helix M7 (203–216). Beginning with the second -NPA- sequence, it returns to the outside. Finally M8 (232–253) returns to the intracellular surface, leaving both N- and C-termini on the inside of the plasma membrane.

Between the two genetic repeats lies a relatively unconserved linking region on the periplasmic side that, in the case of GlpF, is formed by two helices (109–120 and 126–134) that protrude on the periplasmic side (Fig. 2A). They surround a concavity in the center of the tetramer where the shortest helices, M2 and M6, meet near the four-fold axis and on either side of the molecular quasi two-fold axis. An extended chain (137–143) returns to the central channel and contributes three further carbonyls to the periplasmic entry vestibule of GlpF.

The two half-spanning helices, M3 and M7, both in contact with the lipid accessible exterior on the outside of the tetramer along one side, form a coaxial bend (Fig. 1B). This unique contact between the N-terminal ends of the two helices, M3 and M7, plays a key role in maintaining a glycerol-binding site in the center of the bilayer. The conserved -NPA- motifs (68–70 in M3, 203–205 in M7) create this interface, placing the proline rings in van der Waals contact against each other, and cupping each prolyl side chain between the proline and alanine side chains of the opposite helix. Each asparagine side chain is constrained by two hydrogen bonds, one from a main-chain N–H on the same segment, the other to a main-chain carbonyl O from the other genetic segment. This pattern precisely orients the amide side chain NHs towards hydrogen bond acceptor sites on a permeant substrate molecule.

3.2. *Internal symmetry and architecture of entry and exit vestibules*

GlpF contains four remarkable carbonyl oxygens on the periplasmic side, and another four on the cytoplasmic side (Fig. 2B). Entering from the cytoplasmic side, the carbonyls of Gly64, Ala65, His66 are oriented into the cytoplasmic vestibule of the channel, while that of Leu67 follows the same theme, but forms a hydrogen bond to the side chain of conserved Asn203 (of the second -NPA- motif). To achieve this, the conformation of four residues are alternately in the (unusual) right-handed α-helix (Gly64), the (unusual) left-handed (Ala65), right-handed (His66) and then left-handed (Leu67) conformations (Fig. 2B,C).

