Functional selectivity for glycerol of the nodulin 26 subfamily of plant membrane intrinsic proteins

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Abstract The nodulin-like intrinsic protein (NIP) subfamily of water and solute channels in plants is named for nodulin 26 of legume nodules. Two NIPs, soybean nodulin 26 and *Lotus japonicus* LIMP2, show a distinct functional profile with a low intrinsic osmotic water permeability (P_f) and the ability to flux uncharged polyols such as glycerol. NIPs have a conserved signature sequence within the 'aromatic/arginine' region that forms the selectivity filter for major intrinsic proteins. This sequence is a hybrid of glyceroporin and aquaporin residues as well as exhibiting substitutions unique to the NIP subfamily. Site-directed mutagenesis of a conserved tryptophan in helix 2 of LIMP2 shows that this is a major determinant of glycerol selectivity. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Aquaporin; Major intrinsic protein family; Nitrogen fixation

1. Introduction

The major intrinsic protein family of channels is particularly diverse in higher plants, with more than 30 genes present in the sequenced *Arabidopsis* genome [1]. These genes can be divided into four phylogenetic subfamilies: the tonoplast intrinsic proteins, the plasma membrane intrinsic proteins, the nodulin-like intrinsic proteins (NIPs) and the small basic intrinsic proteins [1]. The archetype of the NIP subfamily is soybean nodulin 26 which is specifically expressed during the organogenesis of the symbiosome during the formation of symbiotic nitrogen-fixing root nodules [2]. The symbiosome is a specialized organelle that houses the symbiotic rhizobia bacteroids and mediates the exchange of metabolites between the plant host and the endosymbiont [3]. Nodulin 26 is the major component of the soybean symbiosome membrane and is a target for phosphorylation and regulation by a calcium-dependent protein kinase that resides on this membrane [4].

Biochemical and biophysical studies of nodulin 26 [5] and its *Lotus japonicus* ortholog LIMP2 [6] show that they are aquaglyceroporins that confer upon this membrane a high permeability to water and glycerol [5,7]. The recent elucidation of the atomic resolution structure of a glyceroporin (*Escherichia coli* GlpF [8]) and an aquaporin (mammalian AQP1 [9]) has provided insight into the structural determinants for water and solute selectivity in MIP proteins. In light of these structures, sequence comparisons of NIP family members and site-directed mutagenesis, we have identified residues within the NIP family that appear to confer their unique transport properties.

2. Materials and methods

LIMP2 [6], human aquaporin 1 [10] and soybean nodulin 26 [7] cDNAs were cloned into the *Bgl*II site of pXβG ev-1 and 5′-capped cRNA was generated by in vitro transcription as previously described [6]. Production of the LIMP2 mutant W77H was generated by oligonucleotide-directed mutagenesis using the QuikChange™ Site-Directed Mutagenesis kit (Stratagene). Mutants were verified by automated DNA sequencing on a Perkin-Elmer Applied Biosystems 373 DNA sequencer at the University of Tennessee Molecular Biology Research Facility. Sequencing reactions were done with a Prism Dye Terminator Cycle sequencing kit (Perkin-Elmer Applied Biosystems).

For water and glycerol transport assays, *Xenopus laevis* oocytes (stage VI) were harvested and were injected with 46 nl of either sterile water (control oocytes) or cRNA (0.5–0.75 μg/μl), and were cultured in Frog Ringer's solution (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, 5 mM HEPES, 1000 U/ml penicillin–streptomycin, pH 7.6) at 15 °C for 72 h prior to assay as previously described [7]. 5′-capped cRNA was generated by in vitro transcription of the linearized template with T3 RNA polymerase (Ambion).

Osmotic water permeability (P_f) was measured at 10°C by monitoring the change in cross-sectional area upon hypo-osmotic challenge (10 or 30% Ringer's solution) by video microscopy as previously described [6,7]. After the assay was completed, oocytes were re-equilibrated in full strength Frog Ringer's solution for 30 min. Glycerol uptake assays were performed by measuring the uptake of [^{3}H]glycerol under iso-osmotic conditions (200 mM glycerol, 60 μCi/ml [^{3}H]glycerol, 5 mM $MgCl_2$, 5 mM HEPES, pH 7.6, 210 mosm/kg) as previously described [5,6]. Oocytes were rapidly washed at 0°C with the same solution without isotope and were lysed in 200 μl of 2% (w/v) sodium dodecyl sulfate and scintillation counting was performed.

