Role of C-terminal Domain and Transmembrane Helices 5 and 6 in Function and Quaternary Structure of Major Intrinsic Proteins

ANALYSIS OF AQUAPORIN/GLYCEROL FACILITATOR CHIMERIC PROTEINS*

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We previously observed that aquaporins and glycerol facilitators exhibit different oligomeric states when studied by sedimentation on density gradients following non-denaturing detergent solubilization. To determine the domains of major intrinsic protein (MIP) family proteins involved in oligomerization, we constructed protein chimeras corresponding to the aquaporin AQPeic substituted in the loop E (including the proximal part of transmembrane domain (TM) 5) and/or the C-terminal part (including the distal part of TM 6) by the equivalent domain of the glycerol channel aquaglyceroporin (GlpF) (chimeras called AGA, AAG, and AGG). The analogous chimeras of GlpF were also constructed (chimeras GAG, GGA, and GAA). RNA corresponding to all constructs were injected into Xenopus oocytes. AQPeic, GlpF, AAG, AGG, and GAG were targeted to plasma membranes. Water or glycerol membrane permeability measurements demonstrated that only the AAG chimera exhibited a channel function corresponding to water transport. Analysis of all proteins expressed either in oocytes or in yeast by velocity sedimentation on sucrose gradients following solubilization by 2% n-octyl glucoside indicated that only AQPeic and AAG exist in tetrameric forms. GlpF, GAG, and GAA sediment in a monomeric form, whereas GGA and AGG were found mono/dimeric. These data bring new evidence that, within the MIP family, aquaporins and GlpFs behave differently toward non-denaturing detergents. We demonstrate that the C-terminal part of AQPeic, including the distal half of TM 6, can be substituted by the equivalent domain of GlpF (AAG chimera) without modifying the transport specificity. Our results also suggest that interactions of TM 5 of one monomer with TM 1 of the adjacent monomer are crucial for aquaporin tetramer stability.

The flow of water and small solutes across lipid bilayers is necessary for fundamental cell functions. The first water channel was characterized in 1992 (1) and categorized in major intrinsic protein (MIP)1 family proteins. Based on oocyte swelling assays or proteoliposome osmotic behavior by stopped-flow kinetics, this protein family is now divided in two major functional subgroups: aquaporins (AQP), or specific water channels, and aquaglyceroporins (GlpFs), which are permeated by small solutes such as glycerol and to a lesser extend by water (2). All members of the MIP family present a high degree of similarity in their amino acid sequence and the same topological model: six transmembrane domains (TMs 1–6) connected to each other by five loops (A–E) with two conserved asparagine-proline-alanine boxes in loops B and E (3). Biochemical experiments and three-dimensional analysis by electron crystallography have demonstrated that aquaporins are functional in a tetrameric assembly in biological membranes (4–7) and that aquaporins that do not form tetramers do not facilitate water transport (8). Recent x-ray analysis of three-dimensional crystals of glycerol channel describe an homotetrameric form of the protein within crystals (9). However, several biochemical data indicated differences in quaternary structure between aquaporins and glycerol facilitators. Velocity sedimentation analysis on density gradients demonstrated that aquaporins are homotetrameric when extracted from membranes by non-denaturing detergents such as n-octyl-β-D-glucopyranoside (OG) or Triton X-100, whereas GlpF (the glycerol facilitator of Escherichia coli) exhibits sedimentation coefficient only compatible with a monomeric form (10–12). Freeze fracture analysis data of MIPs expressed in Xenopus oocytes were in agreement with our biochemical observations (13). We postulated that the oligomerization state of MIP proteins could be involved in their transport selectivity. Recent reports have confirmed that the monomeric form of GlpFs exists in non-denaturing detergent. However, oligomeric forms are also found in the same detergents, depending on protein concentration or biochemical environment. This suggests that GlpF could form weak oligomers and that lipid bilayer or other components of the membrane may play a role in protein activity (2, 14). When present in detergent, monomer–monomer interactions of GlpF are less stable than those observed for the aquaporin AqpZ (2). All together, these data could mean that functional specificity is linked to strength of self-association of MIP monomers in membrane bilayers. Nevertheless, the functional native oligomeric state of E. coli glycerol facilitator remains unclear whereas functional water channels such as AQP1, AQPcic (an aquaporin from the homopteran insect Cicadella viridis), or AqpZ are well known to be stable tetramers in lipid bilayers (5, 12, 15, 16).

