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Aquaglyceroporins, one channel for two molecules

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Abstract

In the light of the recently published structure of GlpF and AQP1, we have analysed the nature of the residues which could be involved in the formation of the selectivity filter of aquaporins, glycerol facilitators and aquaglyceroporins. We demonstrate that the functional specificity for major intrinsic protein (MIP) channels can be explained on one side by analysing the polar environment of the residues that form the selective filter. On the other side, we show that the channel selectivity could be associated with the oligomeric state of the membrane protein. We conclude that a non-polar environment in the vicinity of the top of helix 5 could allow aquaglyceroporins and GlpF to exist as monomers within the hydrophobic environment of the membrane.
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1. Introduction

Water is the key solvent for the chemical processes of life and its movement across the plasma membrane accompanies essential physiological functions. All biological membranes exhibit water permeability, but some fast water movements are not fully explained by simple diffusion through the lipid bilayer. Observations of cells able to transport water at greatly accelerated rates led to the suggestion that specialised water transport molecules must exist in membranes. However, the identity of water transport molecules remained elusive until discovery of the aquaporins [1]. Aquaporins are members of the major intrinsic protein (MIP) family, a widespread membrane channel family that has been identified in bacteria, fungi, insects, plants and mammals [2–4]. The MIPs have been classified into three major functional subgroups: aquaporins (AQPs) or specific water channels, glycerol facilitators (GlpFs) permeable to small solutes such as glycerol, and aquaglyceroporins, a class of water channel also permeable to glycerol and small solutes. Based on amino acid sequence analysis, members of the MIP family are predicted to share a common topology consisting of a transmembrane domain formed by six hydrophobic α-helices connected by five loops. Their molecular weights are in the range of 26–34 kDa.

The most ubiquitous aquaporin, AQP1, has been structurally studied and the first three-dimensional reconstruction was derived from electron crystallography at 0.6–0.4 nm resolution [5–8]. It appears that AQP1 is a homotetramer of 28 kDa subunits, each containing six tilted transmembrane helices surrounding two membrane-inserted non-membrane spanning helices, a structure compatible with the "hourglass" model proposed by Jung et al. [9]. Recently, the structure of bovine AQP1 has been resolved at 2.2 Å by X-ray crystallography [10]. At this resolution, the structure of the selective pore is clearly established and reveals the molecular basis for water permeability. Presently, the tetrameric organisation of aquaporins in biological membranes has been demonstrated for other aquaporins by biochemical experiments and electron microscopy (AQP0 [11], AQP2 [12], AQP4 [13], AQPcic [14,15] and AqpZ [16,17]) and seems to be the general form for the aquaporin family.

The glycerol facilitator of *Escherichia coli*, GlpF, is highly permeable to glycerol but is less permeable to water [18].

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Based on their sequence similarities, AQPs and GlpFs were supposed to exhibit a similar structural organisation. This was first confirmed by electron crystallography [19] and later by X-ray crystallography at 2.2 Å resolution [20]. Like AQPs, GlpF, when crystallised, appears organised in tetramers with only subtle structural differences within the selectivity filter. For AQP1, the selectivity filter is located in the centre of the pore and is gated by a narrow constriction region of 2.8 Å in diameter defined by hydrophilic residues, while for GlpF, the constriction region is 3.5 Å wide and defined by more hydrophobic residues. Such structural data provide important clues to understand the mechanism of selective permeability within the MIP channels.

Aquaglyceroporins, such as AQP3, AQP7, AQP9, describe a class of water channels which are also permeable to glycerol [21–27]. Aquaglyceroporins are of particular interest for the investigation of the molecular basis of selectivity of both water and glycerol.

Recently, we have studied glycerol and water transport properties of a microbial MIP from *Lactococcus lactis* in two heterologous expression systems, the bacteria *E. coli* and the *Xenopus* oocyte [28]. This MIP, named Gla$_{Llac}$, has been demonstrated to be permeable to glycerol, like *E. coli* GlpF, and to water, like *E. coli* AqpZ. Thus, Gla$_{Llac}$ is presently the first microbial MIP that possesses such a mixed function. Although GlpF has also been shown to bear water permeability, it is significantly less permeable to water than AqpZ [18] and Gla$_{Llac}$ [28]. On the basis of these unique properties, it appears that Gla$_{Llac}$ presents a real interest to analyse, in the light of the recently published structure of GlpF and AQP1, the nature of the residues which could be involved in the formation of the constriction region of the Gla$_{Llac}$ channel.

