Biochimica et Biophysica Acta 1848 (2015) 622–633

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Review

Folding and stability of the aquaglyceroporin GlpF: Implications for human aqua(glycero)porin diseases

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ARTICLE INFO

Article history:
Received 12 September 2014
Received in revised form 12 November 2014
Accepted 14 November 2014
Available online 20 November 2014

Keywords:
Aquaporin
GlpF
Membrane protein
Protein activity
Protein folding

ABSTRACT

Aquaporins are highly selective polytopic transmembrane channel proteins that facilitate the permeation of water across cellular membranes in a large diversity of organisms. Defects in aquaporin function are associated with common diseases, such as *nephrogenic diabetes insipidus*, congenital cataract and certain types of cancer. In general, aquaporins have a highly conserved structure; from prokaryotes to humans. The conserved structure, together with structural dynamics and the structural framework for substrate selectivity is discussed. The folding pathway of aquaporins has been a topic of several studies in recent years. These studies revealed that a conserved protein structure can be reached by following different folding pathways. Based on the available data, we suggest a complex folding pathway for aquaporins, starting from the insertion of individual helices up to the formation of the tetrameric aquaporin structure. The consequences of some known mutations in human aquaporin-encoding genes, which most likely affect the folding and stability of human aquaporins, are discussed.

Contents

Abbreviations: AQP0–12, aquaporins 0–12; ar/R, aromatic/arginine; CD, Circular Dichroism; CPK, Corey, Pauling, Koltun space-filling models; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; EC, extracellular; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GlpF, glycerol facilitator; IC, intracellular; MD, molecular dynamics; MIM, Major Intrinsic Protein; PIP, Plasma Membrane Intrinsic Proteins; SF, Selectivity Filter; TM, transmembrane; wt, wild-type

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1. Introduction: the aquaporin family

Aquaporins are highly selective polytopic transmembrane (TM) channel proteins with a molecular mass of 26–34 kDa, which facilitate water flux across cellular membranes in a large diversity of organisms [1]. As the passive flux of water is driven by the osmotic gradient, aquaporins play a crucial role for osmoregulation in many tissues [2]. Defects in human aquaporin functions are associated with common diseases,

http://dx.doi.org/10.1016/j.bbamem.2014.11.015
0005-2736/

Table 1
Aqua(glycero)porins with solved structures.

Protein name	Organism	Resolution	PDB-file	Reference
AQP0	*Bovine lens*	2.24 Å	1YMG	[114]
AQP0	*Ovis aries Sheep lens*	3.0 Å	1SOR	[118]
AQP1	*Homo sapiens rbc*	3.8 Å	1FQY	[146]
AQP1	*Bos taurus*	2.20 Å	1J4N	[40]
AQP2	*Homo sapiens*	2.75 Å	4NEF	[147]
AQP4	*Rattus norvegicus*	3.2 Å	2D57	[148]
AQP4	*Homo sapiens*	1.8 Å	3GD8	[149]
AQP5 (HSAQP5)	*Homo sapiens*	2.0 Å	3D9S	[150]
AQPM	*Methanothermobacter marburgensis*	1.68 Å	2F2B	[151]
AQPZ	*Escherichia coli*	2.5 Å	1RC2	[41]
SOPIP2;1	*Spinacia oleracea*	2.10 Å	1Z98	[56]
GLPF	*Escherichia coli*	2.2 Å	1FX8	[23]
PFAQP	*Plasmodium falciparum*	2.05 Å	3C02	[152]
AQY1	*Pischia pastoris*	1.15 Å	2W2E	[153]

such as *nephrogenic diabetes insipidus*, *congenital cataract* and certain types of cancer [3–10]. Although selectively facilitating water flux across membranes is the major physiological function of aquaporins, some family members are also permeable to other molecules, such as urea, nitrate and silicon, to gases such as ammonia, carbon dioxide and nitrogen dioxide and to metalloids, such as arsenic and antimony [1,11–14]. Furthermore, there is also evidence that the human aquaporin AQP1 is permeable to gaseous CO_2 [15], and the gas-conducting channel is postulated to form in the center of an AQP1 tetramer.

Aquaporins are categorized into three subfamilies, based on the conservation of the amino acids flanking two conserved amino acid motifs (NPA motifs) [16,17]. In humans, the classical aquaporins (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8) are only permeable to water, whereas members of the subfamily of aquaglyceroporins (AQP3, AQP7, AQP9 and AQP10) are additionally permeable to glycerol and other small, polar solutes [13,17–19]. AQP11 and AQP12 are categorized as "unorthodox aquaporins", as they do not share a high sequence identity around the conserved NPA motifs, as common for classical aquaporins and aquaglyceroporins (see below). Although AQP1, which is found in erythrocyte membranes and various tissues including the kidney, lung, vascular endothelium, brain and eye, was the first aquaporin to be characterized in 1992 [20–22], the glycerol facilitator GlpF of the bacterium *Escherichia coli* (*E. coli*) is thus far the best studied member of the aquaporin family. GlpF has served as an experimentally accessible aquaporin model for many years, and the GlpF crystal structure was solved more than a decade ago [23]. The protein consists of 281 amino acids and has a molecular mass of 28 kDa. Besides glycerol and other linear polyalcohols it facilitates the flux of urea and antimony-(III)-hydroxide across membranes, whereas sugars are not GlpF substrates. Despite the fact that GlpF was crystallized as a homo-tetramer [23], the oligomeric state of GlpF was the topic of many controversial discussions during the past decade [24–28]. However, by now it is commonly accepted that GlpF forms stable tetramers, which are even highly stable in detergent solutions.

2. The conserved structure of aqua(glycero)porins

In recent years, several structures of aquaporins have been solved, and crystal structures of 14 aquaporins and aquaglyceroporins from 9 different organisms have been published with resolutions varying between 0.88 Å and 3.8 Å (Table 1). Importantly, the overall structures of all aqua(glycero)porins are essentially identical, regardless of the subfamily or the host organism.

An aqua(glycero)porin monomer consists of a total of six TM helices and two reentry loops, which both form a half-spanning helix (Fig. 1A). These half-TM helices, which both contain the highly conserved NPA motif at their individual N-termini, align lengthwise and in this way span the membrane with a helical structure. The eight helices together form a right-handed helix bundle with the channel pore in its center (Fig. 1B), and the overall structure of an aqua(glycero)porin monomer is reminiscent of an hourglass (Fig. 2A) [29]. In the following, we will refer to the helices according to their consecutive number, including both half-spanning helices rather than numbering only the TM helices.

All aquaporin monomers comprise two subdomains with 3.5 TM segments, each with the same tertiary fold but oriented in the membrane at a 180° angle (Fig. 1A). The inverted repeat structure places both the N- and C-termini of the proteins in the cytoplasm [30] and is a common theme among helical bundle membrane proteins [31]. It provides the protein with an internal "quasi-symmetry" with a twofold pseudo axis of rotation. This internal tandem repeat is conserved among all aqua(glycero)porins and probably reflects the evolution of the aquaporins: during the course of evolution a primordial precursor gene likely coded for a protein with dual TM topology, and thus aquaporin precursor proteins were protein dimers [32]. After gene duplication, the individual subdomains evolved separately and eventually fused, resulting in the aquaporin genes/proteins found today. Noteworthy, although characteristic for all aqua(glycero)porins, there are further proteins with an aquaporin fold, albeit they do not belong to the aquaporin family. Prominent examples are the formate channel FocA [33–35] and the nitrite channel NirC [36].

