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Oligomerization State of Water Channels and Glycerol Facilitators

INVOLVEMENT OF LOOP E*

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The major intrinsic protein (MIP) family includes water channels aquaporins (AQPs) and facilitators for small solutes such as glycerol (GlpFs). Velocity sedimentation on sucrose gradients demonstrates that heterologous AQPcic expressed in yeast or *Xenopus* oocytes behaves as an homotetramer when extracted by *n*-octyl β-D-glucopyranoside (OG) and as a monomer when extracted by SDS. We performed an analysis of GlpF solubilized from membranes of *Escherichia coli* or of mRNA-injected *Xenopus* oocytes. The GlpF protein extracted either by SDS or by nondenaturing detergents, OG and Triton X-100, exhibits sedimentation coefficients only compatible with a monomeric form of the protein in micelles. We then substituted in loop E of AQPcic two amino acids predicted to play a role in the functional/structural properties of the MIPs. In two expression systems, yeast and oocytes, the mutant AQPcic-S205D is monomeric in OG and in SDS. The A209K mutation does not modify the tetrameric form of the heterologous protein in OG. This study shows that the serine residue at position 205 is essential for AQPcic tetramerization. Because the serine in this position is highly conserved among aquaporins and systematically replaced by an acid aspartic in GlpFs, we postulate that glycerol facilitators are monomers whereas aquaporins are organized in tetramers. Our data suggest that the role of loop E in MIP properties partly occurs through its ability to allow oligomerization of the proteins.

The MIP[1] family is a rapidly growing group of currently 150 proteins that have been widely identified from bacteria to man. Functional characterization data have distinguished two major subgroups of specific channels: aquaporins (AQPs), which transfer water, and glycerol facilitators (GlpFs), which transfer small neutral solutes.

In the aquaporin subgroup, the eukaryotic AQP0 (1, 2), AQP1 (3, 4), AQPcic (initially called P25 in Ref. 5), and AQP4 (6) have been demonstrated to exist as homotetramers. The procaryotic AqpZ was also recently shown to be tetrameric (7). The role for assembly of AQPs in tetramers is unclear but may be required for stability and function (8–11). According to the projection map of AQP1 obtained by electron crystallography, the protein in its tetrameric native form possesses six transmembrane domains surrounding a high density zone (12, 13). This density area is likely constituted by loops B, C, and E that fold into the membrane through hydrophobic interactions with the transmembrane helical domains according to the "hourglass model" proposed for AQP1 (14). However, the domains involved in aquaporin tetramerization have not yet been identified.

It has been shown that some mutations introduced in aquaporin loops affect water channel function (14, 15), whereas other mutations affect normal routing (16–18) or expression in *Xenopus* oocytes (19). Few data exist about the consequences of mutations on oligomerization of aquaporins. Jung *et al.* (14) reported that following expression in *Xenopus* oocytes, mutants in loop E sedimented with *s* values slightly higher than native AQP1 suggesting an importance of this loop in oligomerization and water channel function.

The second subgroup of proteins in the MIP family is constituted by the glycerol facilitators (20). Although the structural feature of one aquaporin is known (12, 13), no data are yet available about the three-dimensional structure and oligomerization state of GlpFs. However, based on the similarities in their amino acid sequences and hydrophobicity profiles, the monomers of glycerol facilitators and of water channels likely exhibit similar three-dimensional structural organizations. A topological model for MIPs and a comparison of sequences in loop E between an aquaporin, AQPcic of *Cicadella viridis*, and the GlpF of *Escherichia coli* are shown in Fig. 1. We recently detected five amino acid positions that distinguish clearly two groups in MIPs (21). These amino acids have been designated as "discriminant." Interestingly, these two groups correspond to the two functionally different groups within MIPs, namely aquaporins and glycerol facilitators. One of these positions is located in loop C and the four others are found in or close to loop E.

To study the oligomerization state of MIPs, we analyzed the behavior on sucrose density gradients of wild type or mutated AQPcic and of GlpF. We show that the heterologous AQPcic is tetrameric whereas GlpF is in a monomeric form either in OG or in Triton X-100 micelles. Moreover, we demonstrate that the substitution of a conserved amino acid of the aquaporin by a conserved amino acid of GlpF leads to the passage of AQPcic from a tetrameric to a monomeric form.