2. Materials and methods

2.1. Sequence analysis

The protein sequences selected for this study are the following: AQP1 (P29972), AQP2 (P41181), AQP3 (Q92482), AQP4 (P55087), AQP5 (P55064), AQP6 (Q13520), AQP7 (O14520), AQP8 (O94778), AQP9 (O43315) from human, AQPcic (Q23808) from *Cicadella viridis*, GlpF (P11244) from *E. coli* and Gla$_{Llac}$ (Q9CE02) from *L. lactis*. These proteins can be partitioned into three groups according to their main transport properties for water and glycerol. In this study, we considered the aquaporins (AQP1, 2, 4, 5, 6, 8 and AQPcic), the aquaglyceroporins (AQP3, 7, 9, Gla$_{Llac}$) and the glycerol facilitator GlpF.

The sequences were aligned with CLUSTAL W [29]. The resulting alignment is 393 positions in length. The residues discussed in this paper are located at position 80 (RES1), 257 (RES2), 266 (RES3) and 272 (RES4) of the multiple alignment. The polarity assigned to the residues quoted in Table 2 corresponds to the amino acid parameters given by Ponnuswamy et al. [30].

2.2. Sample preparation and freeze-fracture

Studies on osmotic water or apparent glycerol permeability of *Xenopus* oocytes expressing proteins of interest were performed as previously described [31]. After functional measurements, control oocytes (water injected) and oocytes expressing *L. lactis* MIP were fixed between two glass slides and prepared for freeze-fracturing electron microscopy [15]. Freeze-fracture was performed with the Bioetch 2005 freeze-fracture apparatus (Leybold-Heraeus, Cologne, Germany). Electron images were recorded at a nominal magnification of 20,000 using a EM208S Philips electron microscope.

2.3. Measurement of freeze-fracture particles and data analysis

For particle density and particle size determinations, measurements were performed on at least two protoplasmic fracture-faces (P) of membranes from at least two oocytes. Images of the P fracture-face were enlarged to a final magnification of 41,000 and digitised using a flatbed desktop scanner. For particle density measurements, a final pixel size of 1.3 nm was used. For particle size measurements, a pixel size of 0.5 nm was used. The particle size was figured out by measuring the width of the particle perpendicularly to the direction of the shadow. The platinum-carbon thickness was estimated at 1.1 nm [15]. Results were plotted as frequency histograms that were fitted to a multiple Gaussian function [15].

