Switch from an Aquaporin to a Glycerol Channel by Two Amino Acids Substitution*

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The MIP (major intrinsic protein) proteins constitute a channel family of currently 150 members that have been identified in cell membranes of organisms ranging from bacteria to man. Among these proteins, two functionally distinct subgroups are characterized: aquaporins that allow specific water transfer and glycerol channels that are involved in glycerol and small neutral solutes transport. Since the flow of small molecules across cell membranes is vital for every living organism, the study of such proteins is of particular interest. For instance, aquaporins located in kidney cell membranes are responsible for reabsorption of 150 liters of water/day in adult human. To understand the molecular mechanisms of solute transport specificity, we analyzed mutant aquaporins in which highly conserved residues have been substituted by amino acids located at the same positions in glycerol channels. Here, we show that substitution of a tyrosine and a tryptophan by a proline and a leucine, respectively, in the sixth transmembrane helix of an aquaporin leads to a switch in the selectivity of the channel, from water to glycerol.

Based on amino acid sequence, members of the MIP\textsuperscript{1} family are predicted to share a common topology consisting in 6 transmembrane domains connected by 5 loops (A–E). From biochemical and biophysical data, a model representing these proteins as “hourglasses” has been proposed (1) (Fig. 1A). In this model, the channel pore is constituted by the junction of loops B and E that overlap midway between the leaflets of the membrane. Recently, the three-dimensional structure of the first identified aquaporin, AQPI (2), has been obtained and has defined that the protein complex is constituted by four monomers (3–5). Each monomer is formed by six tilted α helices spanning the membrane bilayer and surrounding a central density zone. This zone represents likely the narrowest segment of the water pore and may be constituted by loops B and E according to the hourglass model. As opposed to the increasing amount of data aiming to determine aquaporins structure, no study concerning glycerol channels has been reported, but considering their high level of identity, it was assumed that they had the same structural organization. Using a biochemical approach, we showed recently that an insect aquaporin, AQPCic (6), is tetrameric in cell membrane, like AQPI, whereas the glycerol channel of Escherichia coli (GlpF) is a monomer (7). These results suggest that oligomerization of MIP proteins could be involved in transport selectivity. In order to elucidate molecular mechanisms that are accountable of the channel selectivity, we have developed a strategy consisting in a systematic comparison of the physico-chemical properties of amino acids at each position in multiple sequence alignments (8). We have identified five positions (P1–P5) corresponding to amino acid residues conserved in aquaporins and glycerol channels but with highly different physico-chemical properties in the two subgroups. Interestingly, four positions (P2, P3, P4, P5) highlighted by our sequence analysis are located in, or very close to, loop E, strengthening the idea that this loop is involved in the pore structure and/or selectivity of the protein. In this paper, we performed a functional study combined to an oligomerization state analysis of AQPCic mutants. We demonstrate that substitution of a tyrosine and a tryptophan by a proline and a leucine, respectively, on positions P4P5 of AQPCic abolishes the water transfer and allows the glycerol passage through the protein.

EXPERIMENTAL PROCEDURES

Plasmids Construction and Mutagenesis—The pSP-AQPCic vector corresponds to the full-length AQPCic coding sequence inserted into the pXGl-g-e1 plasmid (6). The AQPCic mutants were obtained by performing a two-step reaction polymerase chain reaction with sets of appropriate primers overlapping in the region of the mutation (9). The mutated AQPCic cDNA were cloned either in pXGl-g-e1 (constructs were termed pSP-AQPCicS205D, pSP-AQPCicA209K, and pSP-AQPCicY222P/W223L) or in the yeast expression vector pYEpD60 (pY60-AQPCic and pY60-AQPCicY222P/W223L).

The pSP-glpF vector has been obtained by cloning the entire coding region of Es. coli glpF (from the pglpF vector, generously given by Dr. Mizuno (10)) into the BglII site of the pXGl-g-e1 plasmid.

AQPCic and GlpF Expression in Xenopus Oocytes—Wild type or mutated cRNA were prepared in vitro with the mRNA capping kit (Stratagene) using either pSP-AQPCic, pSP-AQPCicS205D, pSP-AQPCicA209K, and pSP-AQPCicY222P/W223L or pSP-glpF as templates. The cRNA were injected into stages VI Xenopus oocytes and osmotic water or apparent glycerol permeabilities of oocytes were measured as described in Ref. 6. The [14C]glycerol uptake assays were performed as described in Ref. 11.

Oligomerization State and Protein Analysis—Xenopus total membrane were prepared as described in Ref. 12. Yeast or Xenopus overexpressed proteins were extracted and analyzed on a linear 2–20% sucrose density gradient as described previously (7, 13). Briefly, the protein extracts were submitted to an ultracentrifugation to equilibrium at 100,000 × g for 16 h (5 °C), and the fractions were collected. Xenopus protein extracts or gradient fractions were loaded on SDS gel (14) and electrotransferred onto polyvinylidene difluoride filters (15). Immunodetections were performed using polyclonal rabbit antiserum raised against the native AQPCic (16) or raised against a synthetic C-terminal peptide of GlpF (7).

RESULTS

We have constructed mutants of AQPCic by substituting characteristic amino acids of aquaporins with corresponding glycerol channels amino acids at positions P2 (AQPCic-S205D), P3 (AQPCic-A209K), and P4P5 (AQPCic-Y222P/W223L). Transcripts corresponding to AQPCic and mutants AQPCic and GlpF

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were injected into *Xenopus* oocytes, and the presence of proteins in the oocyte membranes was verified by Western blot (Fig. 2).

