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I am submitting herewith a dissertation written by Nouth Chanmanivone entitled "The Nodulin 26 Aquaglyceroporin Transporter: Influence of Transmembrane Mutations, pH, Calcium, and Phosphorylation." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry, Cellular and Molecular Biology

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The Nodulin 26 Aquaglyceroporin Transporter: Influence of Transmembrane Mutations, pH, Calcium, and Phosphorylation

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Last, but not least, I would like to thank my husband who helped to make this all possible.

I will always be grateful to the all of them.

DEDICATION

This thesis is dedicated to

my parents

Mr. Houat Chanmanivone

and Mrs. Nang Chanmanivone

and my husband

Mr. Lawrence M. Magdovitz II

who have always given me love and support.

ABSTRACT

Bradyrhizobium japonicum bacteria infect the roots of soybeans resulting in the formation of a symbiotic, nitrogen-fixing nodule. Within the nodule, the bacteria are enclosed in a specialized organelle called the symbiosome. The bacteria are separated from the plant cell cytosol by the symbiosome membrane (SM). The formation of nodules is marked by the expression of several nodule-specific proteins called nodulins. In soybean, the nodulin 26 protein constitutes at least 10% of the total membrane protein and has been identified as a member of the major intrinsic protein (MIP) family.

Nodulin 26 is a multifunctional aquaglyceroporin that allows the flux of both water and solutes. Besides its multifunctional transport properties, nodulin 26 has a low intrinsic water transport rate that is 50-fold lower than that of aquaporin 1, the "prototypical" water-transport aquaporin of the MIP family. To determine the factors that may contribute to the multifunctional transport properties of nodulin 26 and its regulation, we used the heterologous *Xenopus* expression system to investigate residues important for the low intrinsic transport property of nodulin 26, we tested the functional roles of AQP1-like mutations of nodulin 26: the glutamine at position 114 of nodulin 26 was substituted by an arginine (Q114R), the cysteine at position 172 was substituted by a histidine (C172F), the valine at position 197 was substituted by a histidine (V197H), and the isoleucine at position 226 was substituted by a histidine (I226H). Findings indicate that two mutants (I226H and Q114R) are expressed and form water channels. Based on comparisons of water and glycerol permeabilities, Q114R is identical to nodulin 26

suggesting that this substitution does not confer higher water permeability or selectivity in aquaporin 1. I226H exhibits a lower permeability even though its expression appears to be normal. The other mutations (C172F and V197H) result in dysfunctional expression and/or targeting

Comparison of the sequences of glycerol and water-selective aquaporins indicate that there are five "discriminant" residues that are invariant within each of these groups. Notable, the nodulin 26 sequence is a hybrid of the consensus sequences for aquaporins and glycerol facilitators. We investigated two of these "discriminant" residues in nodulin 26 to see if they affected its water and glycerol transport properties. The leucine at position 230 was mutated to a tryptophan (AQP-like) (L230W) and the tyrosine at position 229 was mutated to a proline (glycerol transporter-like) (Y229P). Water and glycerol permeability assays revealed that both L230W and Y229P were no longer fluxing water or glycerol above the water-injected control oocytes, again because of defects in targeting. The difficulties with improper expression, folding or trafficking to the plasma membrane made it difficult for us to draw any conclusions about the influences of these residues in the selectivity or single channel rate of nodulin 26.

The low single channel rate observed for noduin 26 suggested that nodulin 26 may undergo regulation via external signals Analysis of oocytes expressing nodulin 26 show that the relatively low water permeability of nodulin 26 is enhanced by reducing the pH. The highest water permeability was observed at pH 5.5 which was increased 3-fold compared to that observed at the standard pH of 7.6. Conversely, calcium was found to be a negative regulator of nodulin 26. A decrease in extracellular calcium increased the

water permeability at least 2-fold. Conversely, an increase in external calcium exerted the opposite effect, with the relative permeability being reduced by 2-fold. Use of the calcium chelator, ethylbis(ocyethylene-nitilo)tetraacetic acid, in the recording bath further elevated the water permeability by 3-fold. The microinjection of another calcium chelator, 1,2-bis(2aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, elevated the water permeability by 4-fold suggesting that the calcium sensor is internal.

Previous studies indicate that nodulin 26 is the major phosphoprotein on the SM. The phosphorylation occurs at serine 262 of nodulin 26 and is catalyzed by a calcium-dependent SM-associated protein kinase of the calcium-dependent protein kinase (CDPK) family. To determine the functional effect of phosphorylation, the effects of a constitutive recombinant CDPK on the ability of nodulin 26 to transport water and glycerol using the *Xenopus laevis* oocytes expression system were determined. Oocytes injected with nodulin 26 alone showed an enhanced rate of oocyte swelling, whereas nodulin 26 oocytes injected with CDPK showed a 2-fold reduction in P_f. To test whether the result is specific for serine 262, a nodulin 26 mutant (serine to alanine at position 262) was generated that was unable to be phosphorylated at the 262 position. In contrast to wild-type nodulin 26, the water permeability of the alanine mutant was not affected by CDPK injection. In addition, glycerol transport properties were also made to determine if phosphorylation by CDPK affects the glycerol permeability of nodulin 26. Similar to the results obtained with the water permeability, nodulin 26 oocytes injected with CDPK showed a significant reduction in glycerol permeability compared to the nodulin 26 oocytes not injected with CDPK. Again, the alanine mutant did not show any significant

reduction in glycerol permeability upon CDPK injection. Western blot analysis revealed that the decrease in nodulin 26 observed in CDPK-injected oocytes appears to be the result of a reduced level of nodulin 26 on the plasma membrane, raising the possibility that phosphorylation of nodulin 26 affected membrane trafficking.

To assay the short-term effects of phosphorylation on the intrinsic transport rate of nodulin 26, the effects of phosphoylation of nodulin 26 by the endogenous *Xenopus* protein kinase C was determined. A synthetic peptide corresponding to the carboxyl terminus of nodulin 26, CK-15, was shown to be a protein kinase C substrate with a K_m of 520 μM, suggesting that nodulin 26 is a protein kinase C substrate. To test the effects of phosphorylation on nodulin 26 activity, we used a protein kinase C agonist (phorbol-12-myristate-13-acetate, TPA) and a protein phosphatase inhibitor (okadaic acid). Both agents were shown to stimulate the water permeability of nodulin 26 by 3-fold. In contrast, mutants of nodulin 26 that do not possess a phosphorylatable residue at position 262 were not affected by either okadaic acid or TPA.

Overall the data show that the water transport property of nodulin 26 was enhanced under conditions of low pH, low intracellular calcium levels, or phosphorylation. The regulation of nodulin 26 by pH, calcium, and phosphorylation may contribute to the regulation of nodulin 26 in the SM The regulation of nodulin 26 by these factors would permit the protein to shift between an activated, higher permeability state to a less active, lower permeability state to allow osmoregulation and possibly adaptation to environmental factors

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LIST OF ABBREVIATIONS

AQP1, 2, 3, etc.	aquaporin 1, 2, 3, etc.
BAPTA	1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
C172F	recombinant nodulin 26 with a phenylanine residue substituted for a cysteine residue at position 172
CDPK	calmodulin-like domain protein kinase
DEPC	diethylpyrocarbonate
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylbis(ocyethylene-nitilo)tetraacetic acid
ENOD	early nodulin gene
GlpF	glycerol facilitator of bacteria
HEPES	N-(2-hydroxyethyl)-piperazine-N'-ethanesulfonic acid
I226H	recombinant nodulin 26 with a histidine residue substituted for a isoleucine residue at position 226
L230W	recombinant nodulin 26 with a tryptophan residue substituted for a leucine residue at position 230
LB	luria broth
LCO	lipo-chitin oligosaccharide
MES	2-N-(morpholino)ethanesulfonic acid
MIP	major intrinsic protein

NLM	nodulin26-like membrane protein
NOD	late nodulin protein
NPA	asparagine, proline, and alanine sequence motif
NodA/ S262A	recombinant nodulin 26 with an alanine residue substituted for a serine residue at position 262
NodD/S262D	recombinant nodulin 26 with an aspartic acid residue substituted for a serine residue at position 262
NodS	wild-type nodulin 26
PBS	phosphate buffered saline
PCR	polymerase chain reaction
P_d	diffusive water permeability
P_f	osmotic water permeability
PIP	plasma membrane intrinsic protein
PMSF	phenylmethylsulfonyl fluoride
Q114R	recombinant nodulin 26 with an arginine residue substituted for a glutamic acid residue at position 114
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SM	symbiosome membrane
TIP	tonoplast intrinsic protein
TPA	phorbol-12-myristate-13-acetate

V197H	recombinant nodulin 26 with a histidine residue substituted for a valine residue at position 197
Y229P	recombinant nodulin 26 with a proline residue substituted for a tyrosine residue at position 229

CHAPTER I

INTRODUCTION

PLANT-RHIZOBIUM ASSOCIATIONS

Ecologically and economically, nitrogen is one of the most important chemical elements that is recycled by living things (Postgate, 1998). In virtually all of Earth's agricultural areas, the availability of inorganic nitrogen in the soil determines its biological productivity (Postgate, 1998). Even though the Earth's atmosphere contains almost 80% nitrogen, only about 0.05% of this is usable by plants (Newton, 1999). Consequently, plants must rely on nitrogen fixation to thrive under limiting nitrogen conditions. Plants of the Leguminosae family obtain the ability to fix atmospheric nitrogen under limiting soil nitrogen conditions through a symbiotic relationship with a diazotrophic (nitrogen-fixing) symbiont. These diazotrophs are soil bacteria that belong to the Rhizobiaceae family (Stacey et al., 1992).

The process of nitrogen fixation in plants begins with the establishment of a symbiotic relationship between the rhizobia soil bacteria and leguminous plants that culminates in the induction of a new plant root organ known as the nodule. The nodule is established through an elaborate series of developmental steps that begins when the

rhizobia bacteria colonize the rhizosphere surrounding the roots of the plant (reviewed in Long, 1989; Franssen et al., 1992). The bacteria then attaches to the root hairs of the host plant, resulting in the deformation and curling of the root hairs (Dazzo & Gardiol, 1984). Meanwhile, the cells of the root cortex located beneath the epidermis begin to divide (Newcomb, 1981). The bacteria multiply and infect the outer epidermal cells of the plant. At the same time, the invaded plant cell is stimulated to produce a cell wall sheath, called an infection thread (Callaham & Torrey, 1981) In addition, cells in the root cortex begin to divide and form the nodule primordium. The infection threads enter the primordium and the bacteria is released into the cytoplasm of the cells of the host plant (Robertson et al , 1978) in an endocytotic process in which the bacteria become enclosed in a plant-derived membrane called the symbiosome membrane (Bassett et al., 1977; Robertson et al., 1978; Roth et al., 1988). Once inside the root cell, the bacteria divide and differentiate into nitrogen-fixing bacteroids. The newly formed organelle within the infected root nodule cell, which encloses these bacteroids, is called the symbiosome (Roth et al., 1988) (**Fig 1**).

The developmental stages involved in the formation of a nodule are orchestrated by an extensive communication system between the bacterial symbiont and the plant host. First, host-plant specific flavonoid compounds from the host plant are secreted (reviewed in Spaink, 1994) The flavonoid compounds induce the expression of bacterial nodulation genes, termed *nod* genes. The only nod gene that is constitutively expressed in the bacteria is the *nodD* gene. The *nodD* gene product is required for the expression of

A *B*

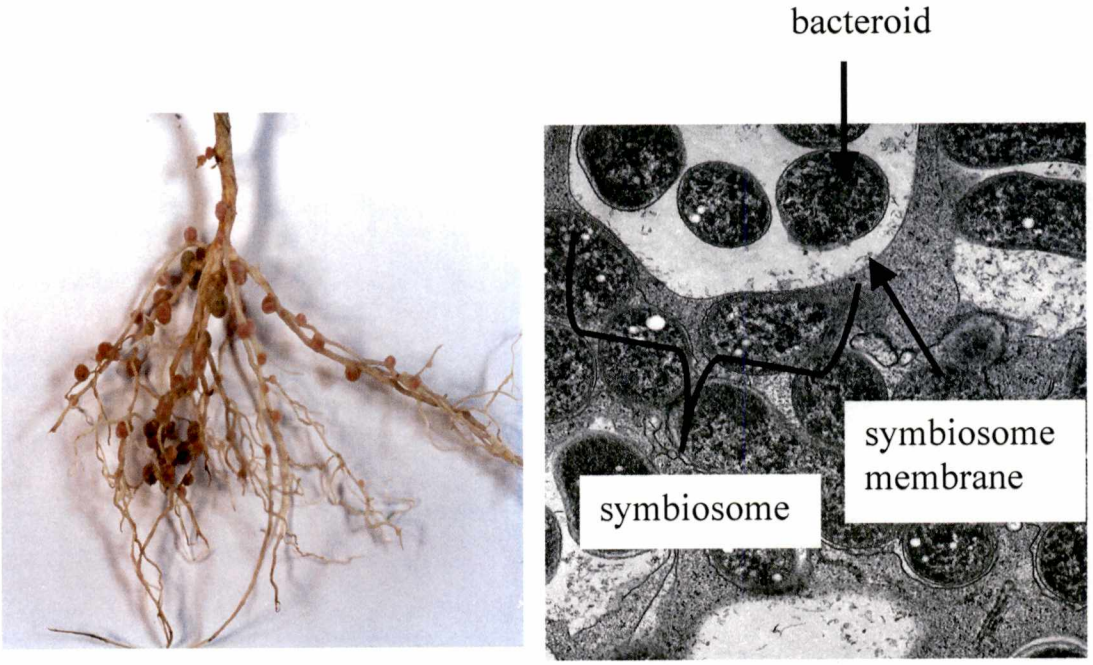


Figure 1. Images of symbiotic, nitrogen fixing nodules. A. Formation of a nodule on a Rhizobia-infected *Lotus japonicus* root. **B**. Electron micrograph of an infected cell from soybean nodules. The rhizobia bacteroids are is enclosed in the symbiosomes. The bacteria are separated from the plant cytosol by the symbiosome membrane. Electron micrograph was provided by Dr. John Dunlap, University of Tennessee, Knoxville. Final magnification is 15,000 x.

the other *nod* genes, and functions as a flavonoid-sensitive transcriptional activator (reviewed in Long, 1989). The *nod* genes encode enzymes which are involved in the processes of fatty acid biosynthesis, fatty acid transfer, chitin synthesis, and chitin modification that lead to the production of lipo-chitin (LCOs) signal molecules (reviewed in Spaink, 1994). These LCOs, also called nod factors (Loh et al., 1999; Peters et al., 1986; Redmond et al., 1986; reviewed in Long, 1989), function as a signal to the plant to initiate nodule organogenesis (Dénarié et al., 1996).

NODULINS

The formation of nodules is marked by the expression of nodule-specific plant genes that code for plant proteins termed nodulins (Legocki & Verma, 1980). Nodulins are involved in the development, structure, maintenance and overall metabolism of the nodule (Verma et al., 1986, Legocki & Verma, 1980). The nodulin proteins can be classified into two groups, depending on the developmental stages at which they are expressed. The nodulin genes that are expressed before the onset of nitrogen fixation are termed early nodulin genes, abbreviated "*enod*" (Govers et al., 1987; Nap & Bisseling, 1990). The nodulin genes expressed shortly before or during the start of the nitrogen fixation are termed the late nodulin genes (Govers et al., 1987, Nap & Bisseling, 1990).