3. Results and discussions

In Table 1 and Fig. 1 are quoted the residues which are structurally involved in the formation of the selectivity filter according to the structures published for AQP1 bovine [10] and for GlpF of *E. coli* [20]. In AQP1, the selectivity to water is made of a steric limit of 2.8 Å and by the chemical properties of the residues forming this structure: three polar residues versus one non-polar. In GlpF, such ratio is inverted, the selective filter contains only one polar residue. The replacement of H182 by a tiny non-polar residue (G191) induces a pore enlargement to 3.8 Å which is compatible with the accessibility to glycerol, and two perpendicular aromatic rings (W48 and F200) form a hydrophobic corner. In the aquaglyceroporin Gla$_{Llac}$ compared to AQP1, H182 and C191 are replaced by small non-polar residues, V223 and P232, respectively. Interestingly, F58 is replaced by Y49 a polar residue. Consequently, the selective filter of Gla$_{Llac}$ appears to be made of two polar and two non-polar residues. These substitutions with small residues, result in an enlargement of the constriction region with a potential aperture larger than the aquaporin one, a size compatible with a glycerol channel, and the switch of F58 to Y49 provides the necessary polar environment for an efficient water channel.
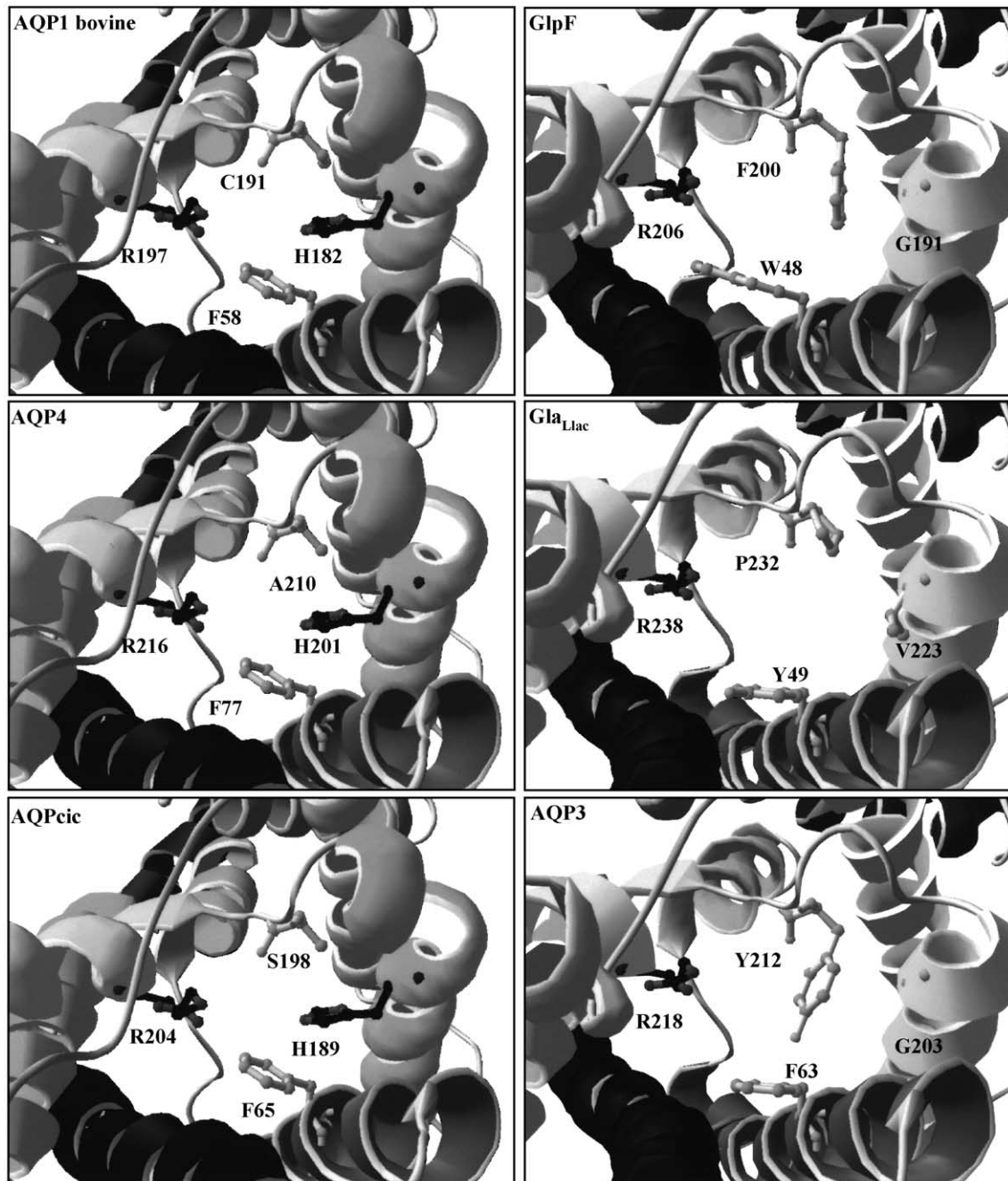
Fig. 1. Three-dimensional models of aquaporins: AQP1 (bovine), AQP4 (human), AQPcic (*C. viridis*) (from structure of the bovine AQP1 monomer in Ref. [10]; PDB accession number: 1J4N) and of GlpF (*E. coli*) and aquaglyceroporins Gla$_{Llac}$ (*L. lactis*) and AQP3 (human) (from structure of the *E. coli* GlpF monomer in Ref. [20]; PDB accession number: 1FX8). Highly polar residues of hydrophobic indices >50 according to Ref. [30] are represented in dark. Molecular models are displayed using SwissPdb Viewer [36], (http://www.expasy.ch/spdbv/).