A similar structure is found from the periplasmic surface

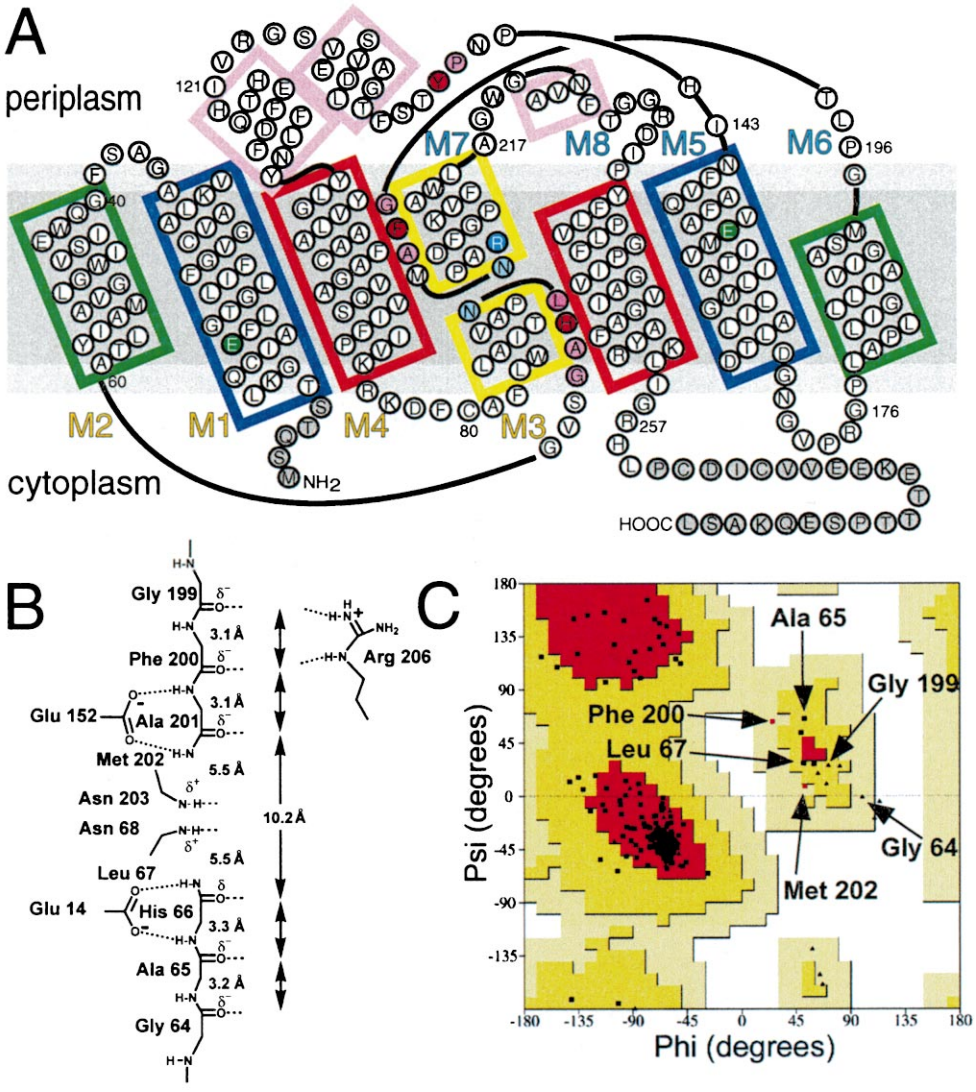


Fig. 2. Topology of the amino acid sequence of GlpF and participation of selected amino acids in substrate permeation. A: Topology as viewed from the inside of the channel. Positions of the helices indicated are arranged around the channel in the order M2, M1, M4, M3, M7, M8, M5 and M6. The -NPA- motifs are in the center and the symmetric relationship between the helices is highlighted by colored rectangles. Residues in pink are main-chain hydrogen bond donors; residues in red are main-chain hydrogen bond donors that participate in substrate interaction; the side chain of Arg 206, donating hydrogen bonds with glycerol G2 is colored dark blue; the two glutamates that form hydrogen bond acceptors within the protein are colored in green. Five residues at the N-terminus and 22 residues at the C-terminus, labelled gray, are not resolved in the structure. B: Schematic drawing of the ladder of consecutive main-chain carbonyls that form the polar line in the channel interior. The symmetric relationship between the two stretches can be seen, and the distances between sites are labelled. This feature is expected to be common to all AQPs, and determines distances that closely correspond to the distances between hydrogen-bonded water molecules in the water channels. The electrostatic triangle formed between conserved Glu152, a site in the channel, and Arg206 may accentuate the polarization of permeant molecules in AQPs. C: The phi and psi angles of residue conformations along the GlpF sequence are analyzed using a Ramachandran plot. The eight residues (64–67 and 199–202) that form extended stretches of peptide project successive carbonyls into the lumen of the channel, as shown in Fig. 2B. Being alternately in right-handed and then left-handed α-helical conformation, residues Gly64, Ala65, Leu67, Gly199, Phe200 and Met202 lie in the area for left-handed α-helices.

related to the one on the cytoplasmic side by quasi two-fold symmetry. In that case, Gly199, the conserved analog of Gly64, provides for the sharp turn off the chain into the lumen, followed by Phe200, Ala201, Met202, Asn203. Both Phe200, which provides a key side chain to the hydrophobic wall of the narrow region of the channel, and Met202 are in the unusual left-handed helical configuration. In this conformation, the side chain makes a close, but not unprecedented, contact with the carbonyl oxygen of the preceding residue.

Maintenance of these two ladders of carbonyls is supported by conserved, buried glutamates. Glu14 accepts hydrogen bonds from the amide NHs of His66 and Leu67, stabilizing an adjacent pair of amides. Glu152 plays the same role with the NHs of Ala201 and Met202. Thus, each glutamate carboxyl orients three carbonyls in the vestibules and a fourth that makes a key interaction between M3 and M7. The conserved, quasi two-fold related Gly64 and Gly199 are symmetrically located near the outer extremities of this carbonyl ladder. The ladder of carbonyls provides adjacent hydrogen bond acceptor sites that are ~3.1 Å apart and line the pathway into and out of the narrow central region of the channel.

The conservation of key glutamates 14, 152, and glycines

64, 199 indicates that this unusual conformation of the extended chains should be common to the entire AQP family as a key element of the fundamental mechanism of substrate permeation.