The *enods* are thought to be involved in the infection process and nodule morphogenesis (van Kammen, 1984; Franssen et al., 1992; reviewed in van de Sande & Bisseling, 1997). Early nodulins can be classified into three broad categories: (1) the

proline-rich cell wall proteins (ENOD2, ENOD5, and ENOD12), (2) the putative metal-binding proteins (ENOD14, ENOD55), and (3) the auxin modulators (ENOD40) and membrane sulfate transporters (ENOD70) (reviewed in Reddy et al., 1999).

Among the most extensively studied early nodulin gene is *enod40*, which is expressed very early in nodulation. *enod40* sequences have been identified in several plant species, such as *Medicago sativa* and *Lotus japonicus* (reviewed in Hirsch et al., 1997). In all of these species, *enod40* contains a small open reading frame (corresponding to 10-13 amino acids) (Vijn et al , 1995) and the ENOD40 is induced in the nodule primordium and in the regions of the pericycle neighboring the primordium (Vijn et al., 1993, Yang et al., 1993) ENOD40 expression in the pericycle was found to precede the induction of cell division in the root cortex (Yang et al., 1997). These findings suggest that expression of ENOD40 in the root pericycle may be involved in the mechanism triggering a local change in auxin/cytokinin balance which ultimately leads to cell divisions in the root cortex These cell divisions are essential for nodule formation (Yang et al., 1997).

Other examples of ENOD genes include RH-42 and RH-44 which have been identified in the root hairs during the preinfection stage of the pea plant (reviewed in Franssen et al., 1992) Their expression in the root hairs of the pea plant suggest that they are involved in curling and deformation of the root hair. Furthermore, the pPsENOD12 and pPsENOD5 genes of the pea plant have also been implicated in the infection process (reviewed in Franssen et al., 1992) PsENOD12 may also be involved in nodule formation (reviewed in Franssen et al , 1992). The PsENOD12 gene is induced in root

hairs, root cortical cells, and nodule cells containing growing infection threads as well as in cells preparing for the growth of the infection thread (Scheres et al., 1990a). In contrast, the PsENOD 5 gene is induced only in cells containing the growing infection thread (Scheres et al., 1990b). Among the early nodulins that have been characterized is the soybean (*Glycine max*) Ngm-75 which is transiently expressed during nodule development (Gloudemans et al., 1987). The expression of Ngm-75 was observed to reach its maximal level at day 13 and then decrease in subsequent days (Gloudemans et al., 1987). Ngm-75 is a proline-rich protein that may be involved in cell wall function (Franssen et al., 1988). Thus Ngm-75 is believed to play a role in the formation of the nodule structure, but not in the infection process (reviewed in Nap & Bisseling, 1989).

While, most early nodulins appear to be involved with processes dealing with early stages of nodule development, the late nodulins appear to be involved with specific processes supporting the maintenance of the nitrogen fixing symbiosis. These late nodulins can be classified into two broad categories: metabolic nodulins and peribacteroid (symbiosome) membrane nodulins (reviewed in Franssen et al., 1992). Metabolic nodulins play a role in the metabolism of carbon, nitrogen, and oxygen in support of the nitrogen fixation and assimilation processes in the nodule. Examples of these metabolic nodulins in the soybean plant, include the oxygen carrier protein leghemoglobin (Brisson & Verma, 1982), uricase which is involved in ureide formation and nitrogen assimilation (Thummler & Verma, 1987), sucrose synthase (Kuster et al., 1993; Legocki & Verma, 1980), and glutamine synthetase (Cullimore et al., 1984, Tingey et al., 1987; reviewed in Schroder et al., 1997; reviewed in Franssen et al., 1992).

Besides these nodulins that play a defined metabolic role, there is a second group of nodulin gene products that are targeted to the symbiosome membrane, including five symbiosome membrane nodulins that have been identified in soybean plants: Ngm-23 (nodulin 23), Ngm-24, Ngm-26, Ngm-26b, and Ngm-53b (Fortin et al., 1987; Katinakis & Verma, 1985, Jacobs et al., 1987; Winzer et al., 1999; Panter et al., 2000; reviewed in Franssen et al., 1992). The function of most of these nodulins has yet to be elucidated. Possibly the most extensively studied symbiosome membrane nodulin is the major integral membrane protein, nodulin 26 (Ngm-26). The properties of this symbiosome membrane nodulin will be discussed in detail below.

THE SYMBIOSOME AND THE SYMBIOSOME MEMBRANE

The symbiosome membrane (SM) separates the symbiosome that encloses the bacteria from the plant cell cytosol (**Fig. 1B**) (reviewed in Udvardi & Day, 1997). The SM plays a vital role in the stability of the symbiosis and the endosymbiont. This is apparent from studies with rhizobia mutants in which degradation of the SM leads to senescence of the symbiosis and elicitation of plant defense responses (Werner et al., 1985). In this study, the SM of soybean nodules that were infected with a mutant *Bradyrhizobium japonicum* showed early disintegration followed by a significant accumulation of the phytoalexin glyceollin I in nodules, to the same extent as if they had been heavily infected with a phytopathogenic fungus Thus, part of the function of the

SM is to protect the bacteria from plant defense. However, the principle role of the SM is to serve as a semi-permeable barrier that controls the primary metabolic exchange between the plant and the bacteroids: reduced carbon influx (from the plant) and reduced fixed nitrogen efflux (from the bacteroid) (reviewed in Udvardi & Day, 1997; Verma & Hong, 1996). The bacteroids catalyze the reduction of N_2 to ammonia using the enzyme nitrogenase, by the following reaction.

$$N_2 + 8H^+ + 8e^- + 16\ ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i \quad \text{(Eq 1)}$$

The energy required for this reaction is derived from reduced carbon from the plant, most likely in the form of C_4-dicarboxylates (reviewed in Udvardi & Day, 1997).

Fixed ammonia, as a small uncharged molecule, was traditionally believed to be transported from the bacteroids through simple diffusion (Howitt & Greshoff, 1985; Jin et al., 1988; Marsh et al , 1984; O'Hara et al , 1985, reviewed in Udvardi & Day, 1997). This idea of dissolved gases simply diffusing through the lipid phase of the membrane has recently been challenged with the identification of a voltage-gated cation efflux channel that is capable of transporting ammonium ions across the SM of soybean (Tyerman et al., 1995). The NH_4^+ currents were also observed to be rectified, meaning that the movement of NH_4^+ was unidirectional (out of the SM) (Tyerman et al., 1995). Recently, a cDNA, GmSAT1, encoding a putative ammonium transporter was isolated from soybean (Kaiser et al., 1998). GmSAT1 was found to be preferentially expressed in the nodules and was also localized to the SM (Kaiser et al., 1998). Besides the transport of ammonium ions through the cation channel, there is a second pathway for neutral ammonia permeation through a mercury sensitive SM channel (Niemietz & Tyerman,

2000). It is possible that this pathway is via nodulin 26, which is discussed in detail further below. Further support for the facilitated uptake of neutral ammonia came from measurements of the energy of activation which was normally 55 kJ mol^{-1}, and 118 kJ mol^{-1} in the presence of mercury (Niemietz & Tyerman, 2000). Furthermore, incubation of SM vesicles with ATP significantly reduces the ammonia permeability suggesting that phosphorylation may play a role in modulating the activity of the channel associated with ammonia permeation (Niemietz & Tyerman, 2000).

Within the infected cell cytosol, ammonia is converted to glutamine by glutamine synthetase The glutamine is then converted to glutamate by the glutamate synthase (GOGAT) reaction (Cullimore & Bennett, 1988; Vance & Heichel, 1991). The glutamate is then converted to aspartate by aspartate aminotransferase. Legumes entering into nitrogen fixing symbioses can be broken down into two groups that either export amides (e.g , pea) or ureides (e g. soybean) In ureide-exporting legumes, the nitrogen from glutamine is channeled into purine biosynthesis in the plastid of the infected cell. The purine nucleotide is then exported into uninfected cells where, in contrast infected cells, peroxisoms contain uricase II which converts uric acid to allantoin. This allantoin is then further converted in the smooth ER by allantoinase into allantoic acid, a form suitable for xylem transport (reviewed in Mellor & Werner, 1990; reviewed in Werner, 1991).

To provide energy for nitrogen fixation, the bacteria utilizes reduced carbon provided by the plant host. Sucrose from the shoot of the plant is transported to the nodule where it is converted to glucose-6-phosphate in the cytoplasm of the infected cells of the nodule via the action of sucrose synthase (Streeter, 1995; reviewed in Day et al.,

1994; Mellor & Werner, 1990, Werner, 1991). Glucose-6-phosphate enters glycolysis and is further metabolized by other glycolytic enzymes to phosphoenolpyruvate (PEP). The phosphoenolpyruvate is converted to malate by PEP carboxylase and malate dehydrogenase (reviewed in Day et al., 1994, Mellor & Werner, 1990; Werner, 1991).

Studies indicate that malate and other C_4-dicarboxylic acids are effective substrates for maintaining bacteroid respiration (Bergersen, 1958; Buris & Wilson, 1939; Tuzimura & Meguro, 1960) and nitrogen fixation (Bergersen, 1977; Bergersen & Turner, 1967; Miller et al., 1988; Peterson & La Rue, 1981). Genetic studies have also provided further evidence for the involvement of C_4-dicarboxylic acids in nitrogen fixation (reviewed in Udvardi & Day, 1997). For example, bacterial mutants that are defective in dicarboxylate transport form ineffective Fix^- nodules (Arwas et al., 1985; Bolton et al , 1986; el Din, 1992; Engelke et al., 1987).

Further support for the role of dicarboxylates in supporting nitrogen fixation was the finding of a dicarboxylate transporter on the SM of soybean that has a preference for malate and succinate (Udvardi et al., 1988) Measured rates of malate transport across the SM have been shown to be sufficient to support the rates of nitrogen fixation estimated from measurements with isolated bacteroids (Day et al., 1989).

Biochemical and biophysical studies of the symbiosome membrane have uncovered the existence of other transporters that may play a key role in nutrient exchange between the plant and the bacteria (Panter et al , 2000). One of these tranporters is a H^+-ATPase that generates a proton motive force across the symbiosome membrane by pumping protons from the cytoplasm to the symbiosome space, located

between the symbiosome membrane and the bacteroid membranes (Blumwald et al., 1985; Domigan et al., 1988; Udvardi & Day, 1989). This proton motive force could be the driving force for many secondary transport processes, including dicarboxylate transport to the bacteroids (Panter et al , 2000).

Nodulins found in the SM are the best candidates for mediating the unique transport functions of this membrane (Winzer et al., 1999). Of the many nodulins identified in the SM (Fortin et al., 1985; Mellor et al., 1989), the most well characterized is nodulin 26. Nodulin 26 was first discovered as a symbiosome membrane nodulin by the work of Fortin et al. (1987) in which symbiosome membrane nodulins were identified by immunoprecipitating polysomes with antibodies against purified soybean symbiosome membranes. They found a partial cDNA that encodes a nodule-specific protein containing an open-reading frame for a 22.5 kDa protein. The full-length sequence of nodulin 26 was later published (Sandal & Marcker, 1988). Subsequent work by Weaver et al (1991) revealed that nodulin 26 is the major protein component of the SM, constituting at least 10% of the total membrane protein (Rivers et al., 1997). Topology studies on nodulin 26 indicated that nodulin 26 is co-translationally inserted into the membrane without any

signal sequence being cleaved and possesses carboxyl and amino termini that face the cytoplasm (Miao et al., 1992).

Upon comparing the deduced sequence of nodulin 26 with other membrane proteins (**Fig. 2A**), Sandal and Marcker (1988) and Shiels et al. (1988) noted that nodulin 26 possesses the structural hallmarks of the major intrinsic protein (MIP) family, an

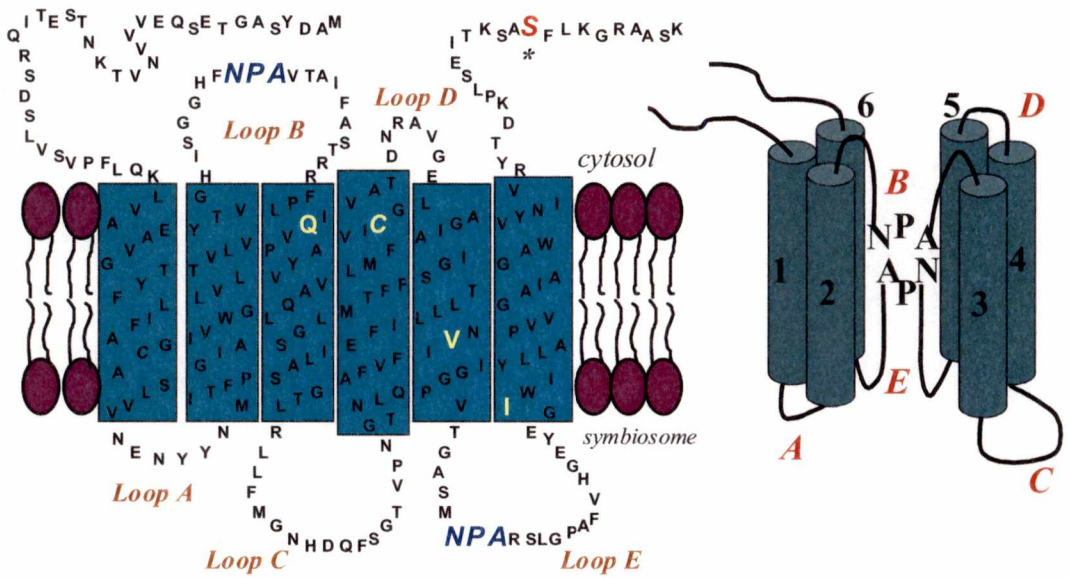


Figure 2. Nodulin 26 and the hourglass model. A. Sequence and topology of nodulin 26. The serine located at position 262 (S262) of the carboxyl terminal domain is asterisked. S262 serves as a site of phosphorylation by a SM-associated CDPK (Weaver et al., 1991; Weaver & Roberts, 1992). Q114, C172, V197, and I226 found in the transmembrane regions are shown in yellow. **B.** Hourglass model for the MIP proteins (Jung et al., 1994b).

ancient channel family. Below, the structural and functional properties of this membrane protein family are discussed, as well as the unique functional properties of nodulin 26 as a MIP

THE MAJOR INTRINSIC PROTEIN (MIP) FAMILY

The MIP family was named after MIP26 protein of the bovine lens fiber cell membrane, the first member that was sequenced and characterized (Takemoto & Hansen, 1981; Gorin et al., 1984). The MIP family consists of integral membrane proteins that are ubiquitous and function in various physiological processes including: fluid secretion, cell volume and osmoregulation, and metabolite (e.g. glycerol) uptake (reviewed in Borgnia et al., 1999; Engel et al., 2000, Park & Saier, 1996). Many or most MIP proteins encode water transport channels termed "aquaporins," though others have solute transport functions as well (reviewed in Agre et al , 1998). All family members share similar structural features including: molecular weights between 23-31,000, 30-40% sequence identity (Reizer et al., 1993), and a similar six transmembrane α-helical topology (**Fig. 2A**) (reviewed in Borgnia et al., 1999, Engel et al., 2000; Park & Saier, 1996). The MIP family is widespread with more than 220 family members identified from full or partial sequences (Froger et al , 1998; reviewed in Calamita, 2000). The diversity of these proteins is apparent by their identification in animals, plants, yeast, and bacteria (Johansson et al., 2000). Nevertheless, their structural and sequence similarity shared by family members suggests that they may possess similar functions.