MATERIALS AND METHODS

Plasmidic Constructions and Mutagenesis—pSP-AQPcic corresponds to the full-length AQPcic coding sequence subcloned into plasmid pXβG-ev1 (22). The pY60-AQPcic contains the AQPcic cDNA coding sequence placed under control of a GAL10-CYC promoter and a phosphoglycerate kinase terminator (19). The AQPcic mutants were obtained by performing a two-step polymerase chain reaction using pSP-AQPcic as a template and sets of appropriate primers that overlap in

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[1] The abbreviations used are: MIP, major intrinsic protein; AQP, aquaporin; GlpF, glycerol facilitator; OG, *n*-octyl β-D-glucopyranoside.

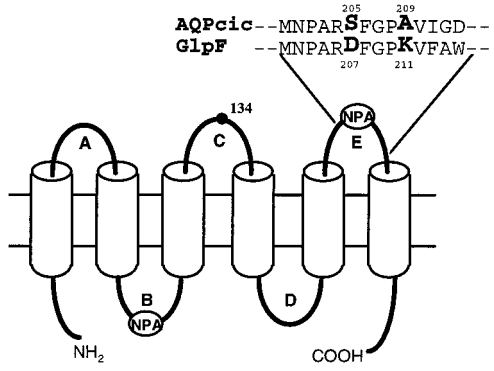

FIG. 1. **Topological model of MIPs and localization of two discriminant amino acid positions between AQPcic and GlpF.** A partial amino acid sequence of loop E in AQPcic and GlpF is shown. *Bold* residues indicate discriminant amino acids between aquaporins and glycerol facilitators as determined in Ref. 21. The positions of the highly conserved NPA boxes (Asn-Pro-Ala) in the MIPs and cysteine 134 of AQPcic are indicated.

the region of the mutation (23). The mutated AQPcic cDNAs were cloned either into plasmid pXβG-ev1 (constructions are named pSP-AQP_{S205D}, pSP-AQP_{A209K}, and pSP-$AQP_{S205D/A209K}$) or into the yeast expression vector pYeDP60 (constructions are named pY60-AQP_{S205D}, pY60-AQP_{A209K}, and pY60-$AQP_{S205D/A209K}$). The constructs pSP-AQP_{C134S} and pY60-AQP_{C134S} were described in Ref. 19. The coding region of *E. coli* glpF was amplified from the pglpF vector, a generous gift of Dr. Mizumo (20), by polymerase chain reaction and cloned either into the bacterial plasmid pUC18 (pUC-glpF) or into pXβG-ev1 (pSP-glpF).

Protein Expression—Yeast cells (W3031B strain) were transformed with pY60-AQPcic, pY60-AQP_{S205D}, pY60-AQP_{A209K}, pY60-$AQP_{S205D/A209K}$, and pY60-AQP_{C134S}. Expression of wild type or mutated forms of AQPcic was then performed as described previously (19). For the production of GlpF in bacteria, the *E. coli* strain GD236 (JM103 (24)) was transformed with pUC-glpF and was grown at 37 °C in LB with ampicillin (100 μg/ml). At midlog phase (A_{600} ~0.6), expression of GlpF was induced by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside in the culture medium for 30 min. Production of proteins in *Xenopus* oocytes and subsequent water and glycerol transport assays were achieved as described (22).

Membrane Preparation and Protein Solubilization—After protein expression, total membrane fractions from yeast cells were prepared as described previously (25). Bacteria and *Xenopus* oocyte membranes were prepared as described in Refs. 26 and 9, respectively. Membranes were incubated in TB buffer (20 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol) containing 2% OG or 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 to equilibrium was performed at 100,000 × *g* for 16 h at 5 °C. 20 fractions were then collected from the bottom of each gradient and analyzed by SDS-polyacrylamide gel electrophoresis (27) and Western blotting (28).

Antibodies and Western Blotting Analysis—AQPcic immunodetection was performed using a polyclonal rabbit antiserum raised against the native *C. viridis* protein (5). GlpF was detected using a polyclonal antiserum raised in rabbit against a synthetic COOH-terminal peptide of GlpF (NH_2-CVVEEKETTTPSEQKASL-COOH coupled to KLH) (Neosystem, France). Western blotting analysis was performed as described (22). Blots were first incubated with either anti-AQPcic or anti-GlpF antibodies (1/1000).