3.3. Comparison with K^+ channels

The arrangement of successive carbonyl oxygens that point into the lumen of the channel deserves comparison with the structure found in the KcsA potassium channel. In that case, the channel is formed in the center of a tetramer of identical subunits. However, beginning from the extracellular side, the carbonyls of Tyr78, Gly77, Val76, Thr75, Thr74 (together with the side chain OH of Thr75) and their four-fold related equivalents form the selectivity filter in the center of the tetrameric ion conducting channel. This fold contributes 20 carbonyls and 4 OHs to the lumen of the channel. The backbone structure is maintained by residues that are alternately in the right, left, right, left and right-handed α-helical configuration, and these residues are highly conserved residues of the selectivity filter in potassium channels.

In this KcsA channel, the residues in left-handed conformation include a glycine (77) and a threonine (75). Thus, only Thr75 is in an unusual conformation. Interestingly, there is a buried carboxyl-containing residue behind the amide NHs Gly77 and Tyr78, namely Glu71, whose side chain is not defined in the structure of the KcsA channel. This glutamate may play the same role as Glu14 and Glu152 in GlpF. If so, it could orient adjacent amide NHs. In the case of KcsA, with our surmised position for the side chain, it would brace the left-handed helical Gly77, rather than the right-handed conformation in GlpF. This buried Glu71 in KcsA, though conserved in several K^+ channels, including Kch, romK, hgirK, is not generally conserved in K^+ channels. Gly79, at the extracellular end of the carbonyl ladder, has an analogous role to that of the conserved Gly64 and Gly199 in GlpF, in allowing a sharp turn away from the central pore at the aqueous surface. However, the glycines in GlpF are at the beginning rather than the end of the carbonyl ladder.

3.4. Glycerol molecules bound in a long, narrow channel

The GlpF channel pathway, defined by three bound glycerol molecules, has a wide vestibule ~15 Å across on the outer surface, and reaches its constriction of ~3.8 × 3.4 Å in size, 8 Å above the central plane of the transmembrane region (Fig. 3A). This constriction lies at the start of a ~28-Å-long selective channel ($r < 3.5$ Å) that subsequently widens out again toward the cytoplasmic surface (Fig. 3B).

The glycerol sites are (G1–G3), as seen by a glycerol molecule moving along its trajectory from the periplasmic side to the cytoplasmic side, and the glycerol OH moieties likewise, are numbered in order from most external to more internal. Conducted molecules may pass through the channel in either direction according to the prevalent concentration gradient.

The O1-H of G1 remains hydrated by water molecules. G1 in the entry vestibule is oriented by G1 O2-H as hydrogen bond donor to the O of Tyr138 (2.7 Å). G2 and G3 are both found in the selective region of the channel. G2 forms a hydrogen bond with one water molecule at its 1′-hydroxyl. Another bridging water molecule is hydrogen bonded both to G2 and G3, suggesting that glycerol and water are co-transported.

The selectivity filter at G2 is strongly 'tripathic' (having three different properties), with two aromatic rings (Trp48, Phe200) forming a corner on two (out of four) sides, two NHs of the guanidinium side chain of Arg206 on the third side, and two main-chain carbonyl oxygens on the fourth side. The OH1 (Fig. 3C) and OH2 (Fig. 3D) of G2 are both hydrogen bond acceptors from NHs of the guanidinium group of Arg206 (2.9 Å and 2.7 Å, respectively), and hydrogen bond donors to the carbonyl oxygens of Gly199 (2.6 Å) and Phe200 (2.8 Å). G2 binds in the Trp and Phe, Arg, carbonyl tetrad leaving no additional space around it, such that van der Waals, hydrogen bond, and electrostatic forces all contribute favorably to stabilization, and hence passage through this site.