FUNCTIONAL PROPERTIES OF THE MIP FAMILY

The principal function that has been described for MIP proteins is that of water transport (reviewed in Agre et al., 1998; reviewed in Borgnia et al., 1999). Until recently, water movement across biological membranes was mainly attributed to simple diffusion and it was proposed that water channels were unnecessary. Nevertheless, many observations suggested that specialized membrane transport molecules must exist in membranes exhibiting intrinsically high water permeability (reviewed in Finkelstein, 1987; King & Agre, 1996). For instance, the activation energy for the simple diffusion of water through pure lipid bilayers was calculated to be greater than 10 kcal/mol. In comparison, the activation energy for the flow of water through some membranes, such as that of red blood cells, was calculated to be lower than 5 kcal/mol, which is equivalent to the diffusion of water in solution (reviewed in Solomon, 1968). These observations led many to speculate about the existence of water pores as the contributing factor for the low energy of activation observed in membranes such as those of red blood cells (reviewed in Solomon, 1968). Nevertheless, such a channel protein was not identified until the 1990s.

While characterizing the Rh blood group antigens in human red cells, a 28 kDa polypeptide was also isolated with the 32 kDa Rh polypeptide (Agre et al., 1987; reviewed in Borgnia et al., 1999). This 28 kDa polypeptide was originally assumed to be a proteolytic fragment of the Rh polypeptide. However, the immunoglobulin resulting from injections with the 28 kDa polypeptide indicated that it was not derived from the Rh

polypeptide (Denker et al., 1988). This observation prompted further biochemical characterization of the polypeptide (Denker et al., 1988). These studies indicated that the protein is composed of hydrophobic amino acids that could span the bilayer and that it also exists in two forms: a nonglycosylated 28 kDa polypeptide and an N-glycosylated polypeptide with an apparent molecular weight of 40-60 kDa (Denker et al., 1988) Interestingly, the core polypeptide of all the forms was observed to be identical, and the protein exists as an oligomeric tetramer (Smith & Agre, 1991). Further analysis of this polypeptide demonstrated its homology (Smith & Agre, 1991) to the major intrinsic protein of the eye lens MIP26 (Gorin et al., 1984), implicating the role of this polypeptide as a membrane channel; it was referred to as CHIP28 (Preston & Agre, 1991). Due to its abundance in red blood cells and kidney (Denker et al., 1988), the polypeptide was proposed to be the sought after water channel.

The *Xenopus* expression system has been used extensively to identify and study water channels (Preston et al., 1992a; Chrispeels & Agre, 1994; Agre et al., 1995). This system is remarkably suited for the study of water channels because oocyte plasma membranes exhibit a particularly low intrinsic membrane water permeability (Fischbarg et al , 1990; Zhang et al , 1990). Upon microinjection of oocytes with the cRNA for CHIP28, the osmotic water permeability (P_f) of the oocytes increased substantially (P_f ~200 x 10^{-4} cm/sec) (Preston et al., 1992a) In contrast, water-injected oocytes exhibited less than one-tenth of this permeability (Preston et al., 1992a) Further studies with proteoliposomes reconstituted with this purified polypeptide, now called Aquaporin-1 (AQP1), also supported its role as a water channel that would permeate water but not

protons, ions, urea or glycerol (Zeidel et al., 1992; Preston et al., 1992b). Water permeation in AQP1 was demonstrated to be bidirectional, with the direction of the water flow determined by the orientation of the osmotic gradient (Meinild et al., 1998). In addition, water permeability through AQP1 was also observed to be reversibly inhibited by mercury chloride and possess a low activation of energy (5 kcal/mol) (Preston et al., 1992a, Preston et al., 1992b; Zeidel et al., 1992), supporting its role as the protein responsible for the water permeability of the red blood cell membranes. Based on these observations and properties, AQP1 and other proteins with similar functions are designated as aquaporins (the official name accepted by the Human Genome Nomenclature Committee) (Agre et al., 1993; Agre, 1997).

Two factors that are often used to describe membrane permeability are the osmotic permeability coefficient (P_f) and the diffusive permeability coefficient (P_d). P_f describes the osmotic water permeability of the membrane, whereas P_d describes the permeability resulting from diffusion. P_f and P_d are useful in distinguishing between lipid- and channel-mediated water flow. If water transport through a membrane is channel-mediated, then P_f exceeds P_d; but, if water transport through a membrane is lipid-mediated, then P_f and P_d are equal (Finkelstein, 1987; Tyerman et al., 1999). Another important factor in describing membrane permeability is the Arrhenius activation energy, E_a which reflects the temperature dependence of the flow rate (Finkelstein, 1987, Tyerman et al., 1999). In lipid-mediated water transport, the E_a is high (10-15 kcal/mol); whereas, in channel-mediated water transport, the E_a is low (4-6 kcal/mol) (Tyerman et al., 1999). Aquaporins, such as AQP1, are characterized by a high

osmotic water permeability (P_f), a low Arrhenius activation energy, an osmotic to diffusive water permeability ratio (P_f/P_d) that is greater than 1, and mercury inhibition (Finkelstein, 1987; Chrispeels & Agre, 1994; Agre et al., 1995; Maurel, 1997). According to Finkelstein's theory, values of this P_f/P_d ratio of greater than 1 indicate that water molecules line up single file within the lumen of the pore. This ratio also provides an estimate of the number of water molecules lined up within the pore at a given time (Finkelstein, 1987; Mathai et al., 1996)

Since the initial discovery and characterizaiton of AQP1, nine other mammalian aquaporins have been discovered, numbered according to the order of their identification as water transporters (reviewed in Borgnia et al., 1999; Verkman & Mitra, 2000; Engel et al., 2000). Based on functional analysis of these and other MIPs, the majority of the MIP proteins identified thus far are aquaporins that selectively transport water (reviewed in Agre et al., 1998; Borgnia et al., 1999). Nevertheless, MIP proteins have also been identified that function as aquaglyceroporins, which facilitate the flux of both water and uncharged solutes. These include AQP3, AQP7, and AQP9 (reviewed in Agre et al., 1998; reviewed in Borgnia et al., 1999) Nodulin 26, as discusssed below, is also a member of this subclass (Rivers et al , 1997; Dean et al., 1999). The third functional subclass is the glyceroporins, which facilitate the flux of glycerol and other uncharged solutes but not water. Among the glyceroporins that have been identified are the glycerol facilitators, GlpF of bacteria and Fps1 protein of *S cerevisiae* (reviewed in Hohmann et al., 2000).

Since the discovery of AQP1, many other water selective aquaporins have been identified. Among these are AQP2, which is localized in the collecting ducts of the kidneys and contributes to urine concentration (Fushimi et al., 1993). AQP2 is a vasopressin-regulated aquaporin that has been implicated in most clinical imbalances of water metabolism and in most clinical problems involving impaired renal water reabsorption such as Diabetes Insipidis (reviewed in Borgnia et al., 1999). AQP4 is the predominant aquaporin in the brain (Hasegawa et al., 1994; Jung et al., 1994b) In the brain, AQP4 has been postulated to function as an osmoreceptor or as an exit port for excess brain water, which can be detrimental in cerebral edema (Nielson et al., 1997). AQP4 has also been identified in the retina, optic nerve (Nagelhaus et al., 1998) and the fast-twitch skeletal muscle fibers in rats (Frigeri et al., 1998). In fast-twitch fibers, lactate accumulates in high concentrations, and the presence of AQP4 may function to restore osmotic equilibrium by permitting the rapid flux of water (Frigeri et al., 1998). AQP5 was isolated as an salivary gland cDNA (Raina et al., 1995) where it is believed to play a role in the regulation of airway humidification and the release of saliva and tears (Nielson et al., 1997). AQP6 has been observed in kidney (Ma et al., 1993) where it is believed to participate in acid secretion (Yasui et al., 1999) AQP0, the original MIP, is expressed only in lens fiber cells where it may function, as the major permeability pathway for the movement of water (Kistler & Bullivant, 1980, Gorin et al., 1984; Mulders et al., 1995; Zampighi et al., 1995, Chandy et al., 1997), to maintain lens transparency and homeostasis (Mathias et al., 1997). AQP0 constitutes over 50% of the total membrane

protein found in the bovine lens membrane and mutations in this lens protein have been associated with cataract development in mice (Shiels & Bassnett, 1996).

Although these aquaporins were observed to selectively flux water, Yang and Verkman (1997) observed that they exhibit very different intrinsic water permeabilites when expressed in *Xenopus* oocytes. In a study of AQP1-5, AQP4 expressing oocytes showed the highest level of water permeability In comparison to the oocytes expressing AQP1 and AQP4, AQP2, AQP3, and AQP5 showed lower water permeabilities. The oocytes showing the lowest permeability to water were those that were expressing AQP0 (Mulders et al., 1995), with a water permeability that was an order of magnitude lower than AQP1 (Yang & Verkman, 1997). In comparison, Chandy et al. (1997) estimated the single-molecule permeability of AQP1 to be 50 times higher than that of AQP0. Similar to AQP0, AQP6 was also observed to form weak water channels, exhibiting very low permeability when expressed in oocytes (Ma et al., 1993; Yasui et al., 1999). The differences in permeability among these structurally similar aquaporins suggest that there may exist variations in their sequences that confer these properties.

In addition to mammalian aquaporins, at least 74 microbial MIP channels have also been identified (reviewed in Hohmann, 2000). The only microbial aquaporins that have been extensively studied are the bacterial AQPZ (Calamita et al., 1995) and the S. *cerevisiae* Aqy1p (Bonhivers et al., 1998; Laize et al., 1999) Analysis of the AQPZ sequence shows that the protein is 28-38% identical to those of other known aquaporins (Calamita et al., 1995). Upon expression in *Xenopus* oocytes, AQPZ exhibits high water

selectivity It is thought that AQPZ may be important for maintaining cell turgor while facilitating volume expansion during cell division in the bacteria (Calamita et al., 1998). AQY1 is another microbial aquaporin that was demonstrated to transport water when expressed in *Xenopus* (Bonhivers et al., 1998; Laize et al., 1999). Interestingly, different laboratory and wild-type strains were observed to possess specific sequence differences, which were also apparent in their observed functional differences (Bonhivers et al., 1998). Nevertheless, the physiological role of AQY1 has yet to be defined; although, Bonhivers et al. (1998) predicted that AQY1 functions to facilitate osmotically directed movements of water.

Besides these water-selective aquaporins, aquaglyceroporins have also been identified that are able to transport both water and uncharged solutes. Among the most well characterized aquaglyceroporins in mammalian tissues is AQP3 of the collecting ducts of the kidneys (Fushimi et al., 1993). Upon expression in *Xenopus* oocytes, AQP3 was demonstrated to transport not only water but also glycerol and to some extent urea (Ishibashi et al., 1994; Echevarria et al., 1994; Echevarria et al., 1996). Although AQP3 is localized to areas where water transport takes place, its physiological function remains unclear. AQP7 is another mammalian aquaglyceroporin that was isolated from testis and is also expressed in spermatids and seminiferous tubules (Ishibashi et al., 1997a). Upon expression in *Xenopus* oocytes, AQP7 was demonstrated to transport water, urea and glycerol (Ishibashi et al., 1997a). The role of AQP7 is speculated to be that of a port for water and glycerol as a carbon source for metabolism by mature sperm (Ishibashi et al., 1997a). AQP7 may also be used to replace water with glycerol during long-term

cryopreservation of sperm (Ishibashi et al., 1997a). In another study, AQP8 was cloned from human adipose tissue (Kuriyama et al., 1997). Analysis of AQP8 suggested that it may be the human homolog of AQP7, an alternatively spliced form of AQP7, or the product of a closely related gene. The presence of AQP8 (temporarily designated AQP7L) in adipose tissue suggests involvement in glycerol export during lipolysis. AQP9 was cloned from human leukocytes and was found to transport urea but not glycerol (Ishibashi et al., 1998). In contrast, a reevaluation of this AQP9 in *Xenopus* indicates that it may be permeable to other uncharged solutes, including carbamides, polyols, purines, and pyrimidines (Tsukaguchi et al., 1999). In addition, Tsukaguchi et al. (1998) cloned a cDNA that was highly related to AQP9 from rat liver and found it to be permeable to a variety of hydrophilic solutes such as those listed for human AQP9.

The third functional subclass consists of glyceroporins, MIP proteins that flux glycerol and other uncharged solutes but not water. These glyceroporins have been identified in numerous microbial organisms. The best-studied microbial glycerol facilitators are GlpF of *E coli* (Heller et al., 1980; Sanders et al., 1997) and Fps1p of *S cerevisiae* (van Aelst et al., 1991; Luyten et al., 1995). Glycerol and other uncharged solutes can move across microbial membranes by simple diffusion as well as through channels such as GlpF (Heller et al , 1980) Mutations of *glpF* in *E coli* were observed to reduce passive diffusion of glycerol, influence a change in lipid composition (Sutherland et al., 1997; Truniger & Boos, 1993), and affect the utilization of glycerol at low concentrations (Chen et al., 1994).

In addition to GlpF of *E coli,* the Fps1 proteins of S. cerevisiae also mediates the transport of polyols, glyceraldehyde, glycine and urea (Maurel et al., 1994; Coury et al., 1999). More specifically, Fps1p mediates the release of glycerol from yeast cells by facilitated diffusion (Luyten et al., 1995, Sutherland et al., 1997, Lages & Lucas, 1997; Tamas et al., 1999). These observations indicate that the primary function of Fps1p is as a glycerol exporter involved in osmoregulation (reviewed in Hohmann et al., 2000). Fps1p mutants were observed to exhibit several developmental phenotypes such as defective cell fusion during mating (Luyten et al., 1995; Phillips & Herskowitz, 1997; reviewed in Hohmann et al., 2000).

THE IDENTIFICATION OF MIP PROTEINS IN HIGHER PLANTS

In addition to animal and microbial systems, it is clear that the MIP/aquaporin family is quite diverse in higher plants (reviewed in Johansson et al., 2000). The first plant aquaporin to be demonstrated was the tonoplast intrinsic protein, γ-TIP in *Arabidopsis* which exhibited water transport properties upon expression in *Xenopus* oocytes (Maurel et al , 1993). γ-TIP was cloned using a cDNA corresponding to α-TIP, a seed-specific tonoplast integral protein. α-TIP was identified due to the fact that it constitutes approximately 2% of the total extractable protein of bean cotyledons (Johnson et al., 1989). A recurring hallmark of MIP proteins is that they constitute the major component of the membranes in which they are found.

Since the discovery of γ-TIP, many more plant aquaporins have been identified (reviewed in Johansson et al., 2000). These plant aquaporins can be classified into three phylogenetic groups (Weig et al., 1997). The first group consists of aquaporins that are found in the plasma membrane, called plasma membrane intrinsic proteins (PIPs). The second group consists of aquaporins that are found in the tonoplast (vacuolar membrane), called tonoplast intrinsic proteins (TIPs). The third group consists of nodulin 26-like membrane proteins (NLMs): nodulin 26, found in the symbiosome membrane of soybean nodules, NLM1 of *Arabidopsis*, and other homologues (Weig et al., 1997; reviewed in Johansson et al., 2000). Water transport activity has been observed for many PIPs and TIPs including at least seven PIPs and three TIPs in *Arabidopsis*, one PIP and two TIPs in tobacco, two PIPs in ice plant, one TIP in kidney bean, one TIP in sunflower, one PIP in spinach, and one TIP in maize (reviewed in Johansson et al., 2000;).