Extending this analysis to 12 other MIP proteins functionally characterised (Tables 1 and 2), it appears that the selective filter of aquaporins is made of two residues having a high polarity (H, R). Analysing 90 sequences from various origins, it appears that position RES2 in the multiple alignment (H182 in AQP1 bovine) always corresponds to a large residue (H or I) for aquaporins and to a small residue for glycerol facilitators and aquaglyceroporins, inducing a polar enlargement. Nevertheless, aquaglyceroporins differ from

GlpF by bearing an extra polar residue (Y or C) instead of an aromatic one (W or F) thus compatible with water flow.

The rationale sustaining the MIP channel specificity deduced from these observations appears sound. However, we have made previous puzzling observations suggesting that oligomerisation could play a role on selectivity. On previous studies using sedimentation velocity on sucrose gradient [32] and freeze-fracture electron microscopy [15], we have shown that GlpF of *E. coli* could behave as a

Table 1
Residues involved in the selectivity filter

Residue position Mip sequence	RES1	RES2	RES3	RES4
AQP CIC	F	H	S	R
AQP1 Bovine	F	H	C	R
AQP1 Human	F	H	C	R
AQP2 Human	F	H	C	R
AQP4 Human	F	H	A	R
AQP5 Human	F	H	C	R
AQP6 Human	F	H	C	R
AQP8 Human	H	I	G	R
GLPF Ecoli	W	G	F	R
AQP3 Human	F	G	Y	R
AQP7 Human	F	G	Y	R
AQP9 Human	F	A	C	R
GLALlac	Y	V	P	R

Polar residues are in grey.

monomer within the membrane, suggesting that, in the MIP proteins, oligomerisation state could be correlated to a functional specificity. In addition, using statistical sequence analysis, we have pointed out that only a few number of key residues distinguish aquaporins from glycerol facilitators and thus could contribute to their functional properties [3,33]. This finding was corroborated by an experimental approach where a substitution of two key residues located in the sixth transmembrane helix of AQPcic abolished water transfer and conferred selectivity to glycerol associated with monomerisation of the protein [31]. Thus the involvement of these two residues in both oligomerisation and in specificity strongly supported the hypothesis that the state of self-association of MIP monomers is linked to functional specificity. Moreover, Borgnia and Agre [18] recently demonstrated by sucrose gradient sedimentation analyses on purified GlpF and AqpZ that, unlike AqpZ, the oligomeric state of GlpF varies according to the ionic strength of the sedimentation gradient. Finally a further argument strengthens this hypothesis: in an analysis conducted on protein chimeras between an aquaporin (AQPcic) and GlpF, we have shown recently that loop E, helices 5 and 6 are important for MIP channel specificity and oligomerisation. We concluded that interactions of helix 5 of one monomer with helix 1 of the adjacent monomer are crucial for aquaporin tetramer stability [34].

Given that aquaporins are tetramers and GlpF is a monomer in the membrane, it appeared interesting to elucidate the oligomerisation state of an aquaglyceroporin like Gla_{Llac}. One way to answer this question is to use recombinant plasma membrane proteins functionally expressed in *Xenopus* oocyte combined with freeze-fracture electron microscopy. Results are presented in Figs. 2 and 3. On freeze-fracture images, the P fracture-face of control oocytes, 72 h following water injection exhibits a population of dispersed particles (346 particles/μm^2) (Fig. 2A). The appearance of the P-face plasma membrane of Gla_{Llac}-