Like many other α-helical TM proteins, aquaporins assemble within biological membranes and form higher ordered oligomers. For a long time it was assumed that all classical aquaporins form homo-tetramers while all aquaglyceroporins are active as monomers [24–27]. In later studies, however, it was found that aquaglyceroporins, too, are present as homo-tetramers in biological membranes, but that their protomer–protomer association seems to be weaker than what is observed in canonical aquaporins [28,37–39].

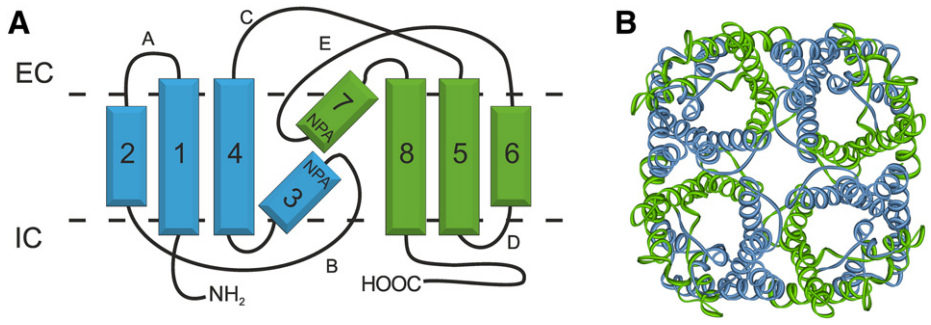

Fig. 1. Aquaporin topology. (A) Indicated in blue and green are the two inverted repeat subdomains found in an aquaporin monomer. Each subdomain consists of three TM helices (1, 2, 4 and 5, 6, 8) and one half-spanning helix (3 and 7), and the two subdomains are orientated in a 180° angle in the membrane. TM helices are connected *via* five extra- and intracellular loops (A–E). Loops B and E contain the two half-spanning helices (3 and 7) with the highly conserved NPA motifs at the interface. (B) GlpF crystal structure (PDB ID: 1FX8) [23]. Periplasmic view on the native tetrameric GlpF structure. The substrate conducting channel is located within each monomer. IC = intracellular and EC = extracellular.

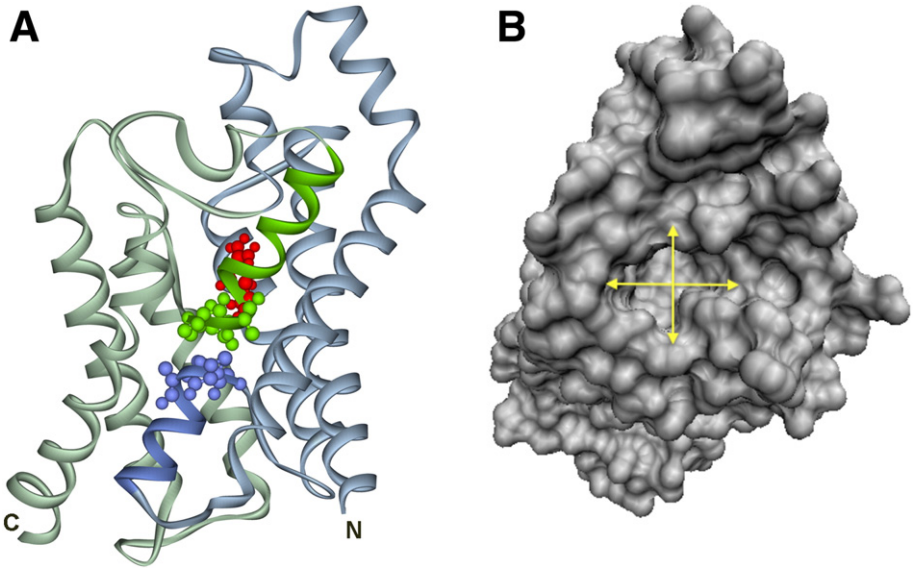

Fig. 2. Structural basis of substrate specificity. (A) A ribbon representation of a GlpF monomer secondary structure. N indicates the amino terminus at the bottom right and C is the carboxyl terminus at the bottom left. Residues 68–78 of the amino terminal half-spanning helix (ribbon) and the NPA motif (CPK) are colored in bright blue and residues 203–217 of the carboxy-terminal half-spanning helix (ribbon) and the NPA motif (CPK) are colored in bright green, except for Arg206 of the ar/R SF, which is colored red (CPK). (B) A surface representation of a GlpF monomer using a 1.4 Å probe. The double-headed arrow indicates an approximate 15 Å wide funnel that narrows to 3.4 Å at the SF. The view is of the GlpF vestibule looking down on the extracellular surface. Glycerol molecules are gradually dehydrated through interactions with the protein. The images were made using VMD (developed with NIH support by the Theoretical and Computational Biophysics group at the Beckman Institute, University of Illinois at Urbana-Champaign), DS ViewerPro and PDB ID: 1FX8 from [23].

3. Substrate specificity and protein dynamics

The substrate specificity of aqua(glycero)porins is determined principally by an aromatic/arginine (ar/R) selectivity filter (SF) (Fig. 2A) that sits at the bottom of a 15 Å-wide vestibule above the extracellular pore entrance (Fig. 2B). The SF forms the narrowest part of the channel, and in classical aquaporins the width of the filter is 2.8 Å, just wide enough to accept a water molecule [40,41]. In GlpF, the filter widens to 3.4 Å to accommodate a CHOH group from dehydrated glycerol or other linear polyalcohols [42], but sugars are much too large to enter [23]. The pore dimensions should easily permit hydroxide ions, hydronium ions and protons to cross the membrane, yet nearly all aqua(glycero)porins are highly selective against the passage of these ions.

In addition to dimensional filtering, the ar/R SF also provides chemical filtering. In the human AQP1, the SF selects for water by providing two H-bond donors (Arg227) and two acceptors (His212, and Ala221) that satisfy all four H-bonding sites in a water molecule [43] (Fig. 3A). Hydroxide ions are selected against water molecules because they cannot H-bond simultaneously with Ala221 and His212 [43]. In a similar fashion, the GlpF SF selects for glycerol using the homologous Arg206 to donate H-bonds to the O1 and O2 glycerol oxygens; Gly199 accepts an H-bond from O1 and Phe200 accepts an H-bond from O2 (Fig. 3B).