Consensus motifs and amino acids have been found to be critical for aquaporins and glycerol facilitators functionality and specificity. In both proteins, part of the channel pore is constituted by the junction of loops B and E that overlap midway between the leaflets of the membrane in a zone delimited by the six transmembrane domains (3, 5). Multiple se-

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1 The abbreviations used are: MIP, major intrinsic protein; AQP, aquaporin; TM, transmembrane domain; GlpF, aquaglyceroporin; OG, n-octyl-β-D-glucopyranoside.
quence alignments of MIP proteins allowed identification of positions corresponding to amino acid residues conserved in aquaporins or in glycerol facilitators with highly different physicochemical properties between the two subgroups. Four of these discriminant amino acids are located in loop E and at the upper part of the sixth TM (17–19). Substitution of tyrosine 222 and tryptophan 223 in the upper part of the sixth TM of AQPcic by the two corresponding amino acids of GlpF (a proline and a leucine, respectively) induced a switch from a water to a glycerol channel and a switch from a tetrameric to a monomeric state in nondenaturing detergent (20). Although the general relevance of this observation is unclear (2), functional analyses of AQP0-AQP2 chimeras have demonstrated that stability of loop E is crucial for MIP channels (21). When TM 5 was replaced in AQP0 by that of AQP2, it was concluded that the distal part of TM 5 is necessary for maximum water channel activity and contributes to formation of the aqueous pore and determination of the flux rate (22). Recent crystallography and molecular modeling data supported the involvement of TM 5 in spatial relative positions of loops B and E for AQP1 and GlpF pore selectivity. These data also suggest that some residues of TM 1 and TM 5 could be involved in interactions between monomers within the tetramer (5, 6, 9, 23).

All together, these observations highlight the importance of the loop E and the fifth and sixth TMs in function and selectivity of MIP proteins. In the present study, we aimed to understand the relation between function, selectivity, and oligomerization of MIPs, by analysis of AQPcic and GlpF chimeras. Three of the chimeras correspond to the aquaporin substituted in loop E and/or C-terminal part by the equivalent domain of the glycerol channel (chimeras called AQA, AAG, and AGG). The three other chimeras correspond to the analogous chimeras of GlpF (GAG, GGA, and GAA). We then analyzed the functional channel properties and the quaternary structure of AQPcic-GlpF chimeras expressed in *Xenopus laevis* oocytes and in the yeast *Saccharomyces cerevisiae*. Our study shows that the C-terminal domain, including half of the sixth TM of the aquaporin AQPcic, can be replaced by the equivalent domain of GlpF without affecting channel specificity and quaternary organization.