expressing oocytes 72 h following cRNA injection is shown in Fig. 3B. When compared to control oocytes, the particle density is largely increased (765 particles/μm^2) and this 2.2-fold amplification can be related to the functional activity measured in these oocytes [31]. Distributions of particle diameters are presented on frequency histograms (Fig. 3). P fracture-faces of control oocytes exhibit a fairly uniform particle population with a mean diameter of 7.8 ± 0.1 nm (Fig. 3A). Oocytes expressing Gla_{Llac} exhibit a bimodal distribution of particles with populations of particles at 6.6 ± 0.1 nm and 8.8 ± 0.6 nm (Fig. 3B). This particle distribution is attributed to exogenous particles and to the expressed recombinant proteins. The histogram corresponding to endogenous particles was superimposed on the histogram of oocyte expressing Gla_{Llac}. Then by determining the area underneath the curve of each population in the frequency histogram and assuming that the 7.8 nm particle population corresponds to endogenous particles (40% of total), we estimated that the increase in particle density was about 2.5-folds. This value is in agreement with the particle density found in Gla_{Llac}-expressing oocytes that demonstrated a 2.2-fold increase over control oocytes. Therefore, the combined analysis of particle density and frequency histogram allowed us to assign the two particle populations of 6.6 nm and 8.8 nm to expressed recombinant proteins. The particle population of 8.8 nm is similar to the AQP1 and AQPcic populations expressed in *Xenopus* oocytes previously described [15,35] and thus could be assigned to tetramers of Gla_{Llac}. The 6.6 nm particle population has a broad distribution which suggests populations of closed size or asymmetric particles. We have previously shown that the GlpF monomer has a mean size of 5.8 nm and that the averaged expected size for a dimer will be 6.7 nm and should display asymmetric particles [15]. Thus, this broad particle population is likely to be a mixed population of monomers and dimers of *L. lactis* MIP. These results corroborate the findings that the transport specificity of the MIP proteins could be closely linked to their oligomeric

Table 2
Polarity of the residues involved in the selectivity filter

Residue position Mip sequence	RES1	RES2	RES3	RES4
AQP CIC	0,35	51,6	1,67	52
AQP1 Bovine	0,35	51,6	1,48	52
AQP1 Human	0,35	51,6	1,48	52
AQP2 Human	0,35	51,6	1,48	52
AQP4 Human	0,35	51,6	2,63	52
AQP5 Human	0,35	51,6	1,48	52
AQP6 Human	0,35	51,6	1,48	52
AQP8 Human	51,6	0,15	0	52
GLPF Ecoli	2,1	0	0,35	52
AQP3 Human	0,35	0	1,61	52
AQP7 Human	0,35	0	1,61	52
AQP9 Human	0,35	0	1,48	52
GLALlac	1,61	0,13	1,58	52

Highly polar residues in grey.

state: glycerol permeation is associated with monomers and water permeation is associated with tetramers [15,31,32]. Moreover, we show that several oligomeric states could exist within the membrane.

In this paper, we have suggested that the specificity for MIP channels can be explained on one side by analysing the polar environment of the residues that form the selective filter. On the other side, we show that the channel selectivity could be associated with the oligomeric state of the membrane protein. Trying to explain these puzzling observations raises many questions. However, we have demonstrated
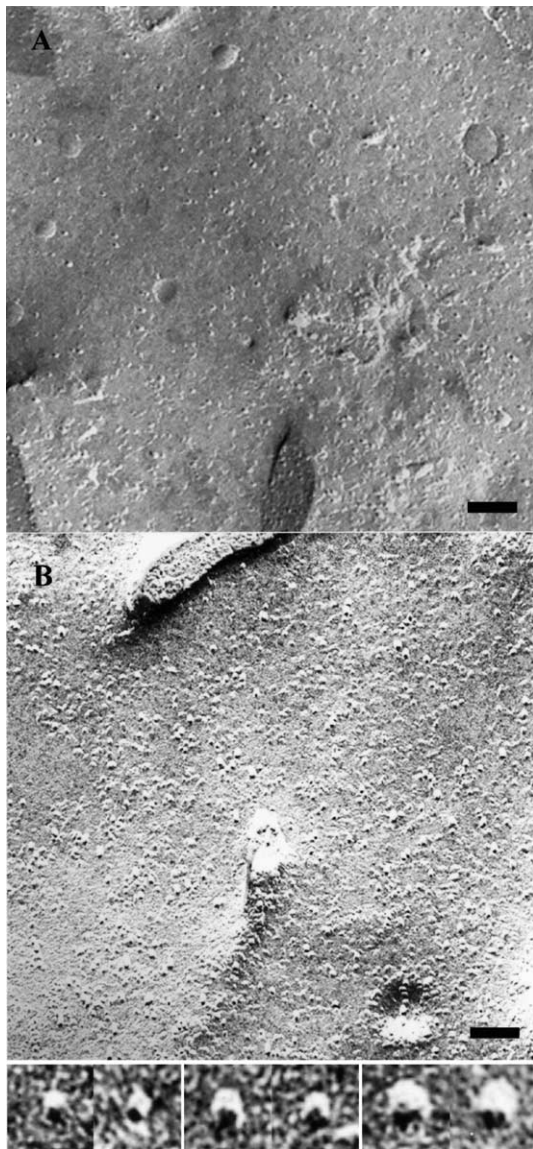