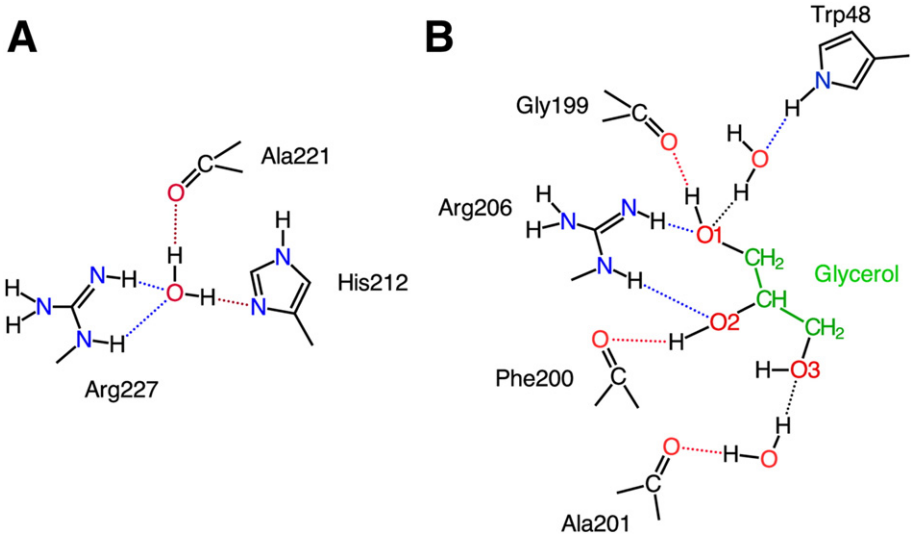

Fig. 3. H-bonding in the Specificity Filter. (A) Protein H-bond donors and acceptors in the AQP1 SF are illustrated. The figure is based on a snapshot from a MD simulation of AQP1 [43] and shows how the protein can satisfy all four H-bonding sites in a water molecule. The H-bond angles are not ideal, ensuring that water does not get trapped in the channel but transits rapidly [43]. (B) H-bond donors and acceptors to glycerol in the SF of GlpF are illustrated based on the X-ray diffraction structure [23]. Arg206 donates H-bonds to the O1 and O2 glycerol oxygens, Gly199 accepts an H-bond from O1, and Phe200 accepts an H-bond from O2. H-bonds from Ala201 and Trp48 to the O3 and O1 of glycerol respectively, are mediated by a water molecule [23]. Trp48 and Phe200 aromatic rings provide a hydrophobic corner to interact with the C–H groups from glycerol (see Fig. 4). The images were made using *ChemDoodle*: Chemical Publishing Software (Version 6.0.1).

H-bonds from Ala201 and Trp48 to the O1 and O3 of glycerol, respectively, are mediated by a water molecule [23]. In GlpF, the SF is amphipathic and the Trp48 and Phe200 aromatic rings provide a hydrophobic corner to interact with the C–H groups from glycerol (Fig. 4).

A third component of the filter is the concentration of positive electrostatic potential that is maximal at the center of the channel [43]. This receives contributions both from the arginine in the ar/R SF [44] and from the two half-spanning helices (3 and 7) that point the positive (amino) ends of their helical macrodipoles at the center of the channel (Fig. 2A) [45]. Molecular dynamics (MD) simulations of both AQP1 and GlpF suggest that proton transport by the Grotthuss mechanism, whereby protons tunnel between H-bonded water molecules, is inhibited predominantly by the large electrostatic barrier (105 kJ/mol) [46] focused on the NPA region. Mutations of the arginine in the SF appear to confirm this hypothesis [44]. Desolvation penalties and configurational barriers also contribute to substrate specificity. Water and glycerol move through the channel pore in a single-file column. As water and glycerol penetrate their respective pores, solvent substrate H-bonds are replaced by channel substrate H-bonds lowering the energy barrier to transport and enabling extremely rapid (ns) transport rates [47]. Protons, hydronium ions and hydroxide ions in the channel are energetically destabilized compared to bulk solvent, because desolvation is not compensated by interactions with the channel wall [46,47]. This is especially pronounced in glycerol channels; they select against hydroxide, hydronium and other ions, and even water, because hydration shell removal cannot be compensated for on the hydrophobic wall of the channel [23] (Fig. 4). A recent 20 ns MD simulation based on a 0.88 Å-resolution X-ray diffraction structure of AQP1 showed water molecules moving in pairs through the SF and the cytoplasmic half of the channel, whereas in the NPA region the correlated motion is highly perturbed owing to interactions with the protein [43]. This result confirmed several earlier MD simulations [48,49] suggesting that Grotthuss proton transport is at least partly inhibited owing to the bipolar orientation of the water molecules in the channel above and below the NPA motifs.

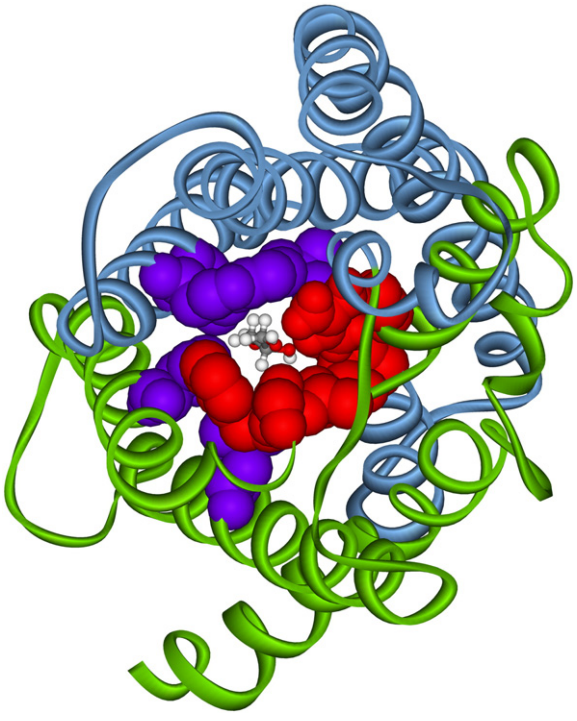

Fig. 4. The amphipathic GlpF pore. Non-polar side-chains that line one face of the GlpF pore and interact with glycerol C–H groups are colored blue. They include Trp48, Val52, Leu67, Leu159, Ile187 and Phe200. Polar side-chains on the opposite face are colored red and include His66, Asn68, Gly199, Phe200, Ala201, and Arg206. Depicted is also a glycerol passing the translocation pore. The view is looking downward onto the periplasmic surface of a monomer. The image was made using the DS ViewerPro software and PDB ID: 1FX8 from [23].

Although there is significant agreement as to the factors that contribute to substrate selectivity in aqua(glycero)porins, further biochemical support is needed [46]. For example, measurements of *in vivo*, *in vitro* and *in silico* substrate flow for selected mutants, designed to inhibit or enhance flow, could contribute significantly to our understanding of how these extraordinary proteins carry out their biological activities [44,45]. This view is supported by an emerging area of research involving putative "dual substrate" aquaporins that transport water or glycerol and also serve as gated ion channels, as has been observed in some aqua(glycero)porin channels (*e.g.* AQP0, 1, 6 and plant aquaporins) under physiological and non-physiological conditions [50].

It is well established that protein dynamics play a key role in the expression of the biological activity of proteins including membrane proteins [51]. With the caveat that X-ray diffraction temperature B-factors may greatly underestimate the conformational heterogeneity of proteins [52], in general, low temperature B-factors suggest that the aqua(glycero)porins are conformationally rigid throughout their structures [53]. Large-scale dynamic fluctuations in the dimensions of the pore would presumably permit a wider range of substrate sizes to cross the membrane, suggesting that the protein in its native membrane is relatively inflexible. However, an early nanosecond MD simulation of GlpF showed that the pore narrowed upon the removal of glycerol, suggesting an "induced-fit" mechanism by which the pore widens in response to interactions with glycerol [48]. An emerging area of interest is the regulation of substrate flow in some aqua(glycero)porins, and this has recently been reviewed [54]. The most convincing physiological evidence for regulated gating is the channel closure that occurs in plant aquaporins (Plasma Membrane Intrinsic Proteins, PIP) upon serine dephosphorylation, pH drop or addition of calcium ions [55]. High-resolution X-ray diffraction structures [56] of open and closed PIP channels show a slightly longer loop D (Fig. 1A) that moves by up to 16 Å occluding the channel on the cytoplasmic side of the protein. MD simulations show that much of the conformational change, including expansion and contraction of the channel pores, occurs within 15 ns. Another example is the allosteric regulation of AQP0 by Ca^{2+}-calmodulin. This is the first example, in which the role of the tetramer has been shown to provide a scaffold for communication between the protomers of the tetramer [57]. Regulation of other aqua(glycero)porins is an active area of research but has been difficult to confirm [54].