**EXPERIMENTAL PROCEDURES**

*Mutagenesis and Plasmid Constructions*—To construct AQPcic-GlpF chimeras, introduction of unique restriction sites on cDNA encoding each protein was necessary. The *NruI* and *AvrII* sites were introduced, respectively, at the beginning and the end of the cDNA encoding the loop E of AQPcic and GlpF. As depicted in Fig. 1, the *NruI* site was introduced at a position corresponding to amino acid 183 of AQPcic and amino acid 186 of GlpF. The *AvrII* site was introduced at a position corresponding to amino acid 228 of AQPcic and amino acid 242 of GlpF. The AQPcic and GlpF mutated vectors were obtained by performing a two-step reaction PCR using pSP-AQPcic or pSP-glpF (20, 24) as a template and sets of appropriate primers (Fig. 1 A) that overlap in the region of the mutation. The mutated CDNAs were cloned into plasmid pXPgel-eva (constructions are named pSP-AQPcic, pSP-AQPcic, pSP-glfp,F, and pSP-glfp,F). These vectors were then used to construct plasmids carrying both *NruI* and *AvrII* restriction sites. The pSP-AQPcic construct was obtained by digesting pSP-AQPcic and pSP-AQPcic by *BamHI*, whereas the pSP-glfp,F construct was obtained by digesting the pSP-glfp,F and pSP-glfp,F vectors by *ApaI* (Fig. 1B). Mutations were confirmed by enzymatic nucleotide sequencing (U. S. Biochemical Corp.). The double mutated vectors were then digested with *NruI* and *AvrII* to obtain plasmids encoding for chimeric AQA and GAG and GGA chimeras were obtained by digestion of pSP-AQPcic and pSP-glfp,F with *NruI*SacI. The chimeras AQA and GGA were obtained by digestion of pSP-AQPcic and pSP-glfp,F with *AvrII*SacI.

The cDNA encoding GlpF and the different chimeras were then cloned into the yeast expression vector pYeDp80 (gift of Dr. Popom). Constructions were named pY60-glpF, pY60-AQA, pY60-AAG, pY60-AQA, pY60-AAG, and pY60-GGA. pDNA bearing the 5’ or 3’ region of AQPcic were amplified by polymerase chain reaction and cloned in pY60 vector as described (25). GlpF cDNA or cDNA bearing the 5’ or 3’ region of GlpF were amplified by PCR using two primers: YG1F, 5’-GGG AGT ACT ATG AGT CAA ACA TCA A-3’; YG2R, 5’-GGG GAG CTC AGT CAT ATT ACA GGG A-3’. The PCR primers contain SacI and SacI restriction sites (underlined).

*After EcoRI digestion of pY60 vector, blunt ends were generated by addition of Klenow fragment polymerase (Eurogene). A SacI digestion allowed us to obtain the pY60 vector blunt end (5’SacI end). GlpF cDNA or cDNA bearing 5’ or 3’ region of GlpF was then cloned following digestion SacI (5’ blunt end) and SacI (3’ cohesive end) of PCR product. Water Activity and Glycerol Permeability Measurements in X. Oocytes—AQPcic, GlpF, and chimeric cRNAs were prepared in *in vitro* using the mCAP mRNA capping kit (Stratagene) and injected into stage VI oocytes. Oocytes were incubated in 5× sucrose, 2× MCl, 1× MCl, 1× MCI, 2× MCI, 10× Hepes/NaOH, pH 7.4, at 18 °C for 8–72 h. Osmotic water and glycerol permeability of oocytes were measured as previously described (24). Xenopus total membranes were prepared as described in Ref. 26.

*Protein Expression in S. cerevisiae*—Yeast cells (*W3031B* strain: *a*, *leu2, his3, trp1, ura3, ade2-1, can1*, *cyr1*) were transformed with all pY60 constructs. The transformed yeast cells were grown at 28 °C in a rich medium (1% yeast extract, 1% Bactopeptone, 0.5% glucose) for 36 h, and induction of protein expression was performed by adding galactose (1%) in the culture medium (20 g/liter) for another 16–20 h (25).

*Membrane Preparation and Protein Solubilization*—After protein induction, yeast cells were homogenized with glass beads and total membrane fractions were prepared as described previously (27). Yeast and *Xenopus* oocyte membranes were incubated in TB buffer (20 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol) containing either 2% OG, 1% Triton X-100 for 12 h at 4 °C or 1% SDS for 12 h at room temperature. Insoluble materials were eliminated by a 100,000 × g centrifugation for 45 min at 15 °C.