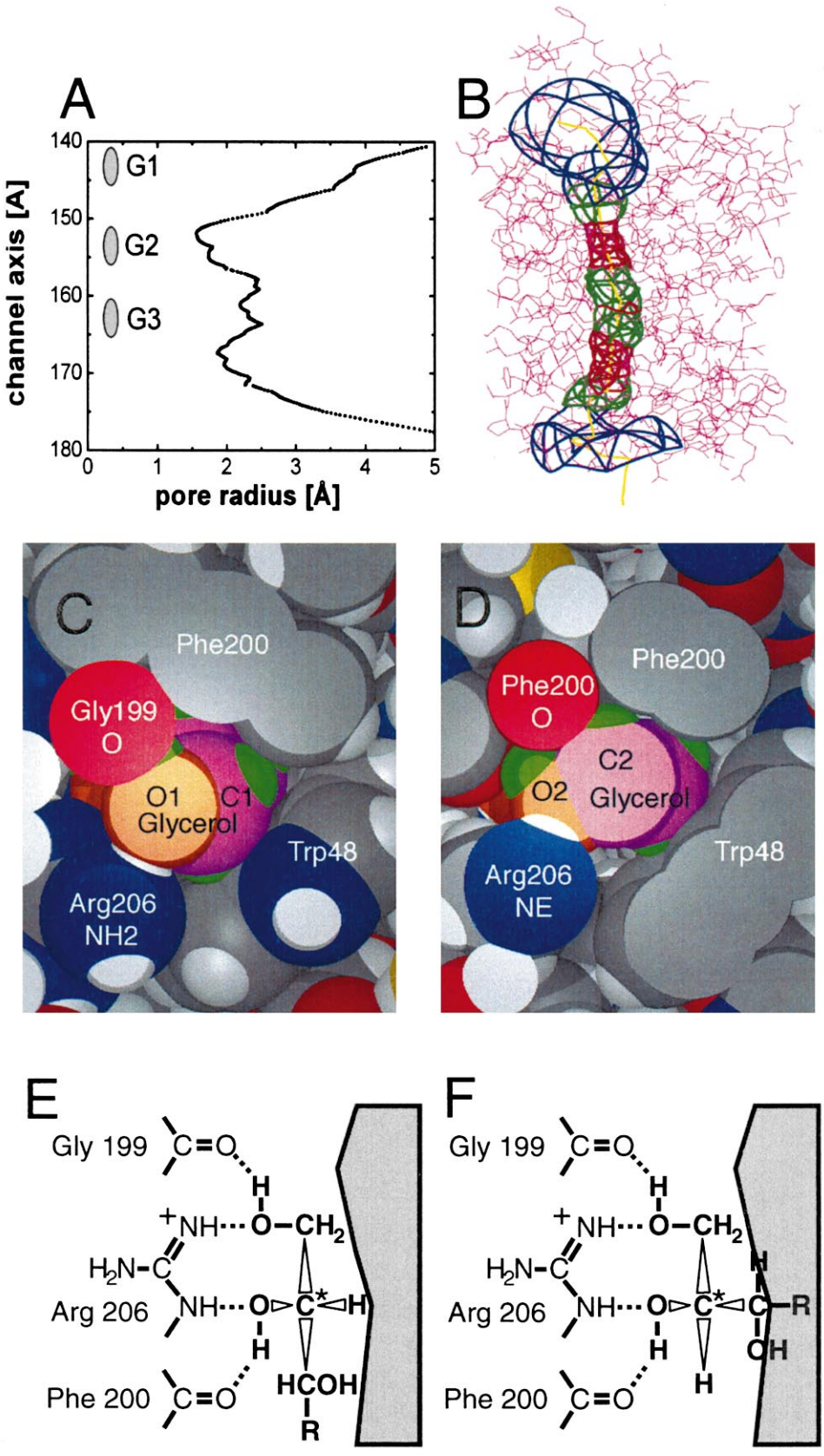
The carboxyl group of Glu152, which is invariant in all AQP family members, and orients the three adjacent carbonyls of Gly199, Phe200, Ala201 in the entry vestibule, may also increase the negative charge on the carbonyl oxygens of Phe200 and Ala201. Thus, the binding of glycerol in the selective filter creates the potential for the negative charge of Glu152 to form an electrostatic interaction with the positive charge on Arg206 via the amides of Phe200, Ala201, and the glycerol OHs. This could accentuate the polarization of the glycerol OHs and imply that a permeant molecule should also be polarizable in cross-section.

The narrowest constriction in the channel lies between G1 and G2, just above G2 OH1, and is barely large enough to allow passage of an HC-OH, the minimum cross-section from the center of the glycerol molecule (Fig. 3C,D). At this narrowest position, the hydrogen bond geometry from the donor NHη of Arg206 to an OH group becomes ideal, helping to specify the only possible orientation of the CHOH group in this putative transition site.

G3 straddles the highly constrained invariants Asn203 and Asn68 at the conjunction of the two -NPA- sequences between M3 and M7. The NH2 of Asn203 is donor to O1 of G3 (3.0 Å) with ideal geometry. The NH2 of Asn68 is donor to O2 of G3 (3.1 Å), also with ideal geometry. OH3 is a hydrogen bond donor to the carbonyl of highly conserved His66 (2.8 Å). Therefore, glycerol molecules can only pass through the region of G2, G3 in single file, separated by a water molecule and with each CHOH within one molecule following its neighbor in single file.