Of considerable interest to aqua(glycero)porin folding and function was a hydrogen-deuterium exchange study of detergent-solubilized GlpF [58] that showed residues in half-spanning helix 7 undergoing a much more rapid exchange than in the rest of the protein. It was suggested that flexibility in this region might be important in permitting rapid substrate flux by a "molecular lubrication" mechanism; the rapid dynamics in this region were confirmed by oxidative labeling experiments [58]. However, it will be crucial to determine whether this region is also flexible in a lipid bilayer or *in vivo*. Ten nanosecond MD simulations of GlpF in octylglucoside micelles and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) bilayers showed a significantly greater loop and helix motion in the detergent-solubilized protein compared to the bilayer-embedded protein [59], although in general the structures remained intact over the course of the simulations. The nanosecond MD data do not contradict the hydrogen exchange measurements since hydrogen exchange is sensitive to protein dynamics on the microsecond and slower timescale. In AQP1 it has been proposed that the half-spanning helix 3 can slide out of the membrane and this has been suggested as an important step in the assembly of the folded protein [60]. Although, there is a paucity of experimentally determined high-resolution dynamics studies of membrane proteins, recent hardware and algorithm advances in MD simulations in bilayers promise to shed light on the long-term MD of these proteins and their role in aqua(glycero)porin folding and function [61,62].

4. Folding of aquaporins

4.1. The GlpF monomer folds in multiple stages

The folding pathway of aquaporins has been the topic of several studies in recent years. Surprisingly, these studies revealed that a conserved protein structure does not guarantee an identical folding pathway. While the folding pathway of human AQP4, a water channel expressed in astrocytes [63], and probably of most of the other aquaporins, is entirely co-translational, AQP1 folding is more complex and involves co- as well as post-translational steps and even includes reorientation of α-helices across the cellular membrane [64–66]. However, a study of AQP1 biogenesis in intact mammalian cells also revealed a completely co-translational folding pathway and it has been suggested that weak signal anchor and stop transfer activities within the protein can be compensated in a natural environment, whereas artificial surroundings might trigger initial misfolding [67]. In general, aquaporin folding pathways appear to exceed formerly proposed two- and three-stage pathways by far [68–70].

While the oligomeric state and the *in vivo* or *in vitro* stability of GlpF have been analyzed to some extent, little is known about folding of the monomeric protein. In line with the two-stage model of membrane protein folding [68,69], the calculated free energies of transfer of all six GlpF TM helices suggest that all TM helices can insert independently into the membrane bilayer and can (conceptually) be described as stable TM helices, which might only be loosely connected *via* interhelical loops or linker regions. The two reentrant half-spanning helices 3 and 7 very likely insert into the membrane only at a later stage. This is supported by the protein's overall topology, as TM helices 2 and 4 as well as TM helices 6 and 8 show an antiparallel orientation and the half-helices could barely act as a stop-transfer signal anchor during the translocation process. Either after or even already during their synthesis and membrane release, individual TM helices will interact to form higher ordered TM helix structures [71]. Especially interactions between the parallel TM helices 1 and 4 and TM helices 5 and 8, as well as interactions between the antiparallel TM helices 2 and 6 are notable. All of these TM helices contain common interaction motifs, such as GxxxA or SxxxGxxxA, and TM helix 8 even contains a perfect glycine zipper motif GxxxGxxxG [72]. In addition to close van der Waals packing interactions mediated by these motifs, the helix dimers are thought to be stabilized by Cα–H···O H-bonds, which are established between the noted helix pairs [73].

As already mentioned above, all aquaporins and aquaglyceroporins show two inverted repeat subdomains. These pseudo-symmetric protein halves show homology in their overall structure and have a high sequence similarity. It appears reasonable to assume an analogous folding pathway with a still retained independent folding capability of the two homologous GlpF protein halves. This implies that helices of the same GlpF subdomain interact before helices, belonging to different subdomains (Fig. 5). Interactions between the parallel helices (TM helices 1 + 4 and 5 + 8) are crucial for folding of the respective GlpF subdomains, while the antiparallel interaction between TM helices 2 and 6 connects the two subdomains (Fig. 5). When a nascent polypeptide chain is inserted into the membrane, the TM helices of the first repetitive unit, *i.e.* TM helices 1, 2 and 4, are the first helices able to interact. In the fully folded monomer, helices 1 and 4, as well as the corresponding helices 5 and 8 of the second subdomain, lie in close contact. According to this proposal, (parallel) packing of helices of a subdomain takes place before helices of different subdomains interact. Interaction of the two antiparallel subdomains is subsequently mediated by interaction of TM helices 2 and 6, involving six putative H-bonds [73]. Therefore, initial folding of individual GlpF subdomains would be a defined stage in an overall GlpF folding pathway.

However, it is still unclear at what stage exactly and in which way the reentrant loops are inserted into the membrane. These half-helices are important both for the structure and function of GlpF, as they contain the highly conserved NPA motifs. As several charged residues are incorporated in both half-helices and as these residues are located at the center of the GlpF monomer within the membrane plane, formation of these half-helices very likely occurs rather late in the folding pathway, after the full TM helices have interacted with one another and have formed an environment that allows formation and integration of the (polar) half-helices [69]. Noteworthy, the TM conformation of the initially formed TM helix bundle might differ from the finally folded GlpF monomer. After folding of the monomer, successive steps lead to the final tetrameric GlpF structure (see below).