*Velocity Sedimentation on Sucrose Gradients*—Linear 2–20% (w/v) sucrose density gradients were prepared from 2% and 20% sucrose stock solutions in TB buffer containing 2% OG, 1% Triton X-100, or 0.1% SDS. Solubilized proteins (1–10 μg) were layered on top of gradients, and ultracentrifugation was performed at 100,000 × g for 18 h at 4 °C. Calibration curves for the determination of the apparent sedimentation coefficient were constructed using cytochrome C (s20w0 = 1.7 S), bovine serum albumin (s20w0 = 4.3 S), and IgG (s20w0 = 7 S). Following centrifugation, 20 fractions were collected from the bottom of each gradient and analyzed by SDS-PAGE (28). Proteins of each fraction were revealed either by Coomassie Blue staining or by Western blotting.

*ultracentrifugation—Water and Glycerol Permeability Measurements in Xenopus Oocyte Membranes*—Yeast and *Xenopus* oocyte membranes were prepared as described in Ref. 26.

**RESULTS**

We constructed chimeras corresponding to the aquaporin AQPcic replaced in the loop E and/or the C-terminal part (including the major part of the sixth transmembrane segment) by the equivalent domain of the glycerol channel GlpF (chimeras called AQA, AAG, and AGG). The analogous chimeras of GlpF were also constructed (chimeras GAG, GGA, and GAA). To be
able to exchange these domains, two restriction sites were introduced in each cDNA (Fig. 1). Constructions obtained were used to create cDNA encoding the following chimeric proteins: AGA, AQPcic with loop E from GlpF including half of the fifth and sixth TMs; AAG, GlpF with C-terminal part from AQPcic (Fig. 2). Transcripts corresponding to AQPcic, GlpF, and chimeric proteins were injected into X. laevis oocytes, and the presence of proteins in oocyte membranes was assayed by Western blot analysis (Fig. 3). All AQPcic-GlpF chimeras are expressed in oocytes membranes except AGA protein. Swelling assays on oocytes injected with AGG, GAG, GGA, and GAA mRNA presented water permeability coefficients similar to control oocytes. Oocytes injected with AGG, GGA, and GAA mRNA present water permeability coefficients significantly higher than control oocytes (Fig. 4A). In both cases, the increase of water permeability is inhibited by the addition of mercury (Fig. 4B). Oocytes injected with AGG, GGA, and GAA mRNA present water permeability coefficients similar to control oocytes. Glycerol apparent permeability (Pgly) measurement show that all chimeras present an apparent permeability coefficient to glycerol similar to that of control oocytes (Fig. 4B). To investigate the possibility that the absence of functionality of AGG, GlpF, AQPcic, GGA, and GAA proteins could be a consequence of misrouting, immunocytochemistry experiments were performed on Xenopus oocyte paraffin sections. Fig. 5 shows that AQPcic is found mostly in the plasma membrane, whereas GlpF is present in the cytoplasmic fraction. The AAG, AGG, and GAG chimeras appear partially targeted to plasma membranes, whereas GAA seems retained in the cytoplasmic fraction. To investigate the oligomerization state of AQPcic-GlpF chimeric proteins, velocity sedimentation on sucrose gradient experiments were performed using proteins expressed in Xenopus oocytes and in yeast. The presence of proteins in yeast membranes was previously verified by Western blot (Fig. 6). All chimeric proteins, including AGA, are fully expressed in the yeast system. AQPcic, GlpF, and chimeras were then solubilized by 2% OG, 1% Triton X-100, or 1% SDS and analyzed on a linear 2–20% (w/v) sucrose density gradient. The results presented in Fig. 7 were obtained in OG; identical data were obtained with samples solubilized in Triton X-100. In both cellular models, oocyte and yeast, AQPcic peaks at sedimentation fractions corresponding to a 6.8 S apparent sedimentation coefficient mean value that fits with a homotetrameric form of the protein, whereas GlpF peaks at ~2.8 S fractions, which correspond to a monomer in detergent (Fig. 7A). Concerning AQPcic-GlpF chimeras, only AAG, which is a functional water channel in Xenopus oocytes, is found tetrameric in sucrose gradients. This chimera sometimes presents a minor monomeric form in addition to the tetrameric form in the oocyte expression system but is always fully tetrameric when extracted from yeast membranes. The AGG, GGA, GAA, and GAA chimeras, which have been found nonfunctional in Xenopus oocytes, are monomeric and/or exhibit intermediary forms that could be dimers in OG. The AGA chimera, unexpressed in oocytes, is monomeric following non-denaturing detergent extraction from yeast membranes (Fig. 7B). When solubilized in
1% SDS, all wild type and chimeric proteins peak at fractions that correspond to monomers (data not shown).