Functional assays were achieved by measuring the water permeability ($P_w$), the apparent glycerol permeability ($P_{gly}$), and the glycerol uptake of oocytes expressing the different proteins. As shown previously, oocytes injected with AQPcic cRNA present a 14-fold increase of $P_w$ compared with control oocytes (Fig. 3A). In contrast, oocytes injected with mutants AQPcic (AQPcic-S205D, AQPcic-A209K, and AQPcic-Y222P/W223L) or GlpF cRNA do not demonstrate any $P_w$ increase (Fig. 3A). In Fig. 3B, apparent glycerol permeability is evaluated for each oocyte batch. Remarkably, oocytes expressing AQPcic-Y222P/W223L behave like GlpF expressing oocytes and exhibit a 8-fold increase of $P_{gly}$ compared with control oocytes. When injected with GlpF cRNA, oocytes exhibit a 9-fold increase of $P_{gly}$, whereas in oocytes expressing AQPcic, AQPcic-S205D, or AQPcic-A209K, no modification of $P_{gly}$ is observed as compared with controls. To reinforce these data, an experiment of glycerol uptake has been performed using [14C]glycerol. The results confirm a significant increase in glycerol uptake of 9-fold for GlpF expressing oocytes as compared with controls. When protein solubilization is performed with SDS, sedimentation occurs at fraction numbers 9–10. These fractions correspond to a 6.8 S apparent sedimentation coefficient mean value that fits with a homotetrameric form of the protein. When protein solubilization is performed with OG, sedimentation fraction number 9–10. These fractions correspond to a 6.8 S apparent sedimentation coefficient mean value that fits with a homotetrameric form of the protein. In opposition, the mutant AQPcic-Y222P/W223L is a monomer (2.8 S).

In parallel to swelling assays, the oligomeric organization of AQPcic and GlpF was resolved by sucrose gradient sedimentation. The experiments were performed using proteins overexpressed in yeast cells and extracted either with denaturing detergent (SDS, sodium dodecyl sulfate) or nondenaturing detergent (OG, n-octyl-$\beta$-D-glucopyranoside). As shown in Fig. 4, AQPcic extracted with OG peaks at sedimentation fraction number 9–10. These fractions correspond to a 6.8 S apparent sedimentation coefficient mean value that fits with a homotetrameric form of the protein. When protein solubilization is performed with SDS, sedimentation occurs at fraction numbers 14 and 15 (mean value of 2.8 S) that correspond to the monomeric form of the protein. In opposition, the mutant AQPcic-Y222P/W223L constantly peaks at fractions corresponding to a monomer whatever the detergent used for the solubilization of the proteins and thus behaves like the GlpF protein (Fig. 4).

**DISCUSSION**

Accumulating data indicate that MIP proteins contain two major physiological subgroups. Recently, we (8) and others have reported that the separation of these two subfamilies...
might be based on few sequence and/or motif distinctions (17–19). In particular, we have identified five positions where amino acids are drastically different in the two subfamilies. Besides aquaporins that are only permeable to water, some homologues are permeated by water, glycerol, and other small solutes: AQP3 identified in kidney (20–22), AQP7 isolated from rat testis (23), and the liver AQP9 (24) belong to this group referred as aquaglyceroporins. Thus, the puzzling question that currently emerged is how the specificity of proteins for water and/or small solutes is achieved. Our data reveal that substitution of amino acids on aquaporins by corresponding glycerol facilitator residues at positions P2, P3, P4P5 abolishes water transport, demonstrating the crucial role of these amino acids in aquaporin function. Furthermore, the AQPCic mutant that has been substituted on positions P4P5 by glycerol channels amino acids gains competence to transport glycerol. Oligomerization analysis further demonstrates that this mutant loses its ability to form a tetramer. The involvement of these two conserved amino acids positions in both oligomerization and specificity of the proteins gives a new important parameter that need to be considered for structural and functional studies on MIP proteins. Indeed, in addition to amino acids strictly involved in the pore structure, one should also take into account amino acids that are necessary for oligomer assembly, without excluding the fact that some residues might be implicated in both mechanisms. Positions P4P5 are located at the top of helix 6 in MIP proteins and correspond to aromatic residues in aquaporins whereas a proline followed by a non-aromatic residue is always found in the glycerol channels group (Fig. 1B). The role of proline in determining local conformation in proteins is well known (25). The presence or absence of such residue in MIP proteins provokes undoubtedly a different conformation of helix 6 and/or influences regions closed to the helix. Since loop E, that is supposed to participate in the pore of the protein, is very close to this helix, the interaction between the loop E and helix 6 may modulates the aperture of the pore and thus be responsible of the protein specificity. Using the three-dimensional model (3) of AQP1, Walz et al. (5) have assigned each helix of the protein by their corresponding numbers in the hourglass model. According to their analysis, each monomer of AQP1 interacts with each other by contacts between helices 4 and 6. Conserved amino acids located in these helices are thus essential to maintain the tetrameric structure of aquaporins. This structural organization of aquaporin monomers explains the crucial role of amino acids at positions P4P5 in the conformation of the protein. In this paper, we report for the first time a double mutation that modifies the protein structure in such a way that it allows a change in the nature of the transported molecule from water to glycerol. We point out two amino acids that are involved in the selective transport by MIP proteins. We further demonstrate that the specificity switch (water to glycerol transport) is correlated to a change in oligomerization state of the proteins. We thus propose that oligomerization state is part of the mechanisms utilized by MIP proteins to ensure selectivity.

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