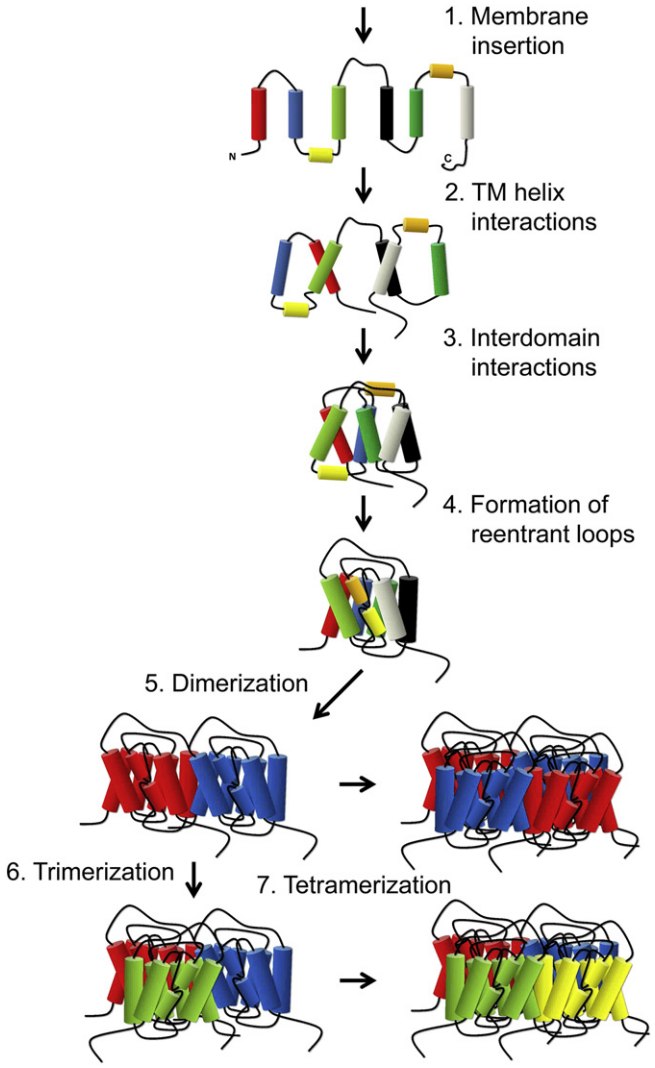

Fig. 5. GlpF folding in multiple steps. Helices are highlighted in different colors. In stage one, helices are inserted independently into the membrane bilayers in an initial six-spanning topology. In a second stage, helices of one GlpF subdomain interact before the inverted repeat subdomains interact with each other in a third stage. Folding of GlpF is completed following membrane integration of the reentrant loops in stage four. Stage five depicts the interaction of two monomers forming a dimer. From then on, two routes are possible. Tetramerization can occur either in one step by dimerization of two GlpF dimers or by sequential addition of two GlpF monomers, involving a trimeric state.

4.2. Oligomerization is crucial for the GlpF function and stability

Ever since its discovery, GlpF has served as a model protein for studying membrane protein folding and assembly, and based on its features and characteristics, several studies on folding and assembly of higher ordered membrane proteins have been performed [28,37,74,75]. Especially its stability during SDS-PAGE, where it maintains its

tetrameric structure [28], has proven to be of great advantage. The stabilities of other aquaporins have already been analyzed to some extent before [76,77], although details of their folding and oligomerization mechanisms still remain mainly enigmatic. In addition to classical aquaporins and glycerol facilitators, many other membrane proteins, too, exist in higher ordered oligomeric structures. In some cases the necessity for oligomerization is clear, as in the case of the potassium channel KcsA or the acetylcholine receptor, where the central pore lies within the oligomer [78–83]. In contrast to such obligate oligomers, the benefits of oligomerization are less clear for other proteins, such as aquaporins and ammonia transporters [83]. Generally, larger proteins are more resistant to denaturation and degradation than smaller proteins [84], suggesting that aquaporin oligomers may form to stabilize the tertiary fold, preventing aggregation and stabilizing the conformation that permits strict substrate selectivity.

Until recently, no information was available about homo-tetramer formation and about the forces and factors that drive oligomerization and further stabilize the assembled state. In addition to the aforementioned folding stages, GlpF tetramerization and assembly can be seen as a separate folding stage, which itself might involve multiple steps.

The crystal structure of the tetrameric GlpF complex revealed two Mg^{2+} ions bound in the central pore of the GlpF tetramer, which are coordinated by residues Trp42 or Glu43 of all GlpF monomers [23]. It is assumed that these ions are not naturally located in the tetramer center of GlpF but are rather the result of the applied crystallization method. In fact, the Mg^{2+} ions are dispensable for *in vitro* tetramerization [28]. In order to assess whether the coordinating residues are somehow crucial for GlpF oligomerization or for stabilization of the protein complex, the oligomeric states of GlpF mutants with substituted residues Trp42 and Glu43 were analyzed [74]. While mutating the Trp residue at position 42 to Ala did not affect oligomerization, a mutation of the neighboring Glu residue to Ala significantly disturbed GlpF oligomerization. In contrast to the wild-type (wt) protein, the E43A mutant appeared mainly as a monomeric protein on SDS gels, and a decreased oligomerization propensity was also observed *in vivo*, using a genetic system [85]. Thus, in contrast to Trp42, the polar residue Glu43 appears to be critical for tetramer assembly. In fact, not an Asp residue *per se*, but a charged or at least a highly polar residue is required at position 43 to ensure proper GlpF tetramerization [74]. Furthermore, the activity of the E43A mutant was ~30% decreased compared to the wt protein, indicating that oligomerization and function of GlpF are ultimately linked. This is probably caused by slight changes in the tertiary structure upon tetramerization, which potentially render the protein fully active. Furthermore, the ΔG_{app} values of wt GlpF and E43A mutant oligomerization were calculated and it was found that the mutation destabilizes the protein tetramer by ~7 kJ/mol [74]. This destabilization also resulted in a reduced *in vivo* stability of the GlpF monomer, which was degraded much faster by cellular proteases and therefore has a much shorter half-life than native, tetrameric GlpF [74]. A higher susceptibility to degradation might also be indicated by an analysis of the B-factor values [86], which are low at the protomer–protomer interfaces, indicating high rigidity [83]. Protomer dissociation weakens the rigidity of the GlpF monomer in the interface region and leads to exposure of flexible parts, which correlates with a decreased *in vivo* stability. The two helices facing the center of the GlpF tetramer, TM helices 2 and 6, are also less hydrophobic than the helices, which contact the lipid environment. An exposure of these more hydrophilic regions to the hydrophobic membrane milieu upon dissociation, too, might account for the increased turnover rate. Thus, besides being crucial for optimal activity, oligomerization affects protein stability and proteolytic degradation.

Noteworthy, one further reason for oligomerization – at least in the case of some aquaporins – could be the formation of an additional pore in the center of the homo-tetramer, as discussed for human AQP1 and the tobacco aquaporin NtAQP1 [15,87].

4.3. GlpF unfolding involves multiple structural transitions

Subsequent to the survey of residues affecting GlpF oligomerization, the formation of GlpF homo-tetramers was analyzed *via* an *in vitro* unfolding assay [75]. Based on experiments in which GlpF was unfolded stepwise, GlpF appears to unfold in a two-step process, which involves an equilibrium of a tetrameric, a dimeric and a monomeric GlpF state. Semi-native SDS-PAGE analyses illustrated the transition from the GlpF tetramer to the monomer. With increasing SDS mole fractions, the intensity of the tetramer band decreased and eventually vanished completely while the protein band corresponding to the GlpF dimer first increased and then decreased again. The intensity of the monomer band reached its maximum at the highest SDS mole fractions, and together these data support a three-state unfolding mechanism. In addition, gel filtration of partly SDS-unfolded GlpF reinforced the SDS-PAGE results. Having a stable dimeric intermediate along the unfolding pathway suggests that at least two phases are involved in GlpF unfolding and the GlpF tetramer forms as a dimer of dimers [75]. Native tetrameric GlpF was purified and after incorporation into DDM micelles, addition of SDS led to the formation of mixed micelles with defined mole fractions. Although the GlpF tetramer is stable and retains its native structure on SDS-PAGE gels [28,74], addition of increasing SDS-concentrations unfolds the protein at least partially. Elevated mole fractions of SDS induce protomer–protomer as well as helix–helix dissociation, resulting in progressive GlpF unfolding. Importantly, after diluting out the SDS, the tetrameric GlpF state could be retrieved. Unfolding under equilibrium conditions was followed by CD and Trp fluorescence spectroscopy. The spectroscopic data suggested that unfolding of GlpF involves multiple transitions. The free energy of GlpF unfolding was determined to be 68.6 kJ/mol, a value that is comparable to other membrane proteins [88–91]. Using CD spectroscopy, two transitions with midpoints at ~0.22 and 0.62 mole fractions of SDS were observed, whereas two transitions with midpoints at ~0.35 and ~0.8 were observed when SDS-induced unfolding was followed by Trp fluorescence spectroscopy (Fig. 6). As the SDS-PAGE analysis had revealed that the tetramer to monomer dissociation has also a transition midpoint at ~0.8, the change observed by Trp fluorescence spectroscopy might represent dissociation of the tetramer. Thus, the tetramer is a rather stable structure and partial unfolding of the protomers appears to precede tetramer dissociation (Fig. 6). Unfortunately, the dimeric GlpF state seen on SDS-PAGE gels could not be clearly linked to any of the spectroscopically observed unfolding intermediates. The dimeric folding intermediate was found to structurally differ, both from the native tetrameric state and the monomer, by having a reduced level of tertiary structure with a looser helix packing but with mostly conserved secondary structure, reminiscent of a molten globule state found in the folding pathway of soluble proteins [75]. This finding, again, demonstrates that an oligomeric state is beneficial for the GlpF stability and function. A potential folding pathway, derived from the spectroscopic and SDS-PAGE analysis, is presented in Fig. 5. However, unfolding of GlpF by heat or urea treatment led to the observation of both, dimeric and trimeric states in SDS-PAGE analyses [28]. These findings strongly suggest that the unfolding pathway depends on the *in vitro* conditions and a sequential GlpF unfolding pathway might involve a GlpF trimer. Assuming both trimer and dimer as true (un)folding intermediates implies that tetramerization