3. Results

Upon expression in *Xenopus* oocytes, LIMP2 and nodulin 26 enhance the osmotic water permeability of the oolemma three- to four-fold, but this increase is much lower than the 30–40-fold increase in P_f conferred by aquaporin 1 (Fig. 1). On the other hand, analysis of the glycerol permeabilities of oocytes expressing LIMP2 and nodulin 26 shows that they are permeable to glycerol whereas aquaporin 1 is not (Fig. 1). Thus, LIMP2 and nodulin 26 both form aquaglyceroporins with a comparatively low water permeability but with a relatively high permeability to glycerol.

To gain insight into the structural features of nodulin 26/LIMP2 that confer these properties, molecular modeling was done using the atomic resolution structures of the *E. coli*

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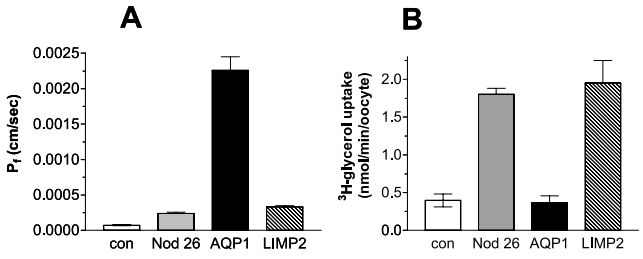

Fig. 1. Comparison of water and glycerol permeability of oocytes expressing MIP proteins. Oocytes were injected with cRNA (46 ng/oocyte) for LIMP2, nodulin 26, AQP1 or with 46 nl of sterile water (con), and were assayed for (A) water permeability or (B) glycerol permeability. Error bars indicate standard error of the mean ($n = 6$).

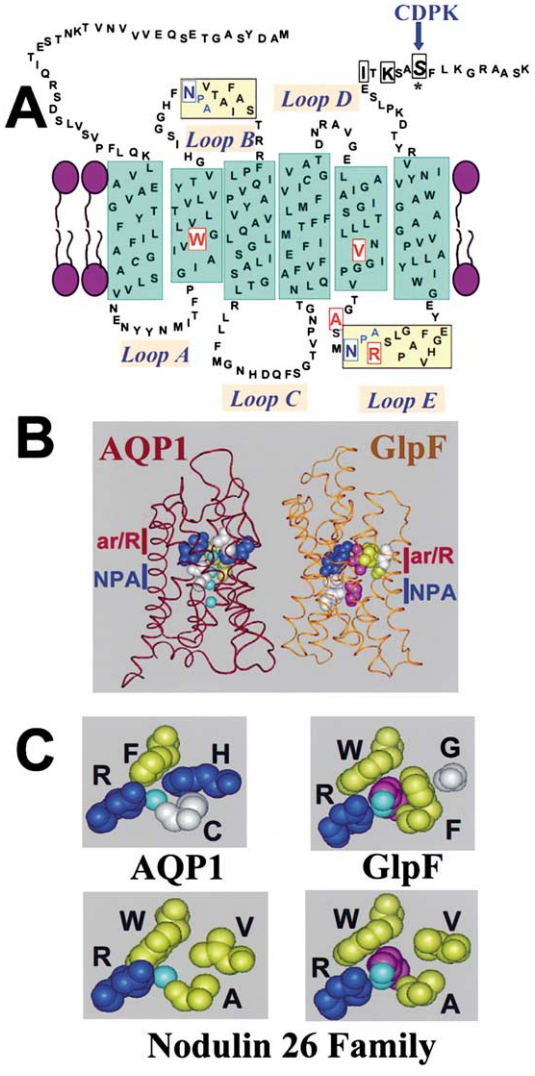

Fig. 2. Molecular modeling of the aromatic/arginine selectivity filter region of aquaporin 1, GlpF and nodulin 26/LIMP2. A: Topology of soybean nodulin 26 with the regions proposed to form the ar/R region highlighted in red. B: Ribbon structure of AQP1 and GlpF with the NPA and ar/R regions of the constricted pore region shown as space filling side chains. Waters in AQP1 are shown in aqua, and the glycerols in GlpF are shown in magenta. C: Comparison of the ar/R tetrads of AQP1, GlpF, and LIMP2/nodulin 26. Water is shown in aqua, glycerol is shown with the carbon backbone in magenta and the hydroxyl groups in aqua. Residues are colored as follows: blue, basic; yellow, hydrophobic; and white, neutral hydrophilic. **A**, ala; **C**, cys; **F**, phe; **G**, gly; **H**, his; **R**, arg; **V**, val; **W**, trp.