3.5. Origin of channel selectivity for linear carbohydrates and against ions

Stereoselectivity of permeation through the glycerol channel has been observed between different chain lengths and different chiralities of linear alditols. As an example, there is a 10-fold difference in transport rate between ribitol and its stereoisomer D-arabitol [18]. The structure shows how this can be explained since two successive CHOH groups are oriented at the G2 site (Fig. 3E). Due to the narrow dimensions of the channel, the carbon backbone must be lined up along the channel axis. Depending on enantiomer, the CH hydrogens attached to the carbons are in contact with the aromatic rings of either Phe200 or Trp48, in similar environments. However, any adjacent CHOH groups to these two will alternately place the carbon in one of two tetrahedrally disposed sites that have quite different environments (Fig. 3F). Therefore, substrates that align along the channel axis are conducted, but less efficiently, if they place neighboring carbon atoms on the side that will most clash with the channel wall. Since ribitol can adapt a conformation similar to the prochiral glycerol (as



shown in Fig. 3E) and its stereoisomer D-arabitol is not able to do so, the permeation of D-arabitol is severely hindered.

The GlpF structure is the first high-resolution structure of a channel that conducts organic molecules and excludes ions. Therefore, by comparison with known ion channels, it helps to define what is required to allow ion conductance in a channel. The channel in GlpF is too small to accommodate a hydrated ion, and by its amphipathic nature, provides no

Fig. 3. Glycerol channel size and location of glycerol molecule G2 in the narrowest channel section. The water- and glycerol-filled channel penetrates GlpF parallel to the membrane normal and reveals two major constrictions. A: Analysis of the channel size using the program HOLE2 [27] and simple AMBER van der Waals radii, ignoring united atom values. The positions of the three glycerol molecules G1, G2, G3 are indicated. B: GlpF monomer shown as a stick model with the channel wall depicted as a grid. The channel radius is color-coded in red where it is smaller than 2.3 Å, in blue where the channel radius exceeds 3.5 Å and in green for radii in between. C: Cross-section through the tripathic selectivity filter and glycerol G2. Interactions of channel residues are shown with G2 OH1, O1 (orange) and its associated CH group (C1, purple), and hydrogen (green). The cross-section shows the donor NH_2 of Arg206, acceptor carbonyl oxygen of Gly199, and the hydrophobic corner formed by Trp48 and Phe200 viewed edge-on to the aromatic rings. D: Cross-section through the glycerol molecule one bond along the glycerol molecule down into in the tripathic selectivity filter. G2OH, O2 (orange) and its associated CH group (C2, purple) are indicated. E: Schematic drawing illustrating the basis of stereoselectivity. The alignment of the substrate molecule is based on the conformer of G2 in the selectivity filter. The asterisk at C2 indicates the chiral carbon center with respect to the channel environment. The guanidinium group and the carbonyls direct the positions of successive OH groups of neighboring substituents on the carbon chain. Thus, the binding of a prochiral glycerol molecule or that of a chiral molecule with a similar chirality at C2 is preferred. F: Binding of a glycerol molecule in a different orientation (R = H) or binding of a chiral molecule (R≠H) with a reversed chiral center (compared to E) is significantly sterically hindered by the opposing hydrophobic wedge of the channel wall.
←

replacement for water of hydration on two (of four) sides. The energetic cost of removing even a single water of hydration from an ion without compensation is vast (on the order of 110 kcal/mol, [28]). Therefore, cation-conducting channels generally provide oriented carbonyl groups that carry a partial negative charge of typically 0.4 electrons. These carbonyls can replace waters of hydration that are displaced within a narrow region that intrudes on the first hydration shell [21–25].

Removal of water from uncharged glycerol and fixation of a specific conformation of glycerol in GlpF are energetically less costly (ca. on the order of 5 kcal/mol) [26]. However, the channel compensates in this case as it makes specific matches with the hydrogen donors and acceptors of each of a series of C–OH groups, replacing the waters of hydration by a precise match on two sides within the channel.

4. Conclusion

The high degree of sequence homology within the AQP family and the close structural alignment of the N-terminal with the C-terminal segment strongly supports the gene-duplication hypothesis. The fact that all residues related by the quasi two-fold symmetry in GlpF are among the most conserved throughout the entire AQP protein family suggests that the structure of GlpF glycerol channel and its suggested mechanism of selective permeation is prophetic of that of other AQP relatives.

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