Fig. 2. Freeze-fracture electron micrographs of P-face oocyte membranes of control (A) and of oocytes expressing Gla_{Llac} (B). Gla_{Llac} expression is correlated with a significant increase of particle density 72 h following cRNA injection (scale bar, 100 nm). Gallery of particles of 5.4, 6.5 and 8.4 nm diameters which could be assigned, respectively, to monomers, dimers and tetramers.
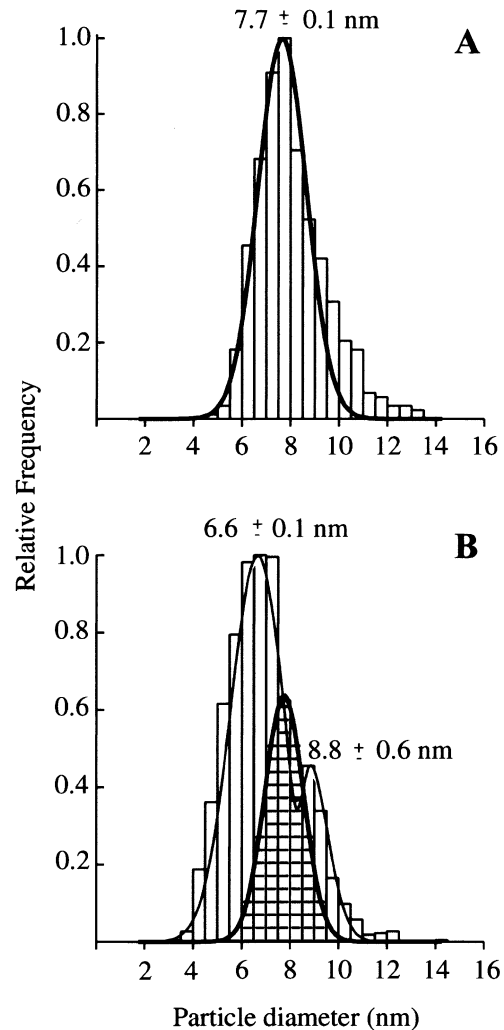

Fig. 3. Particle size distribution of P fracture-face membranes of water-injected oocytes (A), and of oocytes expressing Gla_{Llac} (B). The histogram corresponding to endogenous particles has been superimposed on the histogram of oocyte expressing Gla_{Llac} to differentiate the distribution of the recombinant proteins from the endogenous particles. Values are reported as mean ± S.D.; control: $N = 674$ particles; Gla_{Llac} $N = 1600$ particles; bin size = 0.5.

recently that the top of helix 5 is involved in the oligomerisation process for aquaporin [34]. This region includes RES2, the second residue involved in the selective filter: for aquaporins, it is a highly polar residue, for GlpF and aquaglyceroporins, it is a non-polar residue, thus this polarity difference within helix 5 could be responsible for GlpF and aquaglyceroporins to exist as monomers within the hydrophobic environment of the membrane. Further experiments including site-directed mutagenesis could help to solve this problem.

Acknowledgements

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