Fig. 6. Transitions during SDS-induced unfolding of GlpF. Partial unfolding of the GlpF individual monomers precedes dissociation of the GlpF tetramers, as observed *via* CD and Trp fluorescence spectroscopy as well as by SDS-PAGE analysis. The mole fractions SDS of the respective transition points from one to the next folding intermediate are given.

occurs *via* consecutive addition of single monomers to growing GlpF oligomers (Fig. 5). Together, these results suggest that the tetrameric state is beneficial for the GlpF stability and function.

4.4. Folding of GlpF in multiple steps

Based on the above presented and discussed observations and considerations, folding of GlpF can be described to involve multiple steps, at least conceptually (Fig. 5). After membrane insertion of individually stable TM helices (Step 1), the two homologous GlpF subdomains assemble (Step 2), and interactions of TM helices 1 + 4 or 5 + 8, respectively, are crucial for subdomain assembly. After pre-assembly of the two subdomains, interactions between TM helices 2 and 6 mediate formation of the complete TM helix structure (Step 3), which provides the structural environment for membrane entry of the half-spanning helices and thus for formation of a discontinuous TM helix formed by helices 3 and 7 (Step 4). Thereafter, folded protomers interact to form the oligomeric GlpF structure, and protomer–protomer interactions might induce further structural rearrangements within a monomer. Oligomerization either takes place *via* capturing individual protomers, and thus *via* a progressive growth of the GlpF oligomer (Steps 5, 6, 7), or alternatively, GlpF dimers form and subsequently interact to form the tetrameric structure (Steps 5, 7).

5. Mutations affect the structure and function of human aquaporins

Thirteen aquaporins (AQP0–12) consisting of 261–342 amino acids are expressed in various human tissues where they facilitate the bidirectional flux of water and small polar solutes across cellular membranes. Studies with AQP knockout mice have been particularly helpful in revealing a diverse involvement of human aquaporins in physiological processes, such as the epithelial fluid transport, tumor angiogenesis, cell migration and brain edema [92]. Currently, structures of several aquaporins have been solved, including the human AQP1, AQP4, AQP5 and bovine AQP0 as well as the two bacterial aquaporins AqpZ and GlpF (Table 1).

Several variations in the aquaporin-encoding genes have been identified in human genomes (Fig. 7). Considering the diverse physiological functions of human aquaporins, it is not surprising that single amino acid exchanges can cause severe human diseases. As distinct amino acids define the translocation pore at the center of an AQP channel and establish the selectivity of substrate conductance, direct implications of amino acid substitutions on AQP activity are possible. Indeed, this has been observed in case of the human AQP5, where changes in the pore residues, including the arginine residue of the Ar/R filter, are implicated in diffuse *nonepidermolytic palmoplantar keratoderma* [93]. However, variations in the amino acid composition are generally more