Gradient Analysis—Autoradiographic films were scanned and analyzed using molecular analyst software (Bio-Rad). Curves were obtained using Excel software (Microsoft Corp.).

RESULTS

Expression of GlpF and AQPcic in Heterologous Systems—The presence of wild type and mutant proteins in membrane cells was analyzed by Western blotting. As shown in Fig. 2*A*, yeast cells transformed by appropriate vectors express AQPcic (*lane 2*), AQPcic-S205D (*lane 3*), and AQPcic-A209K (*lane 4*) although no signal is detected in membranes of control yeast (*lane 1*). In membranes of cRNA-microinjected *Xenopus* oocytes, AQPcic (*lane 6*), AQPcic-S205D (*lane 7*), and AQPcic-A209K (*lane 8*) are clearly detected and absent in control oocytes (*lane 5*). Fig. 2*B* shows that the anti-GlpF antiserum reveals a 28-kDa protein in bacteria transformed by pUC-glpF vector (*lane 2*) and in glpF cRNA-microinjected oocytes (*lane 4*). No band is detected in control extracts (*lanes 1* and *3*). The presence of this specific immunoreactive 28-kDa band demonstrates that the GlpF protein is correctly expressed in membranes of both oocytes and bacteria. In addition to the 28-kDa GlpF protein, we observe a 30-kDa protein in bacteria membrane extracts. The presence of this 30-kDa additional immunoreactive protein results from the expression of an additional in-frame sequence corresponding to GlpF cDNA and the NH_2 termini of LacZ cDNA in the pUC-glpF vector.

Functional Assays—To determine the functionality and specificity of exogenous proteins, swelling assays were performed on oocytes microinjected with wild type or mutants of AQPcic and GlpF cRNA (Fig. 2*C*). Osmotic water permeability (P_f) is increased 15–20-fold in wild type AQPcic oocytes when compared with control oocytes. No significant increase of water osmotic permeability is observed in AQPcic-S205D, AQPcic-A209K oocytes, or GlpF oocytes. The functionality of AQPcic-C134S has been previously demonstrated in proteoliposomes (19). On the other hand, apparent glycerol permeability (P'_{gly}) is increased about 10-fold in GlpF-expressing oocytes, whereas no modification of glycerol permeability is observed in oocytes expressing AQPcic, AQPcic-S205D, and AQPcic-A209K. In addition, *in vivo* uptake assays of radiolabeled glycerol have demonstrated that the GD236 *E. coli* strain ($glpF^-$) transformed with pUC-glpF plasmid expresses a functional glycerol channel (data not shown).

Oligomeric Form of Recombinant Proteins—We previously demonstrated that the native AQPcic is a homotetramer in OG and in Triton X-100 and that SDS extraction from insect membranes induces monomerization of the protein (5). Thus we used the same approach to analyze and compare recombinant AQPcic and mutants of AQPcic and GlpF. To determine the oligomeric form of the proteins in the nondenaturing or denaturing detergents, membrane extracts from oocytes, yeast, or bacteria were analyzed by sucrose gradient sedimentation, and localization of proteins in fractions was revealed by Western blotting.

As shown in Fig. 3*A*, the OG-solubilized AQPcic protein peaks at sedimentation fractions 9–10. This corresponds to a 6.8 S apparent sedimentation coefficient mean value and fits with the homotetrameric form of AQPcic (5). When AQPcic solubilization is performed in SDS, sedimentation occurs at fractions 14–15 (mean value of 2.8 S) that correspond to the monomeric form of the protein. These results show that heterologous AQPcic expressed either in yeast or in *Xenopus* oocytes exists in the same tetrameric state as the native protein. We then analyzed on sucrose gradients the 28-kDa GlpF protein overexpressed in *E. coli* and oocyte membranes. In both expression systems, GlpF detergent complexes sediment in OG or in SDS at an apparent sedimentation coefficient of 2.8 S in fractions 14–15 (Fig. 3*B*). This result is representative of three independent experiments. An identical sedimentation profile is obtained when the experiment is performed on *E. coli* GlpF extracted by Triton X-100 (Fig. 3*B*).