DISCUSSION

How various structural motifs influence the divergent functions members of the MIP family is a critical question. Past work suggests that there may be specific motifs conferring either glycerol or water permeability (17–19, 30, 31). This conclusion has been recently reinforced following three-dimensional crystallography and molecular modeling reports. On the other hand, velocity sedimentation experiments reveal that aquaporins and GlpF present different oligomeric states in nondenaturing detergents (2, 14, 20). We have shown that replacement of two amino acids in the upper part of the sixth transmembrane domain of AQPcic by the two corresponding amino acids of GlpF induced a switch from a water to a glycerol channel and from a tetrameric to a monomeric state in non-denaturing detergent (20). This leads us to hypothesize that oligomeric state of MIP proteins and/or strength of monomer association could be involved in functional specificity determinism. Based on knowledge about the importance of loop E in pore formation, we undertook the present study in which loop E and the C-terminal part (including the major part of the sixth transmembrane domain) of AQPcic and GlpF were exchanged. We then assessed expression, targeting, function, and oligomerization state of resulting chimeras in oocytes. Xenopus oocytes have been largely used as an experimental system for expression and functional analysis of heterologous proteins (32) even if expression and targeting mechanisms remain partly unexplained. Unexpressed or misrouted aquaporin mutants for some aquaporins (AQP1, AQP2, and AQPcic) have been previously described in the Xenopus oocytes system (3, 25, 33); in the present study, we observed that one of the chimeras (AGA) was not expressed in oocyte. Yeast cells, which generally allow the easy production of high level of recombinant aquaporins like the AQPcic-C134S mutant (10), appear to be a complementary system to Xenopus oocyte and have been used in this study to express all wild type proteins and chimeras, including AGA.

When proteins were expressed in oocytes, as verified by Western blotting experiments, immunocytochemistry revealed that the major part of wild-type AQPcic is targeted to the plasma membrane. GlpF is shown to be functional in oocytes but is found partially retained into cytoplasmic fraction, as are all chimeras bearing the C-terminal part of the glycerol channel of E. coli (AAG, AGG, GAG). We also observed that chimeras that correspond to GlpF substituted in loop E and/or TM 6 by the equivalent domains of AQPcic (GGA and GAA) are properly expressed but are not fully targeted to plasma membrane.

AGG and GAG are found partially targeted to plasma membrane but do not exhibit water or glycerol channel function. They exhibit a major monomeric state and minor oligomeric forms. AAG is the only chimera evidenced as a functional water channel in oocytes and is the only one with an homotetrameric oligomerization state like wild type AQPcic. This suggests that the distal part of TM 6 and the C-terminal part are not specif-
ically involved in tetramerization and selectivity of the aquaporin. The water permeability of the AAG protein is inhibited by mercury chloride. We previously demonstrated that cysteine 82 in loop B of AQPcic is the unique mercury binding site (25). Loop B has not been replaced in the AAG chimera, and the modification introduced in the aquaporin does not influence the accessibility of mercury ions. Oocytes expressing AAG protein present a lower water permeability than oocytes expressing AQPcic for a saturating amount of injected cRNA. Immunocytochemistry suggests that this difference in measured water permeability is a consequence of protein misrouting in the oocyte system rather than a crucial implication of the substituted domain in channel efficiency. In parallel, we noticed that all chimeras described in this paper and AQPcic, when truncated in its C-terminal part, are more sensitive to proteolysis in both expression systems than are wild type proteins (data not shown). The extramembranous C-terminal part of the aquaporin thus appears unnecessary for pore formation and selectivity, but it may be involved in tetramer folding or protein stability.