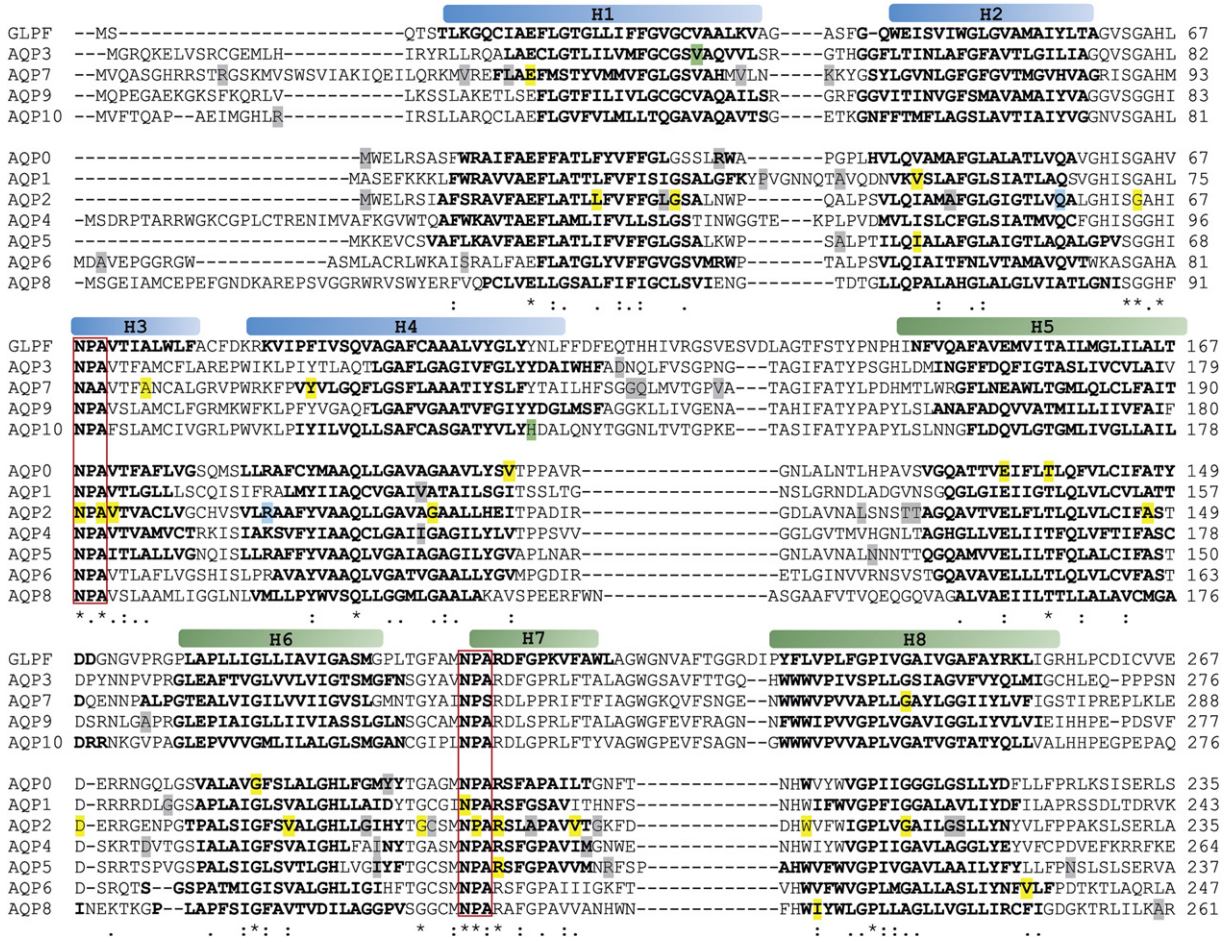

Fig. 7. Sequence alignment of GlpF and the classical human aquaporins and aquaglyceroporins. A multiple sequence alignment was prepared using CLUSTAL 2.1 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). For clarity, the last C-terminal amino acids of the different aquaporins are not depicted. One dot highlights conserved residues, two dots highly conserved residues and a star indicates identical residues. Annotation of TM segments has been performed *via* UniProtKB. The annotation is either experimentally proven (GlpF, AQP0, 1, 2, 4, 5) or has been predicted by UniProtKB applying predictive tools such as TMHMM, Memsat, Phobius, the hydrophobic moment plot of Eisenberg and co-workers and the ΔG_{app} prediction server v1.0 (AQP3, 6, 7, 8, 9, 10). TM segments are highlighted in bold. The highly conserved NPA motifs in the aquaporin sequences are highlighted by a red frame. Residues found to be substituted as a consequence of missense mutations in the various AQP genes have been highlighted with respect to their conservation. Highlighted in light gray are single amino acid substitutions, affecting non-conserved residues, in yellow highly conserved residues between GlpF and the classical aquaporins as well as the aquaglyceroporins, in green residues highly conserved between GlpF and aquaglyceroporins and in blue highly conserved residues within the classical aquaporins. Information about the missense mutations was obtained from the following sources ([133], additionally AQP0 [143], AQP2 [144], AQP4 [145], AQP5 [93], AQP7 [126], AQP8 [141]).

often associated with protein misfolding and misassembly than with direct alteration of protein activity [94,95]. Misfolded AQPs eventually accumulate in the ER membrane, as shown for AQP0 and AQP2 mutants [96–99]. However, it is worth mentioning that, besides improper cellular targeting and ER-retention, mutated AQPs might be functional, and impaired trafficking, rather than malfunctioning, causes an associated disease (*nephrogenic diabetes insipidus* in case of AQP2) [96]. Misfolded and ER-retained proteins are degraded by the ER-associated degradation (ERAD) pathways, and once the mutated proteins are exported into the cytosol, the ubiquitinated proteins are finally degraded by the proteasome [100,101]. Consequently, degradation of some AQP2 mutants remained unaffected by inhibitors of downstream vesicle trafficking and lysosomal proteases [96]. However, other AQP2 mutants, which cause dominant *nephrogenic diabetes insipidus*, form together with the wild-type AQP2 monomers hetero-tetrameric assemblies within the Golgi stacks [102], and from here these proteins are routed to lysosomes for degradation [103]. Both, lysosomal as well as proteosomal protein degradation, are involved in degradation of properly processed wild-type AQPs [104–109], and both appear to be important for regulating the AQP level in a membrane [110,111]. Thus, ERAD and the lysosome can be involved in degradation of unstable or misfolded AQPs.

While it is becoming increasingly clear that the lipid bilayer environment is important for proper folding, assembly and the activity of membrane proteins [112,113], correct folding of α-helical membrane proteins strongly depends on TM helix–helix interactions, which are determined by the amino acid sequence of interacting TM helices [95]. Although the consequences of single amino acid substitutions in human AQP2, associated with *nephrogenic diabetes insipidus*, have been studied in greater detail, the impact of single amino acid exchanges on the function of other aquaporins, and especially on aquaglyceroporins, have not been investigated to the same extent yet. In the following, we discuss the potential impact of a few mutations that have been observed in human genome sequences but have so far not been characterized. These uncharacterized mutations of conserved residues most likely affect folding and stability of aqua(glycero)porins and not substrate conductance directly, as discussed.

5.1. Mutations in GxxxG-like motifs, which mediate tight helix–helix contacts

TM helices 2 and 6 tightly interact in aqua(glycero)porins, and mainly small amino acids line the interface [114]. Thus, it is not surprising that conserved GxxxG-like motifs, which frequently mediate and stabilize TM helix–helix interactions, can be identified in both helices (Fig. 8). In the case of the human AQP0, which mediates water flux and is involved in the formation of junctions in lens fiber cells [115–118], one AxxxGxxxA- and one GxxxA-motif mediate interactions of TM helices 2 and 6 (Step 3 in Fig. 5). The importance of these motifs is evident in the AQP0 G165D mutant, which is associated with the phenotype of a lamellar cataract [99]. The respective Gly is highly conserved at this position (Fig. 7), and the mutated protein is impaired in proper cell trafficking [99]. To determine, whether the mutation affects TM helix membrane insertion, the apparent free energy (ΔG_{app}) of insertion of the affected TM helix into the membrane of the endoplasmic reticulum (ER) was predicted, employing the ΔG prediction server v1.0 [119] (values were converted to kJ/mol (1 kcal = 4.18 kJ)). This web-based server provides a position-dependent prediction of ΔG_{app} for the integration of the wt and mutated TM helices. In fact, the mutation of Gly165 to an Asp decreases the overall hydrophobicity of TM helix 6, and the transfer free energy increases from 2.27 kJ/mol for the wt helix to 8.08 kJ/mol for the mutant helix. Thus, membrane insertion of the mutated helix might already be dramatically impaired when Gly165 is replaced by Asp (Step 1 in Fig. 5).

Gly165 in AQP0 likely mediates close contact and van der Waals packing of TM helices 2 and 6 and might even interact *via* a C_α–H-bond with Gly49 in TM helix 2 [73]. As substitutions of single Gly residues can severely weaken TM helix–helix interactions [120–122], it is very likely that Gly165 is crucial for mediating and stabilizing TM helix–helix interactions along the AQP folding pathway, and in particular for interaction of the two subdomains (Step 3 in Fig. 5), as described above. The substitution of Gly by Asp, which has a charged and bulkier side-chain, in TM helix 6 likely hinders close packing of the two TM helices (Step 3 in Fig. 5), decreases the possibility for formation of C_α–H-bonds and thereby likely causes protein misfolding. Furthermore, by virtue of its polar side-chain, Asp can establish new TM helix–helix interactions [120,123–125], which might also affect the AQP0 folding pathway, yielding misfolded or unstable protein.

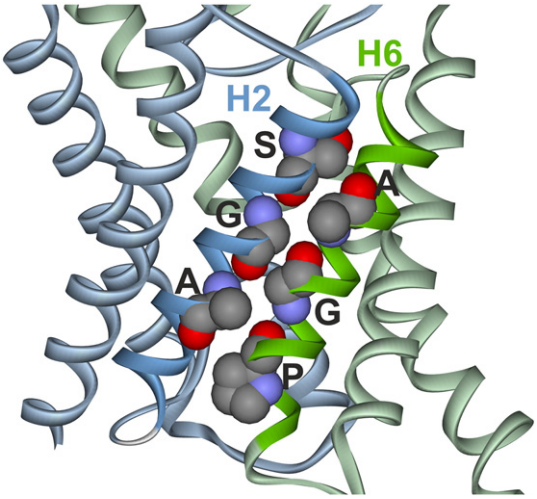

Fig. 8. GxxxG-like motifs. Conserved GxxxG-like motifs frequently mediate and stabilize tight TM helix–helix interactions. Small residues at the interface of the antiparallel GlpF TM helices 2 (blue) and 6 (green) are depicted (PDB ID: 1FX8). The interaction between TM helices 2 and 6 is crucial for the assembly of the two aqua(glycero)porin subdomains.