Thus far, all of the characterized plant aquaporins were found to transport water. A high specificity for water has been reported for some plant aquaporins such as the kidney bean α-TIP (Maurel et al., 1995), the *Lotus japonicus* LIMP1 (Guenther & Roberts, 2000), and the *Arabidopsis* aquaporins γ-TIP (Maurel et al., 1993), PIP1a (Kammerloher et al., 1994), and PIP2b (Kammerloher et al., 1994). However, considering the functional diversity of MIPs in mammals and microbes, it stands to reason that a similar diversity might exist in plants. This was verified by work with soybean nodulin 26, the first plant aquaporin reported to transport both water as well as small solutes such as glycerol and formamide (Rivers et al., 1997, Dean et al., 1999) and thus belongs to the aquaglyceroporin subclass Since then, at least three other plant MIPs

have been reported to transport water as well as solutes. Nt-AQP1, a tobacco PIP, and Nt-TIPa, a tobacco TIP, were observed to transport water, glycerol, and urea (Biela et al., 1999; Eckert et al., 1999; Gerbeau et al., 1999). The nodulin-26 ortholog, LIMP 2 from *Lotus japonicus* was observed to facilitate the flux of both water and glycerol upon expression in *Xenopus* oocytes (Guenther and Roberts, 2000).

The different specificities of the MIP proteins suggest that structural features may be encoded in the sequences, thus contributing to the protein's selectivity. The observation that members of the MIP family fall into different functional categories prompted studies of the conserved sequences among each group (Froger et al., 1998). Comparison of the sequences of glycerol and water selective aquaporins indicate that there are five "discriminant" residues that are invariant within each of these groups (Froger et al., 1998) (**Table 1**). These residues reside in the third α-helix/loop C region (P_1 residue), loop E (P_2 and P_3 residues), and the sixth transmembrane α-helix (P_4 and P_5) (Froger et al., 1998). For the relative location of these structural elements refer to **figure 2**. Support for this hypothesis came from studies in which substitutions of a tyrosine and a tryptophan at positions 222 (P_4) and 223 (P_5) by a proline and leucine, respectively, in

Table 1. Comparison of the "discriminant" residues of Aquaporins and Glycerol facilitators

Protein[a]	P1	P2	P3	P4	P5
Aquaporin consensus	T or A	S	A	Y or F	W
Glycerol consensus	Y or F	D	K or R	P or A	I or L
Soybean nodulin 26	F	S	A	Y	L

[a]P1-P5 consensus sequences for aquaporins and glycerol facilitators are from Froger et al. (1998). P1 is located within loop C, P2 and P3 are located within loop D containing the second "NPA" motif, and P4 and P5 are located within the sixth transmembrane helix.

the sixth transmembrane domain of the insect aquaporin, AQPcic, abolished the water transport property of the protein, while rendering it capable of glycerol transport capabilities (Lagree et al., 1999). The Y229P and the W223L mutants of AQPcic result in P_4 and P_5 residues consistent with other glycerol facilitators. It was suggested that the presence of the substituted proline in the sixth transmembrane helix may result in a different conformation of the helix and possibly affect its interactions with other helices and loop regions (Lagree et al., 1999). This change in conformation or interaction may result in the change of the specificity of the protein for water and/or glycerol.

More recently, Heymann and Engel (2000) have identified two critical conserved hydrophobic residues in the middle of helices 1 and 4. With respect to AQP1, these residues are in position 24 and 149. In most aquaporins, a phenylanine exists at position 24 and a leucine at position 149. In most glycerol facilitators, a leucine exists at position 24 and 149. In addition to the two discriminant residues (P_4 and P_5) proposed by Froger et al. (1998), the two residues proposed by Heymann and Engel (2000) may also contribute to the specificity of aquaporins.

In summary, both plants and animals contain diverse gene families encoding MIP proteins. While sharing an overall similar structural framework, it is clear that functional differences exist between them. In order to understand the basis of these functional differences as well as how aquaporins/MIPs mediate water transport, they have been the subjects of intensive structural analysis Below, the current understanding of aquaporin/MIP structure is reviewed.

AQUAPORIN STRUCTURE

The hourglass model proposed by Jung et al. (1994b) represents the currently accepted model for MIP proteins (reviewed in Borgnia et al., 1999, Heymann et al., 1998, Engel et al., 2000). The basic topology of MIP proteins (**Fig. 2A**) includes six transmembrane α-helices separated by three extracellular loops and two intracellular loops, with hydrophilic amino and carboxyl termini exposed to the cytosolic compartment (**Fig. 2A**) (Preston et al., 1994; Stamer et al., 1996). The two halves of the aquaporins exhibit an internal homology and obverse symmetry, with two characteristic "NPA" amino acid motifs located in the first cytosolic loop (loop B) and the third extracellular loop (loop E) (Preston et al , 1994, reviewed in Borgnia et al., 1999). In the hourglass model, the aqueous pore is formed from infoldings of NPA loops and conserved hydrophilic residues found on the inner faces of the transmembrane α-helices.

Most of the pioneering work on MIP structure has been done on the prototypical AQP1 (reviewed in Borgnia et al., 1999; Heymann et al., 1998). The topology described above was originally proposed based on hydropathy analysis for AQP1 (Preston et al., 1991). Further verification of this topology came from vectorial proteolysis as well as from DNA insertion studies using a cDNA encoding an epitope of the E1 coronavirus. Further analysis identified that loops B and E were essential for the functioning of AQP1 as a water channel (reviewed in Borgnia et al., 1999), leading to the proposal of the

hourglass model as the general model for the topology of AQP1 and related MIP proteins (Jung et al., 1994b) (**Fig. 2B**).

The oligomeric structure of AQP1, based on gel filtration and analytical ultracentrifugation studies, was proposed to be that of a tetramer (Smith & Agre, 1991), consisting of one glycosylated and three non-glycosylated monomers. Support for the proposed structural orientation comes from the determination of the three dimensional structure of AQP1 (Cheng et al., 1997; Li et al., 1997, Walz et. al., 1997). These studies, using electron crystallography at a resolution of 6Å, revealed the presence of AQP1 tetramers with single subunits containing six bilayer-spanning α-helices surrounding a central density that was believed to be the loops containing the 'NPA' motif (Walz et al., 1997). Combining this information with that obtained from the surface topography obtained from atomic force microscopy, the individual subunit was deduced to be a right-handed helix bundle. More recently, crystallographic studies have been successfully resolved to 4.5Å (Mitsuoka et al., 1999; reviewed in Borgnia et al., 1999, Engel et al., 2000; Heymann & Engel, 2000). These studies reveal a barrel formed by six tilted α-helices (**Fig. 3**) (Cheng et al., 1997; Walz et al., 1997; Li et al , 1997, Mitsuoka et al , 1999; Jap & Li, 1995). Two short α-helices, representing the two branches of the central X-shaped structure (presumably formed by the NPA motifs) that extend to both sides of the membrane surfaces, were observed to be in the center of the monomer (Mitsuoka et al., 1999). Two other branches, believed to be loops connecting the short α-helix to a neighboring transmembrane helix, were also observed

A B

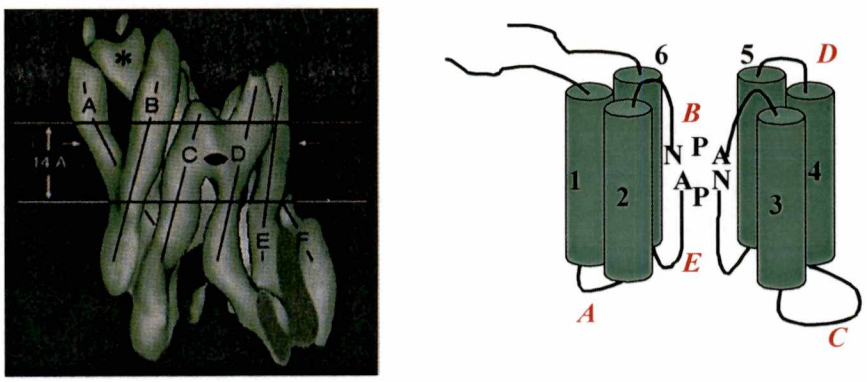


Figure 3. AQP structure and the hourglass model. A. Surface shaded representation of the six-helix barrel viewed parallel to the bilayer with the lines indicating the approximate helix axes of AQP1 (Figure provided courtesy of Dr. Alok Mitra, Scripps Research Institute). The asterisk indicates a portion of density bridging the NPA box to helix F (or helix 6) (Cheng et al., 1997). **B.** Hourglass model for MIP proteins (Jung et al., 1994b).

Further support for the tetrameric organization that was observed with AQP1 came from structural studies with AQP0 (Aerts et al., 1990; Koning et al., 1997), AQP4 (Verbavatz et al., 1997; Hasler et al., 1998), and the insect aquaporin, AQPcic (Beuron et al , 1995; LeCaherec et al., 1996), determined by hydrodynamic and crystallographic analysis. α-TIP is the only plant aquaporin that has been successfully studied using crystallography (Daniels et al,, 1999). It was observed to also form a tetrameric array. The similarity of the structures seen in these five proteins suggest that this tetrameric organization may be a common feature of water-selective aquaporins (reviewed in Engel et al., 2000) and the that tetrameric arrangement is likely to be important for the folding, stability, and/or targeting of the protein to the plasma membrane (Jung et al., 1994b). The functional significance of the tetramer is not certain since each subunit forms a separate, apparently non-interacting, aqueous pore (Preston et al., 1993; Jung et al., 1994b).

In addition to these water-selective aquaporins, the oligomerization state of the glycerol facilitator has also been investigated. Lagree et al. (1998) found that solubilized GlpF was monomeric. Furthermore, to study which residues are involved in the tetramerizaiton of aquaporins, the "discriminant" residue located at position 205 in loop E (P_3, **Table. 1**) (Froger et al., 1998) of AQPcic was mutated from an aquaporin consensus residue (serine) to a glyceroporin consensus residue (aspartic acid). This substitution switched the protein from the tetrameric form observed in water selective aquaporins to the monomeric form observed in GlpF (Lagree et al., 1998). Although this substitution changed the oligomerization of the protein, expression in *Xenopus* oocytes not only showed that this mutation abolished the water fluxing property of this protein,

but also that the protein expressed in oocytes was still unable to flux glycerol (Lagree et al., 1998). These observations suggest that this residue (P_3) is important in the stability of the tetramerization of the protein and not in the selectivity of the protein as previously discussed above.

The 4.5-Å resolution structure obtained by Mitsuoka et al. (1999) was not detailed enough to provide an atomic resolution nor to visualize the water pathway of aquaporin 1. However, enough information was present to speculate on the identification of the water pathway at the pore-like cavity in the center of the aquaporin 1 monomer. The pore size for each side of lipid bilayer was observed to be slightly different. On one side, the narrowest region was about 4.5 Å. This is approximately the size of a water molecule, which would enable the water molecule to come into direct contact with the pore helix, loop, and other side chains. On the other side, the pore size is approximately 12 Å. These differences suggest that one side of the pore is more restrictive than the other side and may serve as the selectivity filter (Mitsuoka et al., 1999).

Particulars about the specific residues involved in transport and pore formation await an atomic resolution model. Despite this, Heymann et al. (1998) proposed a hypothetical model for the aqueous pathway. This model is based on information from water chains in other proteins and sequence analysis of conserved residues. In this model, several highly conserved residues throughout the six transmembrane helices and loops B and E were proposed to function as a chain of polar residues from the cytoplasmic to extracellular sides, participate in the formation of the channel, and/or stabilize the channel (Heymann et al , 1998) (**Table 2**)

Table 2. Residues proposed by Heymann et al (1998) to line the channel pore

Residue	Position in AQP1	Location in AQP1
Q	101	Helix 3
E	17	Helix 1
N	76	Loop B
N	192	Loop E
E	142	Helix 4
T	21	Helix 1
T	146	Helix 4
R	12	Helix 1
S	71	Hinge between helix 2 and loop B
H	74	Hinge between helix 2 and loop B
T	80	Loop B
R	93	Helix 3
Y	97	Helix 3
H	180	Helix 5
T	187	Hinge between helix 5 and loop E
R	195	Loop E
S	196	Loop E
H	209	Helix 6

[a]Residues listed are highly conserved among the MIP family proteins. The position and location of the residues listed are for AQP1.

In addition to those residues proposed by Heymann et al. (1998) to be important in the formation or function of the channel pore, other residues have also been implicated. The sensitivity of aquaporins to mercury reagents may provide information about residues in proximity to the channel pore. Following the incubation of *Xenopus* oocytes in $HgCl_2$, a reduction in osmotic water permeability is observed indicating that the water channel is blocked (Macey, 1984; Verkman, 1992). The mechanism by which this occurs is by the binding of mercury to cysteine residues, which induces a blockade of transport across the channel (Preston et al., 1993; Zhang et al., 1993). Studies on the sensitivity of AQP1-5 to mercury agents reveal that the only one that is mercury-resistant is AQP4 (Fushimi et al., 1993; Ishibashi et al., 1994; Echevarria et al., 1994; Hasegawa et al., 1994; Jung et al., 1994a; Raina et al., 1995). Mutational analysis have identified the mercury-sensitive residues as cysteines located at these positions: 189 of AQP1 (Preston et al., 1993; Zhang et al., 1993), 181 of AQP2 (Bai et al., 1996), and 11 of AQP3 (Kuwahara et al., 1997). Amino acid sequence analysis of AQP1 and AQP2 indicate that these cysteines are located at the same position near the conserved 'NPA' motif found in loop E, suggesting that AQP1 and AQP2 are structurally similar. Furthermore, the substitution of a tryptophan for these cysteines abolishes the transport property of the water channel (Preston et al., 1993; Zhang et al., 1993; and Bai et al., 1996), implicating the importance of these conserved cysteines and also possibly that the large side chain of tryptophan is blocking the pore (Preston et al., 1993; Zhang et al., 1993; Bai et al., 1996).

In contrast to AQP1 and AQP2, the substitution of a tryptophan for the mercury sensitive cysteine in AQP3 did not abolish the transport property of the channel (Kuwahara et al., 1997). A comparison of the sequences for these aquaporins show that cysteine 11 of AQP3 (locate in the hydrophilic amino terminal domain) is not located in the position equivalent to the mercury sensitive residues in AQP1 and AQP2. The position equivalent to 189 of AQP1 (position 212) is occupied by a tyrosine in AQP3. Substitution of a cysteine for tyrosine 212 resulted in an increase in mercurial sensitivity and a substitution of a tryptophan abolished the water transport property suggesting that the residues near the 'NPA' motif contribute to the formation of the pore (Kuwahara et al., 1997). In support of this, the substitution of a cysteine residue at position 211 of AQP4 resulted in mercury sensitivity (Shi & Verkman, 1996).

AQUAPORIN REGULATION BY PHOSPHORYLATION

Aquaporins are regulated by multiple means, including at the transcriptional level, through altered membrane targeting and trafficking in response to hormonal signals, and in response to post-translational modification (reviewed in Borgnia et al., 1999; Engel et al., 2000, Johansson et al , 2000). Post-translational modifications commonly found in MIPs include phosphorylation, glycosylation, and proteolytic processing (Johansson et al., 1998; Weaver et al., 1991, Johnson & Chrispeels, 1992; Miao et al., 1992; Higuchi et al., 1998; Inoue et al., 1995, reviewed in Borgnia et al., 1999; Engel et al., 2000;

Johansson et al., 2000). Of these, phosphorylation appears to have the greatest potential for regulation.