glycerol facilitator GlpF [8] and aquaporin 1 [9] as templates (Fig. 2). The region of selectivity in these two models is formed within the narrowest constriction of the hour glass-shaped pore which is formed towards the extracellular (or symbiosome space in the case of LIMP2 and nodulin 26) vestibule at the confluence of four residues known as the aromatic/arginine (ar/R) region. The ar/R region is formed from two residues in transmembrane α-helices 2 and 5 and two residues of the extracellular NPA loop E (Fig. 2). In the case of GlpF, these positions are occupied by Trp 48 (helix 2), Gly 191 (helix 5), Phe 200 (loop E) and Arg 206 (loop E), whereas in AQP1 these positions are occupied by Phe 58 (helix 2), His 182 (helix 5), Cys 191 (loop E) and Arg 197 (loop E) (Fig. 2C). Nodulin 26/LIMP2 have a unique signature of ar/R residues. Similar to both glyceroporins and aquaporins and most MIP sequences [11], nodulin 26/LIMP2 have a conserved arginine in loop E, but have a tryptophan in helix 2 similar to glyceroporins and possess an alanine within loop E, similar to aquaporins (Fig. 2). A unique feature of the nodulin 26/LIMP2 family is the presence of an unusual valine residue in the helix 5 position of the ar/R region.

To determine whether the conserved tryptophan of nodulin 26/LIMP2 is responsible for determining the glycerol selectivity of this aquaglyceroporin, the LIMP2 mutant W77H was prepared, in which the tryptophan residue was substituted with a histidine, similar to the helix 2 ar/R residue found in the water-selective LIMP1 aquaporin [6]. This mutation did not affect the expression or trafficking of LIMP2 W77H in *Xenopus* oocytes (data not shown) and yielded an osmotic water permeability (2.7×10^{-4} cm/s) that was only slightly

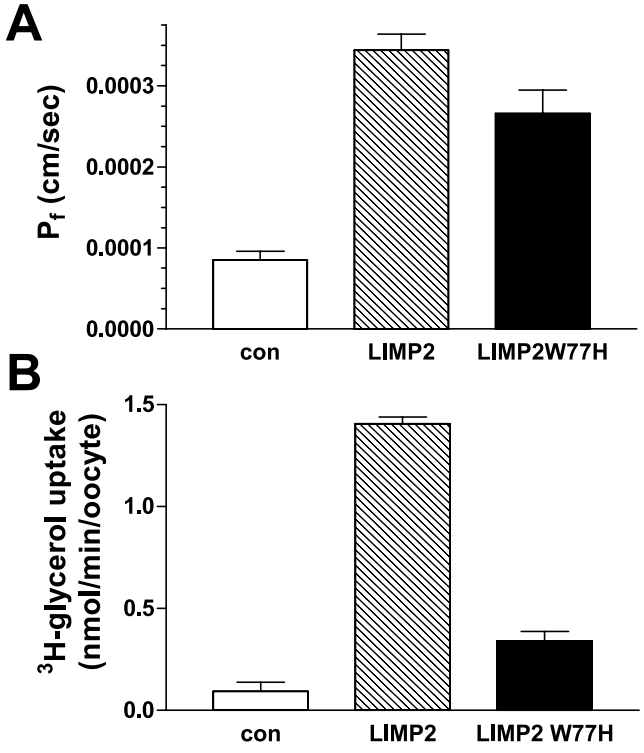

Fig. 3. Water and glycerol permeability of the LIMP2 W77H mutant. Oocytes were injected with cRNA (46 ng/oocyte) for LIMP2 or the LIMP2 W77H mutant or with 46 nl of sterile water (con), and were assayed for (A) water permeability or (B) glycerol permeability. Error bars show the standard error of the mean ($n = 26$ for water determinations; $n = 9$ for glycerol determinations).

Table 1
Comparison of ar/R regions of plant NIP gene family

Protein[a]	*Arabidopsis* gene #[b]	Helix 2[c]	Helix 5	Loop E	
AQP consensus		phe	his	ala/cys	arg
Glyceroporin consensus		trp	gly	phe	arg
Nod 26		trp	val	ala	arg
LIMP2		trp	val	ala	arg
LIMP1		his	ile	ala	val
NIP1;1	4g18030	trp	val	ala	arg
NIP1;2	4g18910	trp	val	ala	arg
NIP2;1	2g34390	trp	val	ala	arg
NIP2;1-like	2g29870	–	val	ala	arg
NIP3;1	1g31880	trp	ile	ala	arg
NIP4;1	5g37810	trp	val	ala	arg
NIP4;2	5g37820	trp	val	ala	arg
NIP5;1	4g10380	ala	ile	ala	arg
NIP6;1	1g80760	ala	ile	ala	arg
NIP7;1	3g06100	trp	val	ala	arg

[a]AQP and glycerol consensus sequences based on [9,10]. Nod26, soybean nodulin 26; LIMP 1 and LIMP2, *L. japonicus* intrinsic proteins 1 and 2 [6]; NIPs, NIP subfamily from *Arabidopsis* arranged in homologous pairs based on nomenclature of [1].
[b]*Arabidopsis* gene # from [15].
[c]Four residues of ar/R region are shown (see Fig. 2).

reduced compared to wild-type LIMP2 (3.4×10^{-4} cm/s) (Fig. 3A). In contrast, analysis of the glycerol permeability characteristics of this mutant shows that it has largely lost the ability to flux glycerol, with a six-fold lower permeability compared to wild-type LIMP2 (Fig. 3B).