Similar effects will most likely occur in the case of the G264V mutation in TM helix 8 of AQP7 [126]. AQP7 is mainly expressed in adipose tissue, where it maintains the efflux of glycerol, but is also found in testis and the kidney [127–129]. The AQP7 residue Gly264 is highly conserved in aquaglyceroporins and classical aquaporins. In the G264V mutant, the glycerol conductance was severely impaired [126], and the mutation was found to correlate with a lower plasma glycerol level during exercise. Normally, the plasma glycerol level increases upon energy demand due to fasting or exercise to maintain hepatic gluconeogenesis. Gly264 is the middle Gly in a conserved GxxxGxxxG glycine zipper-motif, with some Gly being replaced by other small amino acids in some aquaporins (Fig. 7) [72,73]. This motif is expected to be of structural significance, as it allows tight TM helix–helix interactions between TM helices 5 and 8 (Step 2 in Fig. 5) and might be important for proper folding of the second AQP subdomain, as discussed above for the GlpF folding pathway. As mentioned, interactions of the parallel TM helices 1 and 4, as well as of TM helices 5 and 8, contribute to early steps in the folding of the respective GlpF subdomains (Step 2 in Fig. 5). The corresponding Gly residue in GlpF, Gly243, was suggested to be involved in an interhelical C_α–H-bond network, acting both as a hydrogen donor and acceptor [73]. Such networks of C_α–H-bonds are thought to contribute not only to helix–helix interactions but also to the overall protein stability, especially in channel proteins [130,131]. Importantly, prediction of the ΔG_{app} of insertion indicates that membrane insertion of the mutated TM helix is not impaired compared to the wt helix (−0.67 kJ/mol and −4.14 kJ/mol for the wt and the mutated helices, respectively). Therefore, subdomain misfolding or destabilization of the AQP7 structure, caused by the substitution of Gly by Val, very likely causes the observed decrease in protein activity. This has, however, not been tested experimentally yet.

5.2. Insertion of polar residues in transmembrane domains

The AQP7 A100T mutant has not been characterized yet [132,133]. The corresponding residue in GlpF is Ala74 (Fig. 7) that resides in the half-membrane-spanning helix 3 and is highly conserved in classical aquaporins and aquaglyceroporins. Ala74 does not contribute to the lining of the channel pore but packs against Met160 in TM helix 5 and Ala244, Ile245 and Ala248 in TM helix 8. As discussed above, small residues are frequently found at helix–helix interfaces in both globular and membrane proteins, as they allow tight packing of helices, enhancing strong helix–helix interactions *via* van der Waals forces, electrostatic interactions and the formation of H-bonds [134,135]. Small residues (Gly, Ala, Ser, Thr) are also frequently found in aquaporins at helix–helix interfaces [136]. Substituting the AQP7 residue Ala100 by Thr changes a small, nonpolar residue to a slightly larger but polar amino acid side-chain located within a cluster of hydrophobic residues. This might weaken or alter the helix–helix interactions between the half-spanning helix 3 and TM helices 5 and 8. For example, amino acids with hydroxyl side-chains have been shown to be able to form intra-helical H-bonds with carbonyl oxygen atoms (i-3 or i-4), thereby inducing or stabilizing a bending of the particular helix geometry [137–139] possibly leading to a destabilized fold. Alternatively, any interruption of the interactions between TM helix 3 in domain 1 and TM helices 5 and 8 in domain 2, could prevent proper assembly of the tertiary structure at the point where the reentrant loops help assemble the two sub-domains (Step 4, Fig. 5). New electrostatic interactions or H-bonds to Thr100 might alter the folding pathway producing off-pathway intermediates.

5.3. Mutations at helix–helix interfaces

AQP8 is expressed in pancreatic and colonic epithelial cells and is also found in the testis, kidney, small intestine and the liver. Besides having a high water permeability, AQP8 is additionally permeable to ammonia and free radicals [140]. The expression level of AQP8 in colonic epithelial cells is associated with colorectal tumors [10]. A known AQP8 mutation in humans is I229M [141]. Based on the known aquaporin structures, the residue Ile229 is located far away from the conserved NPA motifs in TM helix 8 and is not directly involved in formation of the substrate pore. Thus, if the mutation affects protein activity, this is more probably caused by an impaired protein structure or folding pathway. The hydrophobic nature of the residue substituted in this human AQP8 variant is conserved in aquaporins (in some cases Val is found), and the prediction of ΔG_{app} of insertion indicates that membrane insertion of the mutated TM helix is not impaired (−10.3 kJ/mol and −9.9 kJ/mol for the wt and the mutated helices, respectively). The residue corresponding to I229 in GlpF is V235. This residue resides in a hydrophobic cluster with Phe145, Ala148, Phe149 and the methylenes of Glu152, all belonging to TM helix 5. TM helices 5 and 8 are proposed to form an early intermediate in the formation of the second subdomain (Step 2 in Fig. 5), and weakening of this interaction could result in misfolding or instability. However, the substitution of Ile by Met does not introduce a dramatic change in polarity, and the steric consequences might be minor, suggesting that the I229M mutation might not be deleterious.

6. Conclusions and outlook

Aquaporins are polytopic TM channel proteins that selectively facilitate the flux of water across cellular membranes thereby carefully maintaining water homeostasis in various human tissues and across the biosphere. While in the last decade aquaporin substrate translocation and specificity were studied in greater detail, comprehensive information on aquaporin folding, dynamics and regulation are rather rare. While many of the observations described in recent years allow us to propose a complex folding pathway, especially some differences between the *in vitro* protein folding experiments and the *in vivo* folding process have to be clarified. Further research also needs to be performed concerning aquaporin oligomerization and its implications on the conductance of gaseous substrates.

Various mutations in human genes coding for aquaporins were identified in the past. While some of them are structurally and functionally well characterized, the majority of the identified mutations remain uncharacterized. Importantly, while mutated proteins might still form functional water and/or glycerol channels, the proteins are destabilized, resulting in defective cellular routing and ER-dependent degradation [96]. Inhibition of Hsp90, which is believed to interact with and promote ER-dependent degradation, has already partially rescued the defects in cellular routing of an AQP2 mutant [98]. Thus, modulating the protein quality control system and cellular trafficking might be a promising pharmacological strategy to treat diseases associated with aquaporin misfolding. Similar strategies are *e.g.* currently being followed for the treatment of cystic fibrosis [142]. By understanding the mechanisms underlying aquaporin substrate conduction and folding in greater detail, the impact of aquaporin mutations can be derived and understood. Existing structural and functional data can be utilized to deduce information on the impact of single amino acid substitutions, as shown in this review. In the future, such detailed analyses will facilitate the diagnosis of human diseases implicated with aquaporins and might be the basis for developing drugs targeting human aquaporins.

Acknowledgements

This work was supported by grants from the “Stiftung Rheinland-Pfalz für Innovation” (to D.S.) and the Funds of the German Chemical Industry (to D.S. and N.K.). J.O. is supported by the Natural Sciences and Engineering Research Council of Canada. We thank H. Pearson for carefully reading the manuscript and for helpful suggestions.

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