Several aquaporins have been observed to be phosphorylated in vivo. AQP0 has been observed to be phosphorylated both *in vitro* by both protein kinase A (Garland & Russel, 1985; Johnson et al., 1986; Louis et al., 1985) and protein kinase C (Lampe et al., 1986), and in vivo by protein kinase A (Johnson et al., 1986; Lampe & Johnson, 1989). Despite the fact that this AQP0 is clearly an endogenous substrate for these protein kinases, the role of phosphorylation of AQP0 remains unresolved.

However, recent evidence suggests that protein kinase C is a negative regulator of another mammalian aquaporin, AQP4 (Han et al , 1998). The phosphorylation of AQP4 was observed *in vivo* using rat brain homogenates and protein kinase C activators (phorbol 12,13-dibutyrate and phorbol 12-myristate 13-acetate) (Han et al., 1998). Water permeability assays with AQP4 expressed in *Xenopus* oocytes was used to determine the effect of the phosphorylation on the intrinsic permeability properties of AQP4. In these assays, oocytes were incubated in protein kinase C activators prior to performing the assays The protein kinase C activators were observed to reduce the rate of swelling of oocytes expressing AQP4 (Han et al., 1998). Thus, phosphorylation by this kinase might exert a negative regulation on AQP4, gating the flux of water

Perhaps the most well-studied aquaporin with respect to the effects of phosphorylation is AQP2 of the collecting duct of kidney. Vasopressin, which rapidly increases collecting duct water permeability, is believed to be the factor that modulates AQP2. The binding of vasopressin to an adenylyl cyclase-coupled vasopressin receptor

(V2) on the basolateral membrane of the principal cells results in an increase in intracellular cyclic AMP and subsequent activation of protein kinase A (reviewed in Yamashita et al., 2000; Johansson et al., 2000). Protein kinase A catalyzes the phosphorylation of serine 256 at the cytoplasmic carboxyl terminus of AQP2. This in turn triggers the translocation of the intracellular vesicles containing AQP2 to the apical membrane of the cells in the collecting ducts, raising the cell water permeability (Fushimi et al., 1997; Katsura et al., 1997; Kuwahara et al., 1995; Nishimoto et al., 1999; Marples et al., 1995; Nielsen et al., 1993, Sabolic et al., 1995; Yamamoto et al., 1995). The translocation of AQP2 to the plasma membrane has been demonstrated using immunoelectron microscope (Nielson et al., 1993).

In addition to mammalian aquaporins, phosphorylation has also been observed in plant aquaporins such as α-TIP from kidney bean and PM28A from spinach (Johansson et al., 1998; Johnson & Chrispeels, 1992). α-TIP is a seed specific protein of the MIP family and is found in the vacuolar membrane during the late and early stages of seed maturation and germination (Johnson et al., 1989). α-TIP is phosphorylated at serine 7 in the amino-terminus by a tonoplast-bound protein kinase, tentatively identified as a calcium dependent protein kinase (CDPK) (Maurel et al., 1995; Johnson & Chrispeels, 1992). Furthermore, it was demonstrated that α-TIP expressed in *Xenopus* oocytes was phosphorylated at three separate serine residues by oocyte protein kinase A (Maurel et al., 1995). The phosphorylation of these three serine residues of α-TIP resulted in an increase in water permeability (Maurel et al., 1995).

The aquaporin, PM28A, was identified as the major phosphoprotein of the spinach leaf plasma membrane (Johansson et al , 1996). The phosphorylation of PM28A occurs at serine 274 in the carboxyl-terminus by a calcium-dependent, plasma membrane associated protein kinase (Johansson et al., 1996), and phosphorylation is dependent on the apoplastic water potential, implicating its role in cellular water balance. Protein kinase (K252a) and phosphatase inhibitors (okadaic acid) were used to evaluate the effect of phosphorylation on PM28A expressed in *Xenopus* oocytes (Johansson et al., 1998). Phosphorylation was observed to enhance the water transport activity of PM28A (Johansson et al., 1998). The water channel activity of PM28A is regulated by phosphorylation of two serine residues located at positions 274 and 115 in *Xenopus* that were identified using mutagenesis (Johansson et al., 1998). However, *in vivo* phosphorylation in plants was only demonstrated at serine 274 (Johansson et al., 1998). In addition to these studies, previous work indicates that nodulin 26 is the major phosphoprotein on the SM (Weaver et al., 1991; Weaver et al., 1992), which will be discussed in detail below.

While phosphorylation has been reported in aquaporins found in plants and animals, the functional effects of phosphorylation appears to be diverse, showing either up or down regulation of transport activities, as well as trafficking The current understanding of the aquaglyceroporin nodulin 26, which was previously reported to be the major phophoprotein on the SM of soybean (Weaver et al., 1991; Weaver et al., 1992) is reviewed below

NODULIN 26

Based on activities shown by several other members of the MIP protein family, nodulin 26 was speculated to have channel properties. Earlier functional analyses of nodulin 26 by reconstitution into liposomes for channel studies in planar lipid bilayer revealed that it forms single channels with a maximum unitary conductance of 3.1 nanosiemens (Weaver et al., 1994). A similar finding was originally reported for MIP/AQP0 (Ehring et al., 1990). Furthermore, the channels were found to transport both cations and anions, but possessed a weak selectivity for anions (Weaver et al., 1994). Based on these data it was suggested that nodulin 26 serves as a broad selectivity, high conductance ion channel. However, the function of nodulin 26 as an ion channel was challenged by several observations. First stopped-flow fluorimetric assays of purified soybean SM vesicles show that they have a high rate of water flux which exhibited a low energy of activation (E_a), sensitivity to mercurials, and a high P_f/P_d (Rivers et al., 1997). These properties are characteristic of aquaporin facilitated water transport. SM vesicles also exhibited a mercury-sensitive, facilitated flux of uncharged solutes such as glycerol and formamide (Rivers et al., 1997). To determine whether nodulin 26 was responsible for these activities, its transport properties were investigated by heterologous expression in *Xenopus laevis* oocytes and by reconstitution of the purified protein into proteoliposomes for stopped flow fluorimetry studies (Rivers et al., 1997; Dean et al., 1999). Both analyses show that nodulin 26 is multifunctional and allows the flux of both water and solutes. Furthermore, based on the quantitative stopped flow data and the

density of nodulin 26 molecules in the liposomes, a single channel conductance rate, an estimation of the amount of water that is transported across each channel per unit time, was calculated at 3.8 X 10^{-15} cm^3/s (Dean et al., 1999). In comparison, the single channel conductance rate calculated for aquaporin 1 at 117 X 10^{-15} cm^3/s (Zeidel et al., 1992) was 30-fold higher than that observed for nodulin 26. Nodulin 26 also has a P_f/P_d ratio of 18, which is different from AQP1 (P_f/P_d = 13) (Rivers et al., 1997). The drastically different single channel rate and the different P_f/P_d ratios for nodulin 26 and AQP1 suggest that structural differences in water permeability and in the length of the pore might exist (Finkelstein, 1987).

In addition to its transport abilities, nodulin 26 contains a hydrophilic region on the carboxyl terminus that resembles consensus phosphorylation sites recognized by the calcium-dependent protein kinase of plant and algae (reviewed in Roberts & Harmon, 1992; Harmon et al., 2000). This observation prompted studies of nodulin 26 phosphorylation (Weaver et al., 1991; Weaver & Roberts, 1992). These studies revealed that a synthetic peptide (CK-15), containing the 14 amino acids residues of the carboxyl terminus of nodulin 26 was phosphorylated by a calcium-dependent protein kinase in nodule extracts (Weaver et al., 1991). In addition, it was shown that a CDPK exists on symbiosome membranes that phosphorylates native nodulin 26 both *in vitro* and *in vivo* (Weaver et al., 1991). Phosphoamino acid analysis of labeled CK-15 showed phosphoserine as the only detectable product (Weaver et al., 1991). The serine at the 262 position of the carboxyl terminus of nodulin 26 was identified as the site of phosphorylation by using phosphopeptide mapping of endoproteinase Lys-C-digested

nodulin 26 and automated and manual peptide sequence analysis (**Fig. 2**) (Weaver et al., 1992).

CDPKs have been identified in higher plants, algae, and protists (reviewed in Roberts & Harmon, 1992; Harmon et al., 2000). The activity of CDPKs are dependent on submicromolar to micromolar concentrations of Ca^{2+} and they bind to Ca^{2+} without the involvement of effector molecules, such as phosphatidylserine and diacylglycerol or calmodulin (reviewed in Roberts & Harmon, 1992; Harmon et al., 2000). This requirement is due to the presence of its three functional domains: catalytic (kinase), autoinhibitory and a calmodulin-like regulatory domain that contains four calcium-binding EF-hands (reviewed in Roberts & Harmon, 1992; Harmon et al., 2000).

While it is clear that nodulin 26 is an endogenous target for CDPK, the effects of phosphorylation on the activity and its role in the symbiosome membrane remain less certain. Lee et al. (1995) pursued this question by using planar lipid bilayer recording techiniques, similar to the approach used by Ehring et al. (1990; 1991). By using recombinant DNA techniques nodulin 26 with serine, alanine, or aspartate at position 262 was produced by expression in *Escherichia coli* (Lee et al., 1995). Upon reconstitution into planar lipid bilayers, the recombinant proteins displayed large single channel conductance (3.1 nanosiemens) and weak anion selectivity, similar to the wild type nodulin 26 described in Weaver et al., (1994). This study showed that phosphorylation of S262 nodulin 26 affected its voltage sensitivity. Whereas unphosphorylated nodulin 26 is completely open and pore-like, upon phosphorylation it shows voltage-dependent gating and partial to full closure (Lee et al., 1995). Whether phosphorylation exhibits the

same effect on nodulin 26 aquaglyceroporin activity, and what other factors might control this activity still remain an open question.

From the discussion above, it is clear that nodulin 26 is functionally distinct from other aquaporins in that. 1) It is a multifunctional transporter of water, glycerol, and uncharged solutes; 2) it shows low intrinsic water transport properties; and 3) it is a major target for CDPK in the SM. In this present study we wished to investigate. 1) The unique residues in nodulin 26 that confer these transport properties; 2) factors such as pH and calcium that may affect the gating of the nodulin 26 channel; and 3) The functional effect of phosphorylation on these properties.

CHAPTER II

MATERIALS AND METHODS

MOLECULAR CLONING AND SITE-DIRECTED MUTAGENESIS METHODS

The full-length nodulin 26 cDNA (Zhang & Roberts, 1995; Sandal & Marcker, 1988) was first cloned into the *Bam*HI site of the pRSETA expression vector (Lee et al., 1995) The construct was removed from pRSETA by digestion with *Bam*HI. The nodulin 26 fragment was separated by electophoresis on a 0.8% (w/v) low melting point agarose gel and was isolated as described in Sambrook et al. (1989). The nodulin 26 cDNA was inserted into the *Bgl*II site of the *Xenopus* β-globin gene downstream of the T3 promoter of the pXBG-ev1 *Xenopus* expression vector (Preston et al., 1992) as previously described (Zhang & Roberts, 1995; Rivers et al., 1997). The pXBG-ev1(nod26) plasmid was then transformed into *E coli* strain JM101.

The pXBG(nodulin 26) construct was used as the template for PCR mutagenesis by using the QuickChange™ Site-Directed Mutagenesis kit (Stratagene) The pXBG-nodulin 26 vector (27 ng) was combined with two synthetic oligonucleotide primers (0.125 μg) containing the desired mutation (**Table 3**). The two primers were designed to be complementary to opposite strands of the nodulin 26 cDNA. For amplification the

Table 3. Primers used in generating mutants of wild-type Nod26.

Primers[a]	forward primers[b]	reverse primers[b]
Q114R	5'gattccccttgatc**cgg**gtaccagcttatgt3'	5'acataagctggtac**ccg**gatcaaggggaatc3'
C172F	5'tcatgttcgtcata**ttc**ggggttgccaccga3'	5'tcggtggcaaccccg**aa**tatgacgaacatga3'
I226H	5'cggtgaatacgaagga**cac**tggatatatttgttg3'	5'caacaaatatatcca**gtg**tccttcgtattcaccg3'
V197H	5'acattactgctgaat**cac**attattggagggcca3'	5'tggccctccaataat**gtg**attcagcagtaatgt3'
L230W	5'ggaatatggatatat**tgg**ttggcacctgttgtgg3'	5'ccacaacaggtgccaa**cca**atatatccatattcc3'
Y229P	5'cgaaggaatatggata**ccc**ttgttggcacctgttgtgg3'	5'ccacaacaggtgccaacaa**ggg**tatccatattccttcg3'
S262A	5'gatcaccaagagtgct**gct**ttcctcaaaggcc3'	5'ggcctttgaggaaagc**agc**actcttggtgatc3'

[a]The abbreviation shows the residue mutated and the new amino acid introduced. Thus, Q114R represents the substitution of an arginine for glutamine at position 114. C172 F represents the substitution of a phenylalanine for cysteine at position 172. I226H represents the substitution of a histidine for isoleucine at position 226. V197H represents the substitution of a histidine for valine at position 197. L230W represents the substitution of a tryptophan for leucine at position 230. Y229P represents the substitution of a proline for tyrosine at position 229. S262A represents the substitution of an alanine for serine at position 262.

[b] Nucleotides in **bold** correspond to amino acids that were affected by the mutagenesis. Underlined nucleotides indicate the nucleotides that were changed in the mutagenesis.

following parameters were used: 30 seconds of strand separation at 94°C, 1 minute of annealing at 55°C, and 8 minutes of elongation at 68°C. PCR was done for 14 cycles with *PfuTurbo* DNA polymerase. The final product was treated with *Dpn*I, which digests the parental DNA template, leaving only the PCR-amplified DNA containing the desired mutations. The quality of the PCR products was determined by electrophoresis on 1% (w/v) agarose gel in Tris-acetate/disodium ethylenediaminetetraacetate (EDTA) electrophoresis buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) for 1 hour (Sambrook et al , 1989). The pXBG vector bearing the nodulin 26 mutants was transformed into XL1-Blue *E coli* (Sambrook et al., 1989).