The effect of discriminant amino acid substitution in loop E on oligomerization of MIPs was then analyzed. We substituted the serine 205 or the alanine 209 of AQPcic with the amino acids located in the corresponding positions in GlpF, an aspar-

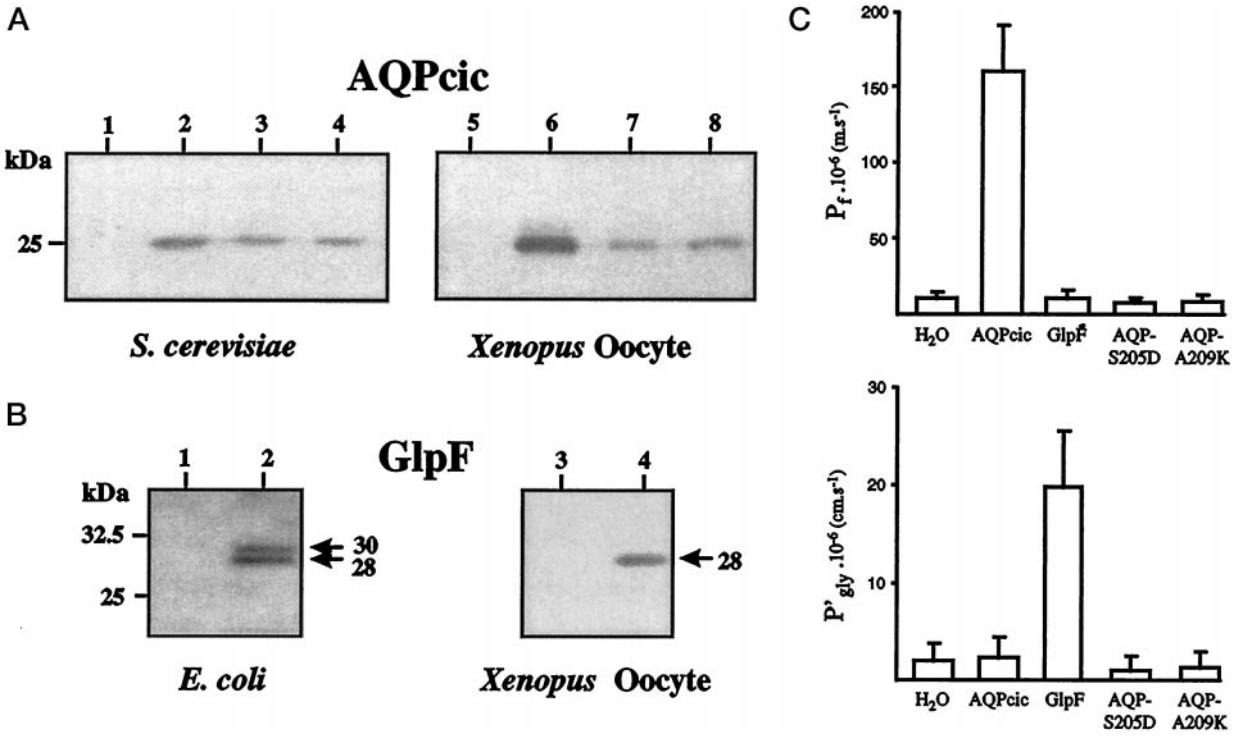

FIG. 2. **AQPcic and GlpF are produced in heterologous expression systems.** *A*, expression of wild type or mutated type AQPcic in yeast or in *Xenopus* oocyte. Western blots were performed using total membrane extracts from yeast cells transformed with pY60 alone (*lane 1*), pY60-AQPcic (*lane 2*), pY60-AQP$_{S205D}$ (*lane 3*), or pY60-AQP$_{A209K}$ (*lane 4*), or using membrane extracts from *Xenopus* oocytes injected with water (*lane 5*) or with AQPcic-cRNA (*lane 6*), AQPcic-S205D cRNA (*lane 7*), and AQPcic-A209K cRNA (*lane 8*). *B*, expression of GlpF in *E. coli* or in *Xenopus* oocytes. Immunoblots were performed with membrane extracts of *E. coli* transformed with control vector (*lane 1*), pUC-glpF vector (*lane 2*), or with membrane extracts from control *Xenopus* oocytes (*lane 3*) or from GlpF cRNA-injected oocytes (*lane 4*). The 28-kDa GlpF is specifically detected in both expression systems. The 30-kDa immunoreactive band in bacteria results from the plasmid construction. *C*, functional analysis of AQPcic, GlpF, AQPcic-S205D, and AQPcic-A209K in *Xenopus* oocytes. Swelling assays were performed 48–72 h following cRNA injections. For water permeability (P_f) measurements, oocytes were submitted to an osmotic shock by diluting one-third of the OR2 medium. For glycerol apparent permeability (P'_{gly}) measurements, oocytes were submitted to an iso-osmotic shock in OR2 containing 120 mM glycerol. Control oocytes were injected with water. Values are mean ± S.E. (n = 7–20).

tic acid and a lysine, respectively (Fig. 1). Density gradient sedimentation analysis of these mutants expressed in yeast and in *Xenopus* oocytes is shown in Fig. 4. In both expression systems, the AQPcic-S205D mutation leaves the protein in a monomeric state in OG, whereas the mutation A209K in AQPcic does not modify the tetramer in the same nondenaturing detergent. We have also demonstrated that both AQPcic-S205D and AQPcic-A209K mutants are monomeric in SDS and that the double mutant AQPcic-S205D/A209K is monomeric in OG and in SDS (data not shown). In contrast, in yeast membrane extracts solubilized in OG, the mutant AQPcic-C134S, which is a functional aquaporin (19), is found tetrameric.

DISCUSSION