**FIG. 5.** Immunolocalization of AQPcic, GlpF, and AQPcic-GlpF chimera proteins expressed in *Xenopus* oocytes. Three days following water or cRNA injections, oocytes were fixed and embedded in paraffin. Immunofluorescence on oocyte sections were achieved by using preimmune sera (A and C) or polyclonal anti-AQPcic (B) or anti-GlpF (D) antisera as primary antibodies, and anti-rabbit IgG conjugated with fluorescein isothiocyanate as secondary antibody.

**FIG. 6.** Expression of AQPcic, GlpF, and chimeric proteins in *S. cerevisiae*. Yeast cells were transformed with expression vector pY60 containing cDNA encoding AQPcic, GlpF, and chimera proteins. After expression, yeast total membranes were prepared and immunodetections were achieved with anti-AQPcic (A) or with anti-GlpF (B) antibodies. All chimeras, including AGA, were detected.

**FIG. 7.** Oligomeric state of AQPcic-GlpF chimera proteins in nondenaturing detergent. Proteins were extracted from *Xenopus* oocytes (--) or *S. cerevisiae* (----) membranes with 2% OG and submitted to velocity sedimentation analysis on sucrose gradients as described under "Experimental Procedures." Gradient fractions were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. A, autoradiograms of AQPcic and GlpF. Apparent sedimentation coefficient mean values of 2.8 and 6.8 S correspond to monomers and tetramers in 2% OG, respectively; B, sedimentation profiles of wild type and chimera proteins. Curves are representative of three independent experiments. Only AQPcic and the AAG chimera are tetrameric.
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is stabilized by interactions between N- and C-terminal domains, which extend on the cytoplasmic side in close proximity in adjacent monomers (36). Putative equivalent interactions between GlpF monomers in three-dimensional models have, however, not been described. In the AAG chimera, the replacement of C-terminal part and the lower part of TM 6 does not modify helix-helix interactions between adjacent monomers, whereas it abolishes C-terminus-N terminus putative interactions at the intracellular side. This suggests that interactions between helices are more crucial for aquaporin tetramer stability than interactions between extramembranous C- and N-terminal domains. In the AGA and AGG chimeras, the native interacting surfaces between TM 5 of one aquaporin monomer and TM 1 of the adjacent monomer are substituted. Even if some interactions remain possible at the cytoplasmic surface in chimeras, lack of TM 1-TM 5 interactions appears sufficient to abolish tetramer formation or stability. In contrast, in none of the chimeras deriving from GlpF (GGA, GAG, GAA) was a putative TM 1-TM 5 AQp-like interaction introduced and none of these chimeras clearly tends to form oligomers. These observations lead us to the conclusion that the role of interactions between TM 1 and TM 5 at the extracellular surface of the tetramer is crucial for AQp quaternary structure stability.

All together, the present results corroborate previous data showing that loop E, including the proximal part of the fifth transmembrane helix, is crucial for MIP protein channel formation and that the structure of this loop is important for oligomeric assembly or tetrameric stability. We show that the C-terminal domain, including half of the sixth transmembrane helix of the aquaporin AQPCic can be substituted by the equivalent domain of GlpF without affecting channel specificity and quaternary organization. Another point is that the functional native oligomeric state of GlpF still remains to be clearly demonstrated whereas it has been suggested that an equilibrium of monomers, dimers, or tetramers might exist with stabilized specific oligomeric form of GlpF, depending on membrane environment (2, 14). Nevertheless, the present AQPCic-GlpF chimera oligomerization state study strengthens the hypothesis that, in the MIP family, aquaporins and GlpF do not exhibit identical monomer self-assembly mechanisms. Recent crystallographic and molecular modeling data have considerably increased our knowledge on water and glycerol pore formation structures and conduction mechanisms in MIP proteins. Knowledge about interactions between amino acids or domains in quaternary structure of MIP proteins still remains to be improved to fully elucidate the molecular mechanisms responsible of MIP proteins’ functional specificity.

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