The plasmid was purified by using the Qiagen® plasmid miniprep kit. Mutations were verified by automated DNA sequencing on a Perkin Elmer Applied Biosystems 373 DNA sequencer at the University of Tennessee Molecular Biology Research Facility (Knoxville, Tennessee, USA). Reactions were prepared using a Prism Dye Terminator Cycle sequencing kit (Perkin Elmer Applied Biosystems, Foster City, California, USA).

cRNA PREPARATION

E coli clones expressing the nodulin 26 proteins were streaked onto a Luria Broth (LB) plate containing 100 μg/mL ampicillin, and were grown overnight at 37°C. Single colonies from the plate were grown by shaking overnight at 37°C in 10 mL of LB containing 100 μg/mL ampicillin

Isolated plasmids were linearized by digestion with *Xba*I. Capped nod26 cRNA's were synthesized by in vitro transcription of linearized plasmids by using T3 RNA polymerase (Stratagene, La Jolla, CA, USA). cDNA (1 μg) was incubated in a reaction buffer containing 40 mM Tris-HCl, pH 7.5, 50 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine, 2 mM rUTP, 2 mM rCTP, 2 mM rATP, 0.6 mM rGTP, 0.6 mM Cap analog (5' 7MeGpppG 3'), 30 mM DTT, 10 Units T3 RNA polymerase for 30 minutes at 37°C. The 5' cap structure helps to stabilize the RNA during synthesis and enhances the translation efficiency of RNA transcripts by microinjected *Xenopus* oocytes. RNase-free DNase I (10 Units) was added to the reaction mixture and incubated for another 10 minutes at 37°C to remove the DNA template. The reaction was stopped with 100 μL of diethylpyrocarbonate (DEPC)-treated deionized water and was extracted with phenol-chloroform-isoamyl alcohol (24:24:1) and then with chloroform. Sodium acetate (pH 5.0) was added to a final concentration of 0.3 M and the RNA was precipitated by addition of 2.5 volumes of absolute ethanol and incubation at –20°C overnight. The precipitated RNA was collected by sedimentation in a Marathon 16KM model microfuge (Fisher Sci., Pittsburgh, PA) for 20 minutes at 4°C The pellets were washed with 80% (v/v) ethanol and were dried under vacuum. The RNA was resuspended in 25 μL of DEPC-treated deionized water The concentration of the final RNA product was determined by measurement of the absorbance at 260 nm and the following calculation:

final concentration = A_{260} x dilution factor x conversion factor (Eq 2)

The conversion factor for RNA is 0.040 μg/μL/OD_{260} unit.

The quality and integrity of the RNA samples was determined by electrophoresis on 1% (w/v) agarose gel in Tris-acetate/EDTA electrophoresis buffer for 1 hour (Sambrook et al., 1989) as described previously RNA samples were stored at –80°C at a concentration of 1 μg/μL in 3 5 μL aliquots.

XENOPUS OOCYTE CULTURE AND cRNA MICROINJECTION

Oocyte positive female *Xenopus laevis* frogs were obtained from either Xenopus One (Dexter, MI, USA) or Xenopus Express (Ann Arbor, MI, USA) and were housed in the animal facility at the University of Tennessee (Knoxville, TN, USA). For oocyte removal, frogs were anesthetized by immersion in a tricaine solution (1g/L tricaine methanesulfonate [MS-222, Argent Finquel®, Redmond, Washington], 0.5 g/L baking soda) at room temperature for 5-10 minutes. The anesthetized frog was placed ventral side up on a wet cloth and a small incision (0.5-1 cm) was made in the lower abdomen. An ovarian lobule with oocytes (5 mL) was surgically removed and then placed in sterile calcium-free frog Ringer's solution (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, 5 mM Hepes-NaOH, pH 7.6, 200 mOsm/kg). The ovarian lobules were cut up into small pieces and defolliculated with collagenase at room temperature (1 mg/mL, Sigma, St. Louis, MO, USA) for 2 h in 15 mL calcium-free frog Ringer's solution. The collagenase treatment was stopped by washing the oocytes 4-5 times with frog Ringer's solution (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, 5 mM Hepes-NaOH, pH 7.6, 0.6 mM $CaCl_2$).

Stage V and VI oocytes were identified under a dissecting microscope by a distinct light-colored vegetal pole and a dark-colored animal pole separated by a white-colored animal-vegetal axis.

Injection pipettes were constructed by pulling 3.5 inch RNase-free glass capillaries (Drummond Scientific Co., Broomall, PA, USA, 3-000-203G/X) on a microelectrode puller (Flaming/Brown micropipette puller Model P-87, Sutter Instruments Co.) and then breaking off the tip under a microscope with a P-200 pipette tip. The capillaries were made RNase-free by baking at 220°F for at least 8 hours. The setting used on the microelectrode puller was heat at 375, pull at 200, velocity at 150, and time at 120. A small amount of mineral oil was used to backfill the pipette. The pipette was secured onto the plunger of a "nanoject" automatic injector micromanipulator (Drummond Scientific Co., Broomall, PA. USA) and was dialed down until the oil emerged from the tip. The cRNA sample was placed onto a Parafilm sheet and was drawn up into the injection pipette. Stage V and VI oocytes were placed on a specialized platform containing frog Ringer's solution and were injected with 46 nl of cRNA (1 μg/μL) or DEPC-H_2O (negative control) The platform was constructed by fixing a plastic grid into a plastic petri dish. Proper injection can be observed in the swelling of the oocytes as well as movement of the oil-RNA solution interface within the pipette. Following injection, the oocytes were transferred to individual wells of a 96-well microplate filled with frog Ringer's solution supplemented with 100 μg/mL penicillin-streptomycin, 50 μg/mL tetracycline. The oocytes were cultured at 18°C for three days in frog Ringer's solution supplemented with 100 μg/ml penicillin-streptomycin, 50 μg/ml

tetracycline as previously described (Rivers et al., 1997) with daily changes of fresh media.

OSMOTIC WATER PERMEABILITY (P_f)

The osmotic water permeability was determined at 18°C as previously described (Rivers et al., 1997; Guenther & Roberts, 2000). Briefly, the oocytes were transferred from a 100% isoosmotic frog Rınger's solution (200mOsm/kg) to a 30% hypoosmotic (60mOsm/kg) frog Ringer's solution (prepared by diluting frog Ringer's solution in deionized water), and the rate of swelling was assayed by video microscopic imaging as described previously (Guenther & Roberts, 2000). Oocyte images were collected every 5 seconds over a 70 second time interval by a Pro-Series High Performance CCD camera attached to a Nikon Alphaphot YS microscope. The oocyte images were digitized using a frame grabber (Image-Pro Plus™ software, Media Cybernetics, Silver Spring, MD, USA) attached to the CCD camera. The cross-sectional area (A) of the oocyte at a particular time was used to calculate the relative volume by:

$$(A/A_o)^{3/2} = V/V_o \qquad \text{(Eq 3)}$$

where $\mathbf{A_o}$ represents the initial cross-sectional area, $\mathbf{V}$ is the volume at a specific time, and $\mathbf{V_o}$ represents the initial volume. The relative oocyte volume (V/V_o) measured over tıme was used to establısh the rate of swelling, $(dV/V_o)/dt$. The rate of change of oocyte volume is related to the rate of water uptake. There was nearly a linear increase in oocyte

volume over the first few minutes corresponding to osmotic water flux. From the rate of swelling, the osmotic water permeability was calculated·

$$P_f = \frac{V_o(dV/V_o)/dt}{(S_{real}/S_{sphere})V_w(osm_{in} - osm_{out})} \quad \text{(Eq 4)}$$

Where $\mathbf{V_o}$ is the surface area of the oocyte at time zero, $\mathbf{osm_{in}}$ is the osmolarity in the oocyte, $\mathbf{osm_{out}}$ is the osmolarity of the bath media, $\mathbf{V_w}$ is the partial molar volume of water (18 cm^3/mol), $\mathbf{S_{real}}$ is the actual area of the oolemma and $\mathbf{S_{sphere}}$ is the area calculated by assuming that the oolemma is a sphere. S_{real}/S_{sphere} is considered to be 9 for all permeability measurements (Zampighi et al., 1995, Rivers et al., 1997). S_{real}/S_{sphere}, derived from morphological measurements, corrects for the increase in area due to the existence of folds and microvilli in the oolemma (Zampighi et al., 1995). In the analysis of the osmotic water permeability, the early time data were used (first 70 seconds). Later time data were not generally used because the osmotic gradient would be reduced

The osmolality of all solutions was determined by freezing point depression using the Osmette S Automatic Osmometer (Precision Systems, Inc., Natick, MA, USA). The osmolarity for the 100% frog Ringer's solution ranged from 195-215 mOsm/kg and the 30% frog Ringer's solution ranged from 50-60 mOsm/kg. To facilitate our comparison of such a low water fluxing protein, relative permeabilities were calculated by subtracting the background of the water control and standardizing to the P_f obtained at the standard recording experimental conditions:

$$\text{relative } P_f = \frac{P_{exp} - P_{water}}{P_{std} - P_{water}} \quad \text{(Eq. 5)}$$

where P_{exp} refers to the P_f of the oocyte sample measured under the experimental conditions, P_{water} refers to the P_f of the water-injected oocytes under the experimental conditions, and P_{std} refers to the P_f of nodulin 26-injected oocytes under the standard conditions.

To assay the effects of varying extracellular calcium levels, cultured oocytes were incubated in 100% Ringer's solution at the experimental Ca^{2+} concentration (1 mM ethylenebis (oxyethylene-nitrilo) tetraacetic acid (EGTA), no added Ca^{2+}, 0 6 mM Ca^{2+}, or 10 mM Ca^{2+}) for 15 minutes and the assay was performed in 30% Ringer's solution at the same experimental Ca^{2+} concentration. 0.6 mM Ca^{2+} is the standard concentration used in most measurements. To assay the effects of intracellular calcium buffering, microinjection of the calcium chelator BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, Sigma, St Louis, MO) was performed as previously described (Nemeth-Cahalan & Hall, 2000). 46 nL of 40 mM BAPTA were injected into the oocytes and they were incubated for 30 minutes in frog Ringer's solution. Assays were then performed under standard conditions of 0.6 mM calcium-containing frog Ringer's solution. The final BAPTA concentration in the oocyte was estimated to be approximately 2 mM (Nemeth-Cahalan & Hall, 2000).

To assay the effects of changing the extracellular pH, cultured oocytes were incubated in 100% Ringer's solution at each experimental pH for 15 minutes prior to the assay at the same pH. HEPES was used to buffer the frog Ringer's solution at pH 7.6 and pH 7.0. MES was used to buffer the frog Ringer's solution at pH 6.5 to pH 4.5.

To assay the effects of phosphorylation by protein kinase C, cultured oocytes were incubated in 100% Ringer's solution containing either 10 nM phorbol-12-myristate-13-acetate (TPA, a protein kinase C activator, Calbiochem, San Diego, CA, USA) or 5 μM okadaic acid for 30 minutes prior to performing the assays in 30 % Ringer's solution. To assay the effects of phosphorylation by CDPK, 46 nL of recombinant CDPK (Harper et al., 1994) (12 pmol/μL/min, see below) was microinjected into the oocytes 24 hours before the oocyte swelling assay.

GLYCEROL PERMEABILITY

The glycerol transport permeabilities were assayed by using a modified version of an isotopic glycerol uptake assay (Maurel et al., 1993). At least 6 oocytes were incubated in 13 X 100 mm borosilicate culture tubes containing 20 μL of assay medium (2 mM KCl, 5 mM $MgCl_2$, 600 μM $CaCl_2$, 5 mM HEPES-NaOH pH 7.6, 60 :Ci/mL ^{3}H-glycerol [80 Ci/mmol, NEN, Boston, MA, USA], and 150 mM cold glycerol, 210 mOsm/kg) per oocyte at room temperature for 10 minutes The uptake reaction was terminated by two washes with 6 mL ice-cold frog Ringer's solution. The oocytes (in groups of two) were lysed overnight in 2% (w/v) SDS at room temperature and the amount of ^{3}H-glycerol uptake was determined by liquid scintillation counting (Dean et al., 1999).

PROTEIN KINASE ISOLATION

E. coli clones that express a recombinant CDPK (KJM AK23-6H2, Harper et al., 1994) were streaked onto a Luria Broth (LB) plate containing 34 μg/mL chloramphenicol and 100 μg/mL ampicillin, and were grown overnight at 37°C. Single colonies from the plate were grown by shaking overnight at 37°C in 50 mL of LB containing 34 μg/mL chloramphenicol and 100 μg/mL ampicillin. The 50 mL overnight culture was then used to inoculate 450 mL of LB containing 34 μg/mL chloramphenicol and 100 μg/mL ampicillin, and was grown for 2.5 hours while shaking at 37°C. Isopropyl β-D-thiogalactopyranoside was added to the culture to a final concentration of 1 mM and the culture was incubated with shaking for 2 hours at 37°C. The cells were collected by centrifugation at 5,000 x g for 10 minutes at 4°C. The cells were lysed with 2 mg/mL of lysozyme (Sigma, St. Louis, MO, USA) in 20 mL of 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 14 mM β-merceptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF) for 15 minutes on ice. Triton X-100 was added to a final concentration of 0.4% [v/v]. The sample was sonicated on ice by using a Sonic Dismembrator Model 301 (Artek Systems Corp , USA) equipped with the microprobe in 6 bursts, each lasting 15 seconds with the output between 0.6 and 0 8, followed by 45 seconds of rest The sample was centrifuged for 10 minutes 12,000 x g at 4°C. The supernatant fraction was collected and was centrifuged for 30 minutes at 100,000 x g at 4°C.

The recombinant CDPK possesses a histidine tag sequence at the carboxyl terminal end of its coding sequence to facilitate purification by metal chelate

chromatography as well as a glutathione S-transferase (GST) sequence at its amino terminal end to facilitate purification by affinity chromatography on glutathione resins (Harper et al., 1994). Using a 10 mL syringe, the sample was applied to a Ni^{2+}- column (250 µL iminodiacetic acid coupled to Sepharose 6B fast flow resin, Sigma, St. Louis, MO) twice. The nickel column was prepared by washing with 5 mL of distilled water, followed by 2.5 mL of 0 1 M $NiSO_4$. Residual $NiSO_4$ was removed by washing with 5 mL of distilled water, and the resin was then equilibrated with 10 mL of equilibration buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl). After sample application, the column was washed with 30 mL of equilibration buffer. CDPK was eluted with 10 mL of elution buffer (300 mM imidazole, 20 mM $NaPO_4$, pH 6.0) and was collected in 500 µL fractions. Fractions containing CDPK were identified by a protein kinase assay as described below.

Samples containing high CDPK activity were pooled, and were diluted (1:5) with GST binding buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1 mM dithiothreitol (DTT), 0.4% [v/v] Triton X-100). The pooled fractions were incubated with 200 µL of glutathione Sepharose 4B for 30 minutes at room temperature with gentle shaking. The unabsorbed fraction was collected and reapplied twice. The column was washed with 200 mL of the GST binding buffer and then washed with 25 mL of 50 mM Tris-HCl, pH 7.4. CDPK was were eluted with 10 mL of 10 mM glutathione in 50 mM Tris-HCl, pH 8.0 and was collected in 500 µL fractions. The kinase activity of the proteins in the fractions was assayed as described below and the purity was assessed by

SDS-polyacrylamide gel electrophoresis on 15% (w/v) polyacrylamide gels (Laemmli, 1970).

PROTEIN KINASE ASSAY

CDPK activity was assayed by its ability to phosphorylate a synthetic peptide, either CK-15 (based on the carboxyl terminus of nodulin 26, Weaver et al., 1991) or KM-14 (based on the MLCK phosphorylation sequence of the smooth muscle myosin light chain, Roberts, 1989). KM-14 (6 μg) or CK-15 (7.2 μg) was incubated at 30°C for 5 minutes in the presence of 1 mM [γ-^{32}P]ATP (500 dpm/pmol), 25 mM MOPS-NaOH, pH 7.0, 10 mM magnesium acetate, 0.5 mM $CaCl_2$. The assay was initiated by addition of the protein kinase sample (CDPK) and incubation was continued at 30°C for 30 minutes. The reaction was terminated by spotting 25 μL of the assay mixture onto a 1 cm square of phosphocellulose paper. After 2 minutes, the squares were placed in 75 mM phosphoric acid (10 mL per phosphocellulose square). The phosphocellulose squares were washed 4 times with 75 mM phosphoric acid with periodic swirling for 5 minutes. The filters were then washed with 95% ethanol and allowed to dry under a stream of air. The phosphocellulose squares were placed in 10 mL of scintillation cocktail (Bio-Safe NA™, Research Products International Corp , Mt. Prospect, IL, USA) and counted by using a Beckman scintillation counter.