In the present work, we studied the oligomerization state of representative proteins of the two functional subgroups of the MIP family. For this purpose, we analyzed the insect aquaporin AQPcic expressed in yeast or in *Xenopus* oocytes and the glycerol facilitator of *E. coli* expressed in bacteria or in oocytes. We also determined the oligomerization state of AQPcic when characteristic residues of aquaporins are substituted with characteristic residues of the glycerol facilitator family and showed that the amino acid in position 205 is involved in tetramerization or stability of the AQPcic tetramer.

The native tetrameric state has been previously demonstrated for AQP0 (1, 2), AQP1 (3), or AQPcic (5) in part by hydrodynamic studies, on the basis that native MIP tetramers are maintained in solution with nondenaturing detergents such as OG or Triton X-100. Additionally, functional AQP1 (29) and AQPcic (19) solubilized in OG have been successfully reconstituted in liposomes. Thus we performed protein extraction and sedimentation gradient analysis in 2% OG or 1% Triton X-100, values above critical micellar concentrations, and in 1% SDS, a percentage of the denaturing detergent sufficient to monomerize native AQPcic (5).

We previously showed that AQPcic is a functional water channel when expressed in oocytes (22) or in yeast (19). The present study demonstrates that in both heterologous expression systems, the functional wild type aquaporin AQPcic is tetrameric. In yeast, the functional aquaporin mutant AQP-C134S is also tetrameric. To analyze GlpF oligomerization, we used the same technique as for AQPcic. We verified the functionality of the protein following expression in *Xenopus* oocytes and in bacteria. By oocyte swelling assays, the specific glycerol transport through GlpF has been demonstrated. The monomeric state of GlpF that we have observed in oocytes is not likely to correspond to a mistargeted form of the protein. Furthermore, uptake assays in bacteria expressing GlpF strengthen our statement about a monomeric GlpF protein.

To analyze the relationship between the sedimentation coefficient and the oligomerization state of a membrane protein in detergent micelles, the amount of bound detergent should be known. Although the amount of OG bound to GlpF in our samples remains undetermined considering the highly conserved hydrophobicity profiles of MIPs, we assume that OG has the same behavior on GlpF as on other members of the MIP family. Moreover, exposure of hydrophobic transmembrane regions to the detergent should likely induce the monomeric forms of MIPs to bind more detergent/protein (w/w) than the