4. Discussion

The nodulin 26/LIMP2 proteins belong to the NIP subfamily of plant MIPs. Based on previous work [5,7] as well as the findings here, these proteins form aquaglyceroporin channels that facilitate the flux of both water and glycerol, but have an intrinsic water transport rate that is substantially lower compared to the archetype of water-selective aquaporins, AQP1 [5,7,12]. Based on the high resolution structures obtained for GlpF and AQP1, we have modeled the residues within the selectivity filter of the pore (the ar/R region) and find that they consist of a hybrid sequence of residues between aquaporins and glyceroporins, as well as possessing substitutions unique to the subfamily (Fig. 2, Table 1). Interestingly, this NIP ar/R 'signature' is conserved in virtually all subfamily members and includes: tryptophan in the helix 2 position, valine or isoleucine in the helix 5 position, Ala and Arg in the two loop E positions. This suggests that the transport properties of nodulin 26 (an aquaglyceroporin with a low intrinsic water permeability) are likely to be conserved in other NIP family members. This is supported by data with at least two other family members, NIP1;1 and NIP1;2 [13,14].

Two features are proposed to mediate glycerol vs. water permeability in MIP proteins: (1) size selectivity based on the aperture of the pore; and (2) the presence of amino acid side chains that control the hydrophobicity and hydrogen bonding potential within the ar/R filter [8,9]. In the case of GlpF, the ar/R is amphipathic with Trp 48 and Phe 200 on one side of the pore forming a planar aromatic wedge that forms van der Waals contacts with the carbon backbone of glycerol, while arginine 206 found on the opposite side of the pore provides hydrogen bonding stabilization and orientation of the hydroxyl groups 1 and 2 of glycerol [8]. The residue on helix 5 is a glycine that is highly conserved in glyceroporins and serves to (1) allow the close approach of phenyl alanine in loop E with the glycerol backbone (Fig. 2) and (2) to provide a wider aperture (3.7 Å) at the ar/R constriction to accommodate the glycerol molecule. In the case of AQP1, the key conserved residue is His 182 on helix 5 which, along with the phenylalanine from helix 2, serves to constrict the diameter of the ar/R region to 2.8 Å, providing a size exclusion filter for larger solutes [9]. In addition, the His 182 side chain and Cys 191 peptide backbone carbonyl provide hydrogen bonds at the ar/R filter that are proposed to compensate for the energy cost of breaking the hydration shell around each water molecule transported single file through the pore [9].

As shown in Fig. 2, the unique ar/R signature of nodulin 26 and LIMP2 could explain its transport selectivities and rates. The presence of a valine in helix 5 rather than the highly conserved histidine found in most water-selective aquaporins would provide a wider pore aperture and increase the hydrophobicity of the ar/R region in NIP proteins (Fig. 2). The loss of the hydrogen bond donor provided by His 182 could increase the energetic cost of water transport and could account for the lower intrinsic transport rate of nodulin 26 compared to AQP1. Indeed, the higher hydrophobicity of the GlpF selectivity filter has been proposed to be responsible for the selectivity for glycerol over water despite the wider aperture of the pore [9]. The replacement of Phe/Gly conserved in glyceroporins with the unusual Val/Ala pair in the NIP family would appear to conserve the wider pore aperture to allow the flux of glycerol. Further, the valine and alanine pair, along with the conserved tryptophan and arginine, would appear to provide an amphipathic surface to interact with the hydrocarbon backbone of glycerol as well as the hydroxyl groups (Fig. 2). Our site-directed mutagenesis studies with W77 support this model and show that the substitution of a histidine at this position selectively affects glycerol transport while water permeability is only slightly affected.

Further molecular dynamics simulations with GlpF suggest a 'gating motion' in which the pore radius varies and affects the permeability of water through the GlpF channel [16]. Preliminary data with nodulin 26 show that water transport rate can be enhanced approximately three- to four-fold by pH and phosphorylation [17]. Thus, it is possible that changes in the position and orientation of residues within the pore/selectivity

filter of NIP proteins may modulate water and solute permeability.

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