The ability of protein kinase C (α) (Panvera, Madison, WI, USA) to phosphorylate the synthetic peptide, CK-15, was also assayed. Various amounts of CK-15 (10 μg – 48 μg) were incubated at 30°C for 5 minutes in the presence of 0.2 mM [γ-^{32}P]ATP (500 dpm/pmol), 25 mM MOPS-NaOH, pH 7.0, 10 mM magnesium acetate, 0.2 mM $CaCl_2$, 0.2 mM ATP, 20 μg/mL L-α-1,2 dioleoylglycerol, and 100 μg/mL phosphatidylserine. Lipids were prepared by solubilizing L-α-1,2 dioleoylglycerol (1 mg) and phosphatidylserine (5 mg) in chloroform, drying the solution under a stream of nitrogen, resuspending in 25 mM MOPS-NaOH, pH 7.0, 10 mM magnesium acetate and sonicating in a bath sonicator for 3 hours. The assay was initiated by addition of protein kinase C (4 5 ng) and incubation was continued at 30°C for 30 minutes The reaction was terminated by spotting 25 μL of the assay mixture onto a 1 cm square of phosphocellulose paper. The washing was done as described above for the CDPK assay.

OOCYTE LYSATE AND PLASMA MEMBRANE ISOLATION

To test whether expressed nodulin 26 was properly targeted to the plasma membrane, oocyte lysates (Mulders et al., 1998) and plasma membranes (Wall & Patel, 1989) were isolated from oocytes that had been cultured for three days. Lysates were prepared by homogenizing eight oocytes in 160 μL of homogenization buffer A (20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 5 mM NaH_2PO_4, 80 mM sucrose, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 5 :g/mL leupeptin and 5 :g/mL pepstatin A) at 4°C. Homogenates

were centrifuged for 10 minutes at 125 x g to remove the yolk proteins, and the supernatant fractions were removed and stored at -80°C

To isolate plasma membranes, fifty oocytes were gently homogenized with 20 strokes by a plastic pestle in 500 µL of homogenization buffer (250 mM sucrose, 10 mM HEPES, pH 7 4, 1 mM EGTA, 2 mM $MgCl_2$, 1 mM PMSF, 2.5 µg/mL pepstatin A, 1 µg/mL leupeptin) at 4°C in a 1.5 mL eppendorf tube. The homogenization yielded large sheets of plasma membrane complexes (PMC's) as well as adjoining pigment granules and organelles that quickly settled to the bottom of the tube within 5 minutes. The buffer was removed, and the settled PMC's were washed by resuspension in 500 µL of homogenization buffer and then mixed by pipetting up and down. The PMC's were allowed to settle to the bottom of the tube for 15 minutes. The washing was repeated 4 times. The amount of time needed for the PMC's to settle increased with the number of washes. The washing was used to remove the yolk, pigment granules, and contaminating organelles (Wall & Patel, 1989). At the end of the washing regime, PMC's were centrifuged at 100,000 x g for 1 hour at 4°C. The pellet was resuspended in 1% (v/v) Triton X-114. 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, and was shaken on ice for 45 minutes. During this time, the sample was also sonicated on ice using a Sonic Dismembrator Model 301 (Artek Systems Corp., USA) equipped with the microprobe for 1 minute with the output between 0.6 to 0.8, followed by 14 minutes of rest. The mixture was centrifuged for 5 minutes at 10,000 x g. The supernatant fraction, which contains the solubilized plasma membrane proteins, was removed (Wall & Patel, 1989) and was stored at -80°C.

The protein content of the oocyte lysates and plasma membranes was determined using a modification of the Lowry Protein Assay (Lowry et al., 1951) as described by Peterson (1977). Bovine serum albumin was used as a standard.

IMMUNOBLOTTING

Lysates (5 μg protein) and plasma membranes (20 μg protein) were resolved by SDS-PAGE on 12.5% (w/v) acrylamide gels (Laemmli, 1970). Proteins were transferred to polyvinylidene fluoride (PVDF) membranes overnight at 4°C at a constant current of 100 mA for Western blot analysis. The membrane was blocked with 50 mL 10% (w/v) non-fat dry milk in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 9.6 mM NaH_2PO_4, 1.5 mM K_2HPO_4, pH 7 2) at 37°C for 2 hours with two changes. The membrane was washed with 50 mL PBS at room temperature for 10 minutes. The solution was decanted and the wash was repeated twice. The membrane was incubated with affinity purified anti-nod26 IgG at a 1:1000 dilution in PBS, 0.01% (v/v) Tween-20 at 37°C with shaking for 1 hour. The membrane was washed three times at room temperature for 10 minutes each with 50 mL PBS, 0.01% (v/v) Tween-20. The membrane was incubated with horseradish peroxidase coupled goat-anti-rabbit IgG secondary antibody (Southern Biotechnology Associates, Inc., Birmingham, AL) at a 1:10,000 dilution in PBS, 0.01% (v/v) Tween-20. Subsequently, the membranes were washed three times with 50 mL of PBS, 0 01% (v/v) Tween-20 at room temperature (10

minutes per wash) followed by three washes with 50 mL of PBS, 0.01% (v/v) Tween-20, 0.05% (w/v) SDS, 0 01% (v/v) Triton X-100. Chemiluminescence detection was performed by incubating the blot for 2-5 minutes at room temperature in a 1·1 mixture of solution I (0.25 mM luminol, 0 4 mM p-coumaric acid, 100 mM Tris-HCl, pH 8 5) and solution II (0 018% [v/v] H_2O_2, 100 mM Tris-HCl, pH 8.5). The blot was wrapped in plastic and exposed to film (Kodak Biomax light film, Eastman Kodak Co., Rochester, NY, USA) for 5 seconds to 2 minutes

Antibodies against purified nodulin 26 were prepared as described in Zhang & Roberts (1995) For affinity purification, the antibody was adsorbed to soybean nodulin 26. Soybean SM from 24-35 day old nodules were isolated as previously described (Weaver & Roberts, 1992) SM samples (60 μg) were resolved by SDS-PAGE on 15 % (w/v) acrylamide gels (Laemmli, 1970) The proteins were transferred to nitrocellulose membrane for 3 5 hours at 100 mA and the membrane was incubated in PBS overnight at 4°C The 26 kDa region of the blot was excised and the remaining regions were cut into small pieces. The nodulin 26 antibody serum was diluted in PBS (1:5) and incubated with the blot pieces that did not contain the 26 kDa region for 2 hours at room temperature The unabsorbed antibody solution was diluted in PBS (1·2) and incubated with the blot pieces containing the 26 kDa region for 2 hours at room temperature The blot pieces containing the absorbed antibody were then eluted by incubation in 600 μL of 100 mM glycine, pH 2.5 for 10 minutes at room temperature. 0.1 volume of 1 M Tris-HCl (pH 8.0) was added to neutralize the glycine eluant, which contains the affinity, purified antibody solution. The eluant was adjusted to 2 mL with PBS and stored at -80°C in 500 μL aliquots.

CHAPTER III

RESULTS

MEASUREMENTS OF WATER PERMEABILITY IN XENOPUS OOCYTES

While many assays exist for measuring protein-mediated water flux (Agre et al., 1999), one of the most facile methods for measuring and comparing the water permeability of recombinant DNA derived aquaporins is the *Xenopus* oocyte swelling assay pioneered by Preston et al. (1992a). This assay involves the expression of the protein of interest by microinjection of its corresponding cRNA into *Xenopus* oocytes. After a suitable period of culture to allow expression of protein, the water permeability of the oolemma is determined by using video microscopy to measure the rate of oocyte swelling upon hypoosmotic challenge (Rivers et al., 1997; Guenther & Roberts, 2000). A representative assay is shown in **Fig. 4A**. As seen in water-injected controls, the permeability of control *Xenopus* plasma membranes is extremely low However, upon injection of nodulin 26 cRNA the rate of swelling increases. From the rate of swelling ([dV/Vo]/dt), the osmotic water permeability (P_f) was determined (**Fig. 4B**). For this study, the P_f was determined at 17-18°C at pH 7.6 and a free calcium concentration of 0.6

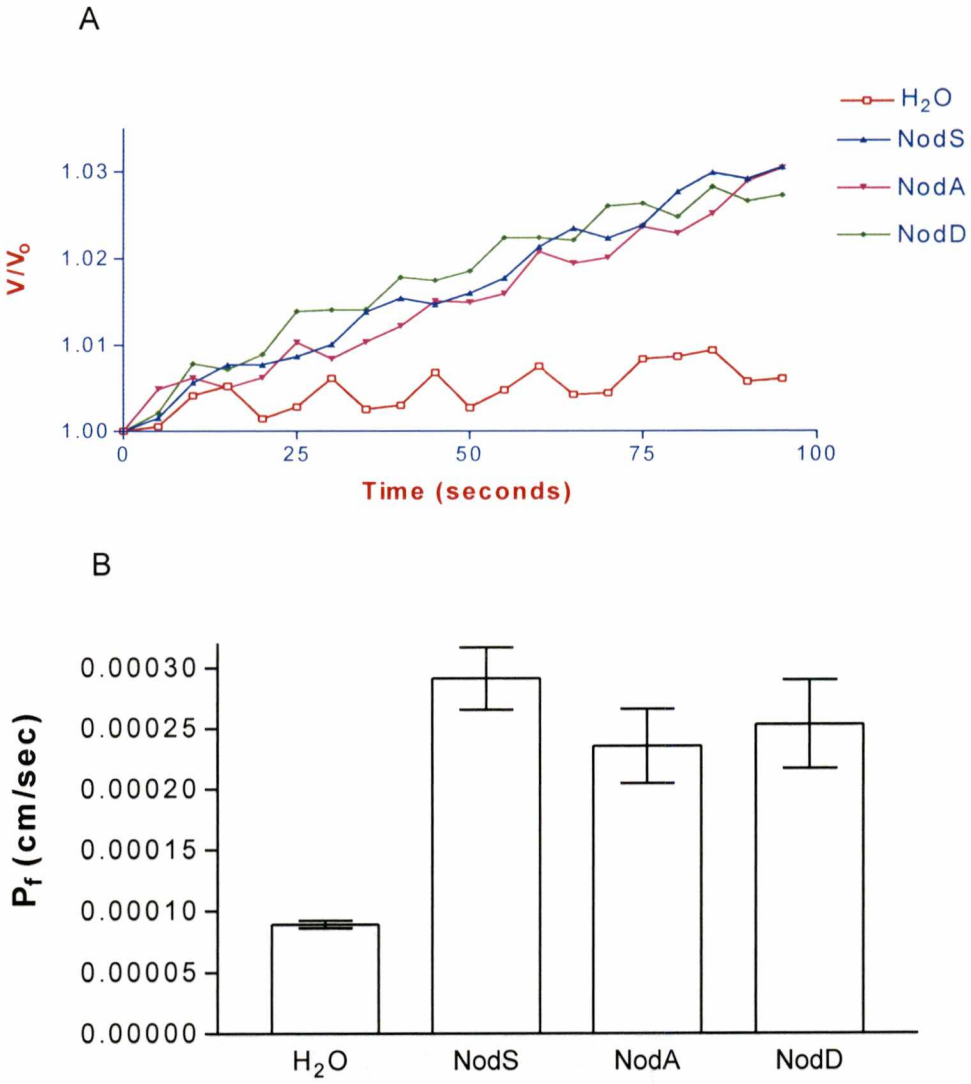


Figure 4. Water permeability measurements of NodS, NodA, and NodD. Ooctyes were injected with 46 nL of 1 μg/μL cRNA or DEPC-treated water (control): H_2O (n=12), **NodS** (n=12), **NodA** (n=15), **NodD** (n=13). NodS describes the wild-type nodulin 26 with a serine residue located at position 262. NodA describes a recombinant nodulin 26 with an alanine residue substituted for a serine residue at position 262. NodD describes recombinant nodulin 26 with an aspartic acid residue substituted for a serine residue at position 262. **A.** The increase of the relative oocyte swelling (V/Vo) of individual oocytes was determined and measured as a function of time by placing the oocytes in hypoosmotic Ringer's solution. The relative volume was assessed at 5 second intervals for a total of 95 seconds. **B.** Osmotic water permeability (P_f) of oocytes. Error bars represent SE.

mM upon dilution from 200 mOsm/kg to 60 mOsm/kg. These are referred to as standard conditions.

MUTATIONS DESIGNED TO ENHANCE WATER PERMEABILITY

Aquaporin 1 (AQP1) and nodulin 26 are two members of the MIP family that reflect the diversity that exists among this structurally homologous protein family. For example AQP1 is a much more efficient water transporter, with a single channel rate for water that is 30 times greater than that of nodulin 26 (Rivers et al., 1997; Dean et. al., 1999). Furthermore, aquaporin 1 is very specific for water transport whereas nodulin 26 transports water and solutes (Zeidel et al., 1992; Zeidel et al., 1994, Rivers et al., 1997; Dean et al., 1999). This difference in the transport rates of these two MIPs is reflected in the relative differences in the P_f of AQP1 and nodulin 26-injected oocytes (**Fig. 5**).

While a crystal structure exists for AQP1 (Mitsuoka et al., 1999; reviewed in Borgnia et al., 1999, Engel et al., 2000; Heymann & Engel, 2000) at 4.5Å (Mitsuoka et al., 1999), it is still difficult to predict what specific residues are important since atomic resolution of the structure has yet to be obtained. However based on information

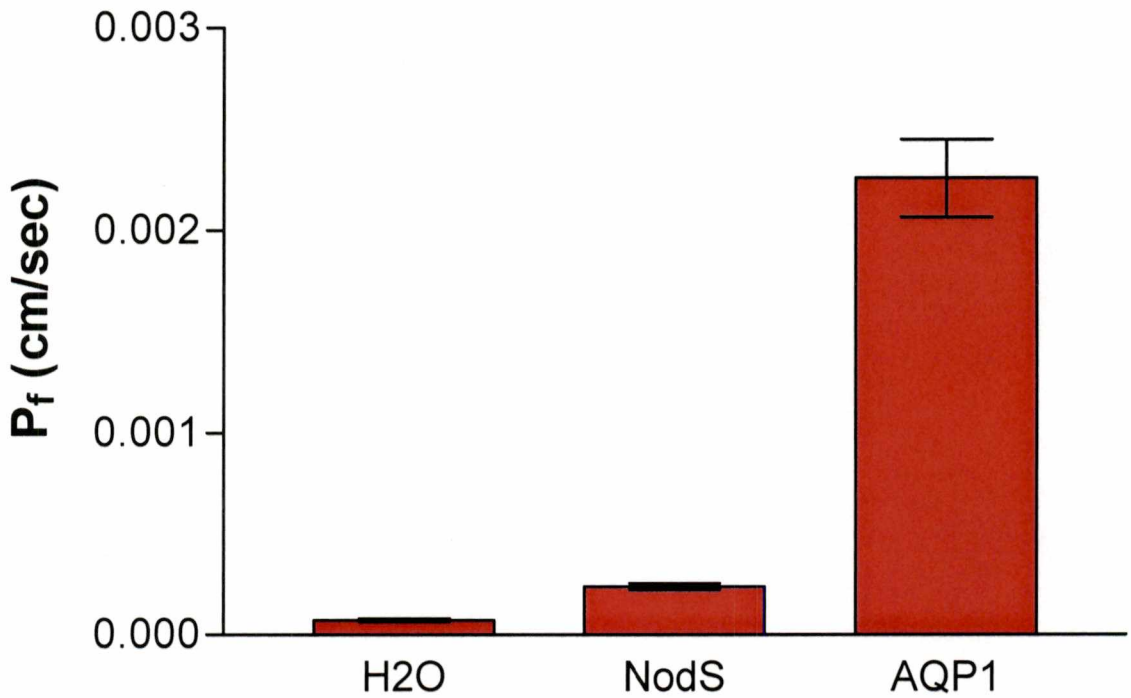


Figure 5. Water permeability for H_2O, NodS, and AQP1. Oocytes were injected with 46 nL of 1 μg/μL cRNA or DEPC-treated water (control): H_2O (n=6), NodS (n=6), and AQP1 (n=5). Error bars represent SE.

gathered from aqueous pores formed by transmembrane α-helices in other membrane channels, combined with information obtained from three-dimensional electron microscopic studies of AQP1, and from sequence conservation analysis of MIP proteins, Heymann et al. (1998) proposed a pathway for the course of the water through the aquaporin 1 protein pore The residues important for establishing a contiguous water flow pathway should be highly conserved in aquaporin proteins. The six transmembrane helices and the two functional loops (B and E, **Fig. 2A**) are observed to be highly conserved throughout the MIP protein family (Heymann et al., 1998) The hourglass model (**Fig. 2B**) and the Heymann model provides us with a starting point for the rational design of mutants to test the role of specific residues in nodulin 26 function that could confer unique properties on the protein.