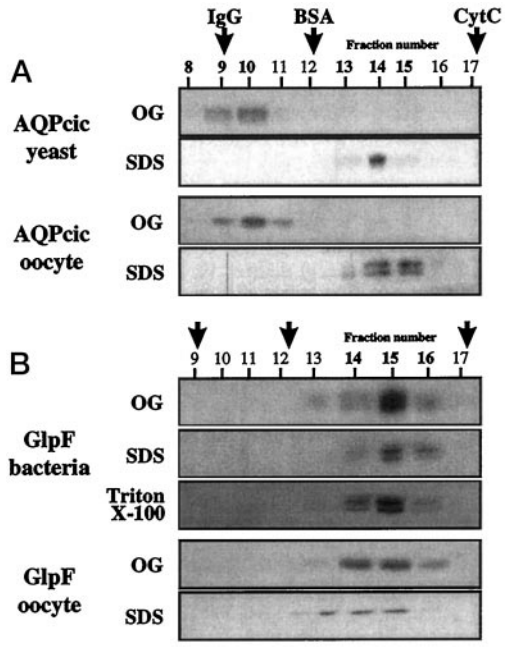

FIG. 3. **Analysis of fractions from sucrose density gradient centrifugation of AQPcic and GlpF in different detergents.** Total membranes were solubilized by 2% OG, 1% SDS, or 1% Triton X-100 as described under "Materials and Methods." Solubilized extracts were layered on top of 2–20% (w/v) sucrose gradients in the same detergents and centrifuged for 16 h at 100,000 × *g*. Gradient fractions were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. Positions of marker proteins cytochrome *c* (*CytC*, 1.7 S), bovine serum albumin (*BSA*, 4.3 S), and IgG (7 S) detected by Coomassie Blue staining of acrylamide gels are indicated at the *top*. *A*, AQPcic expressed in yeast and oocyte is tetrameric (6.8 S) in OG and monomeric (2.8 S) in SDS. *B*, *E. coli*-expressed GlpF is monomeric (2.8 S) in OG, Triton X-100, and SDS. Oocyte-expressed GlpF is monomeric in OG and in SDS.

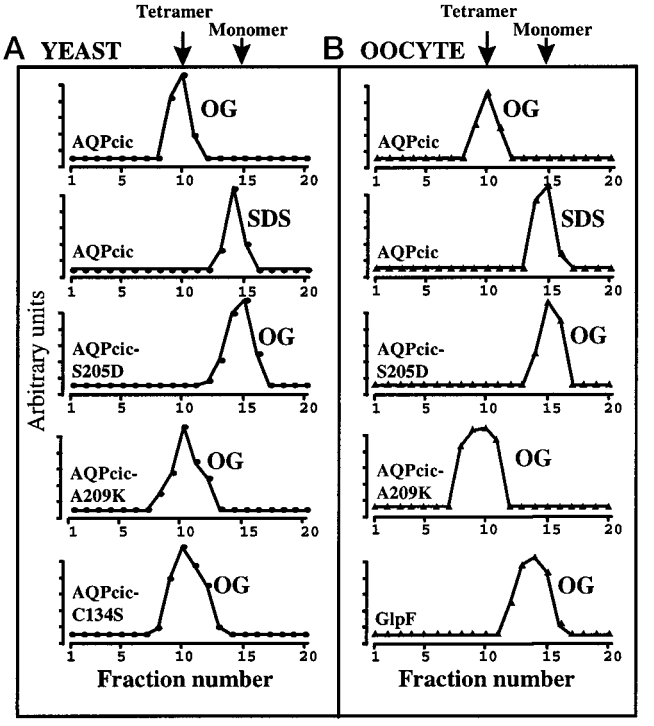

FIG. 4. **Sucrose density gradient centrifugation analysis.** *A*, sucrose density gradient centrifugation of wild type AQPcic or mutants expressed in *S. cerevisiae*. Membrane proteins from yeast expressing wild type or mutated AQPcic were solubilized by OG or by SDS and then analyzed on sucrose gradients as in Fig. 3. Wild type AQPcic is tetrameric in OG and monomeric in SDS. In the nondenaturing detergent OG, AQPcic-S205D is monomeric, and AQPcic-A209K and AQPcic-C134S are tetrameric. *B*, sucrose density gradient centrifugation of wild type or mutants of AQPcic and GlpF expressed in *Xenopus* oocytes. 48 to 72 h following cRNA injections, oocyte membrane proteins were solubilized by OG or SDS and analyzed on sucrose gradients as in *A*. Wild type AQPcic is tetrameric in OG and monomeric in SDS. GlpF and AQPcic-S205D are monomeric in OG, whereas AQPcic-A209K is tetrameric.

homotetrameric forms in detergent micelles. The amount of OG bound to AQP0 was found from 0.71 to 0.80 g/g of protein (1, 2). For AQPcic, we previously determined an OG binding of 0.87 g/g of protein (5). Thus, if GlpF was in a dimeric or trimeric form in OG micelles, with an assumed amount of OG bound of 0.80 g/g of protein, the averaged molecular mass of the complex should be at least 90–100 kDa. This value is incompatible with the 2.8 S sedimentation coefficient measured in sucrose gradients for GlpF-OG micelles, and only fits with a monomer of the protein with bound detergent. We also observe that in yeast membranes, the wild type or the mutants of AQPcic are expressed in quite identical amounts, and no significant variations in the OG/protein ratio could have interfered with the results. Extraction of GlpF was performed with two nondenaturing detergents from membranes of two different organisms; we nevertheless always observe that GlpF is in monomeric form. Additionally, the mutation S205D systematically induces monomerization of AQPcic. All together, these data support our hypothesis that the monomeric state of GlpF is not a detergent-induced phenomenon.

The archetypal aquaporin AQP1 had been previously shown to solubilize in Triton X-100 as heterotetramers presumably constituted by one glycosylated and three nonglycosylated molecules (9). Schulte and van Hoek (30) recently obtained OG heterodimers of AQP1 and glycosylated AQP1 from various mammals. The authors mention that different qualitative and quantitative glycosylation states could influence the behavior of some aquaporins in nondenaturing detergents. In the present work, we studied AQPcic, which is a nonglycosylated aquaporin,[2] and GlpF for which no report exists concerning any sugar association. For that point, our Western blots using the anti-GlpF antiserum did not evidence any high molecular mass putatively glycosylated forms of GlpF in *E. coli* or in oocyte membranes.

Interestingly, this study shows that the serine residue in position 205 of the putative loop E in the aquaporin AQPcic might be essential for tetramerization, whereas both Ser-205 and Ala-209 residues are found essential for the water transfer function. The AQPcic-S205D mutant does not increase oocyte swelling in response to an osmotic shock. This lack of apparent functionality could be attributed to the change in the oligomerization state in the plasma membrane or to a mistargeting process. The mutation S205D was designed following our previous sequence analysis of MIPs (21). The 205 and 209 positions correspond, respectively, to a negatively and a positively charged residue for the glycerol facilitators and two small uncharged amino acids for the aquaporins (Fig. 1). The failure of AQPcic-S205D to form a tetramer can be attributed to the presence of the negatively charged aspartate residue in position 205 rather than the small uncharged serine. The mutation S205D modifies the net charge of loop E, and this single point could explain the structural changes. However, in the same experimental conditions, the mutation A209K that also changes the net charge of loop E did not modify the tetrameric form of the heterologous protein. On the basis of the structural

[2] M. T. Guillam and J-F. Hubert, unpublished results.

map of AQP1 determined by electron crystallography (12), in the tetrameric native form of aquaporins, the six transmembrane domains surround a zone that is attributed to some infoldings of loops B, C, and E. In this model, the serine 205 and the alanine 209 localized in loop E likely do not reside at the interacting site between monomers. We do not have enough information to understand how the S205D mutation affects oligomerization and why the A209K substitution does not. The intracellular folding, assembly, and transport of MIPs and the pathway for tetramer formation of aquaporins in various cell types have to be better understood.

In the present data, we demonstrate that the substitution of a single amino acid in an aquaporin by the residue located at the same position in GlpF induces the aquaporin to adopt the oligomerization state of GlpF. Because the serine and the aspartic acid in this position are highly conserved in aquaporins and in GlpFs, respectively, we postulate that glycerol facilitators are monomers in membranes, whereas aquaporins are organized in tetramers. Our results further suggest that loop E plays a major structural and functional role in the MIP family proteins.

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