An examination of the MIP protein database shows that nodulin 26 possesses unique substitutions at otherwise highly conserved residues in MIP proteins, including residues proposed to be in the aqueous pore (Reizer et al., 1993; Park & Saier, 1996; Froger et al., 1998, Heymann et al , 1998) For example, in most aquaporins, the cysteine at position 172 within the fourth helix of nodulin 26 is either a tyrosine or a phenylalanine, the glutamine at position 114 of the third helix is an arginine, the valine at position 197 of the fifth helix is a histidine, and the isoleucine at position 226 of the sixth helix is a histidine (**Fig. 3A**). The glutamine, valine, and isoleucine are residues proposed by Heymann et al. (1998) to be in the aqueous pathway. The cysteine at position 172 is significant because it is only one of two cysteines located in all six helices and it has been shown that nodulin 26 is sensitive to mercury inhibition. To test their functional role,

mutants of nodulin 26 with aquaporin 1-like residues at each position of the mentioned residues were made to determine if selectivity or activity of the channel was altered. Since these substitutions are similar to aquaporin 1, it is proposed that if they lie along the aqueous path, they will confer a higher water permeability.

To test this hypothesis, site-directed mutagenesis of nodulin 26 was done and the water fluxing activity of the mutant proteins was assayed upon microinjection of *Xenopus* oocytes (**Fig. 6**). Nodulin 26 (NodS) showed its usual low transport rate relative to AQP1. However, contrary to the hypothesis, microinjection of the C172F, I226H, Q114R and V197H nodulin 26 mutants did not increase the water permeability of the oolemma relative to nodulin 26. Among the mutants tested, Q114R showed the highest activity, but this was equivalent to nodulin 26-injected oocytes. The water permeability for I226H was lower than that of nodulin 26 oocytes. C172F and V197H (not shown) oocytes did not transport water above the control, water injected, oocytes (**Fig. 6**).

Glycerol transport properties of oocytes injected with C172F, I226H, Q114R, and V197H were also determined by assay of ^{3}H-glycerol uptake (**Fig. 7**). Uptake assays indicate that the glycerol permeability of Q114R oocytes was equivalent to nodulin 26. The glycerol permeability for I226H was lower than that of nodulin 26 oocytes. C172F and V197H oocytes did not transport glycerol above the control oocytes (**Fig. 7**).

All nodulin 26 proteins that gave a positive water and glycerol flux result also showed expression in oocyte lysates and were trafficked to plasma membrane (i.e.,

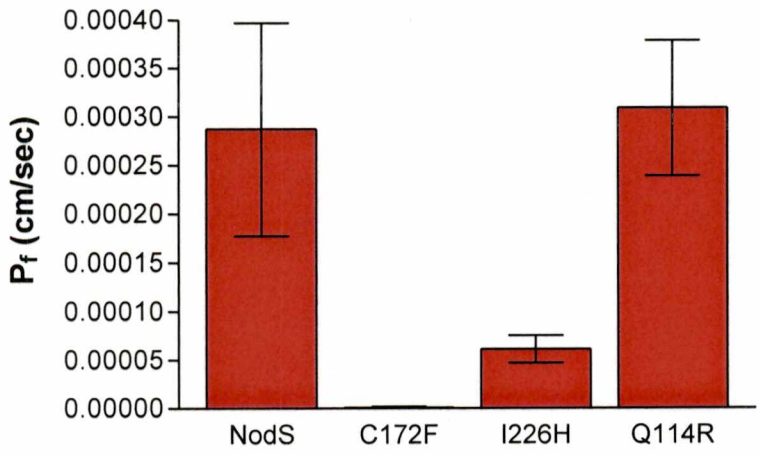


Figure 6. Water permeability of NodS, C172F, I226H, and Q114R. Oocytes were injected with 46 nL of 1μg/μL cRNA or DEPC-treated water (control): **H_2O** (n=7) (not shown), **NodS** (n=7), **C172F** (n=7), **I226H** (n=7), **Q114R** (n=7), and **V197H** (n=7) (not shown). The data for each sample was corrected for H_2O background. Error bars represent SE.

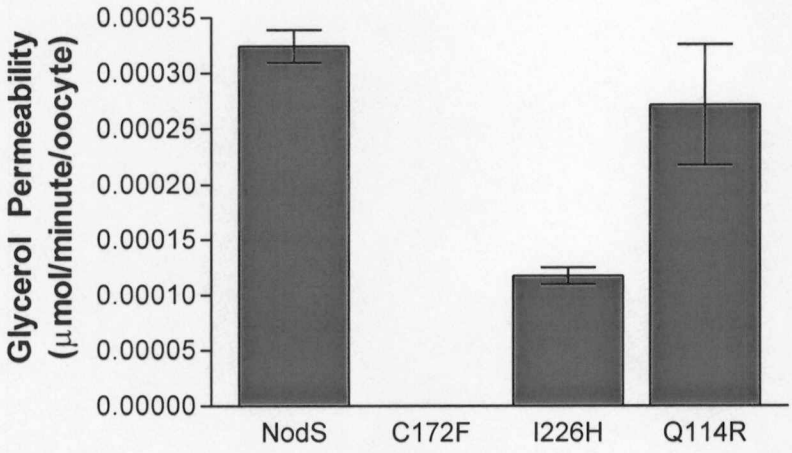


Figure 7. Glycerol permeability of NodS, C172F, I226H, and Q114R. Oocytes were injected with 46 nL of 1μg/μL cRNA or DEPC-treated water (control); **H_2O** (n=6) (not shown), **NodS** (n=6), **C172F** (n=6), **I226H** (n=6), **Q114R** (n=6), and **V197H** (n=6) (not shown). The data for each sample was corrected for H_2O background. Glycerol uptake of oocytes incubated in 150 mM glycerol. Error bars represent SE.

nodulin 26, I226H, and Q114R) (**Fig. 8**). However, the two mutants that gave a negative result showed no apparent expression, either in whole oocyte lysates or in isolated plasma membranes (**Fig. 8**). Thus, these mutations in nodulin 26 apparently result in dysfunctional, and possibly misfolded proteins, that were not expressed or were degraded. In the case of the two remaining mutants, I226H and Q114R, normal expression is observed but the proposed increase in water permeability was not observed, and in the case of I226H, water permeability was actually impaired.

MUTATIONS TO IDENTIFY DETERMINANTS FOR SOLUTE (GLYCEROL) TRANSPORT

Based on comparison of all available amino acid sequences of glycerol and water selective aquaporins, it was proposed that the two functional classes can be distinguished by five "discriminant" residues that are conserved within each of these groups (Froger et al., 1998) (**Table 1**). These residues reside in the third α-helix/loop C region (P_1 residue), loop E (P_2 and P_3 residue), and the sixth transmembrane α-helix (P_4 and P_5) (**Fig. 2A,** Froger et al , 1998). A comparison of the sequence of nodulin 26 shows that it has a hybrid sequence with P_1 and P_5 residues showing the characteristics of glycerol facilitators, and P_2,P_3, and P_4 residues showing characteristics of aquaporins (**Table 1**). Substitutions of a tyrosine and a tryptophan at positions 222 (P_4) and 223 (P_5) by a

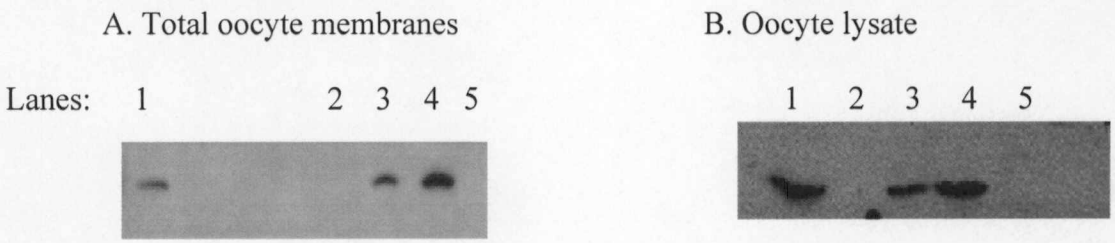


Figure 8. Western blot analysis of NodS, C172F, I226H, Q114R, and V197H. The equivalent of 5 oocytes (total oocyte membranes, **A**) and 0.5 oocytes (oocyte lysate, **B**) injected were loaded into each lane: **lane 1** (nodulin 26 wild type), **lane 2** (C172F), **lane 3** (I226H), **lane 4** (Q114R), and **lane 5** (V197H). Immunodetection was achieved with affinity purified anti-nodulin 26 antibodies (Zhang & Roberts, 1995).

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proline and a leucine, respectively, in the sixth transmembrane of the water specific aquaporin, AQPcic from insects, abolished the water transport property of the protein, but the protein acquired glycerol transport capabilities (Lagree et al , 1999). The Y222P and the W223L mutants of AQPcic results in P_4 and P_5 residues being converted from aquaporin-like signature to a glyceroporin sequence (**Table 2**). The presence of the substituted proline in the sixth transmembrane helix may result in a different conformation of this helix and possibly its interactions with other helices and loop regions. This change in conformation or interaction may result in the change of specificity of the protein for water and/or glycerol.

We investigated these corresponding residues in nodulin 26 to see if they affected the water and glycerol transport properties of nodulin 26. The leucine at position 230 was mutated to a tryptophan (AQP-like) and the tyrosine at position 229 was mutated to a proline (glycerol transporter-like). If these are determinant residues then we propose that the L230W mutation would result in a more specific water transport and that the Y229P mutation would result in a protein that could no longer flux water but would still retain its glycerol transport property.

Upon injection into oocytes, water and glycerol transport assays were done. Both analyses revealed that neither L230W nor Y229P showed water or glycerol transport rates above those of water-injected control oocytes (**Fig. 9 and 10**). Western blot analysis of oocyte lysates from L230W and Y229P-injected oocytes and their plasma membranes shows that both mutant proteins are expressed in oocytes (**Fig. 11B**), but that

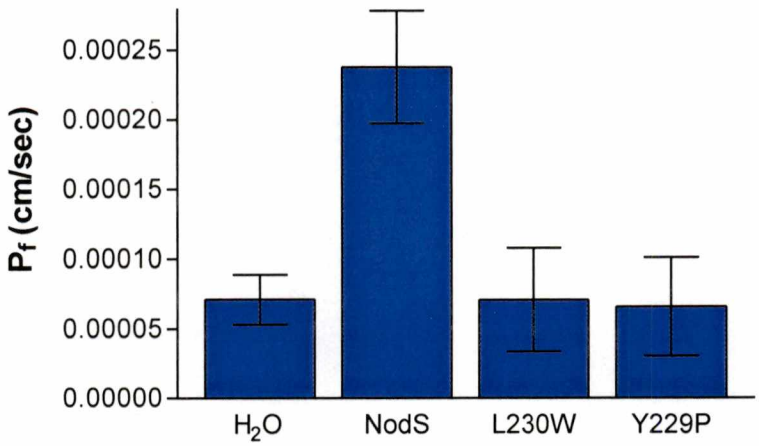


Figure 9. Water permeability of NodS, L230W, and Y229P. Oocytes were injected with 46 nL of 1μg/μL cRNA or DEPC-treated water (control): **H_2O** (n=6), **NodS** (n=6), **L230W** (n=6), and **Y229P** (n=6). Error bars represent SD.

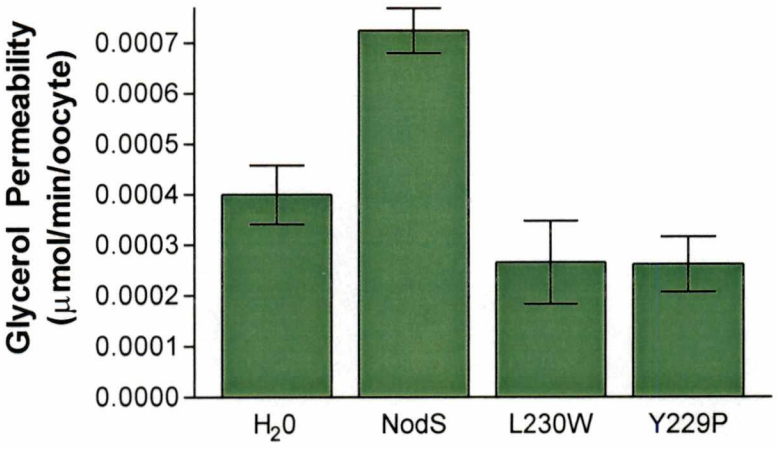


Figure 10. Glycerol permeability of NodS, L230W, and Y229P. Oocytes were injected with 46 nL of 1μg/μL cRNA or DEPC-treated water (control): **H_2O** (n=6), **NodS** (n=6), **L230W** (n=6), and **Y229P** (n=6). Glycerol uptake rates of oocytes incubated in 150 mM glycerol. Error bars represent SD.

A. Immunoblot of plasma membrane

B. Immunoblot of oocyte lysate

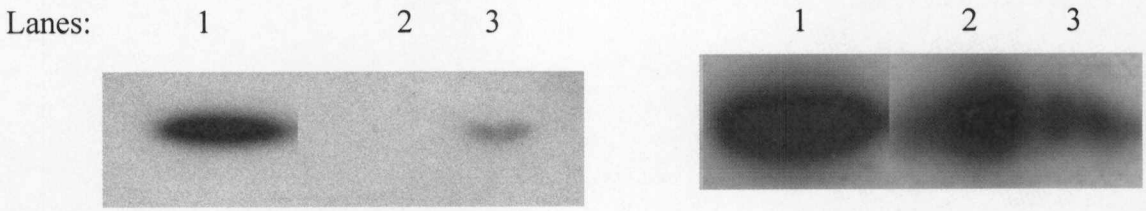


Figure 11. Western blot analysis of NodS, L230W, and Y229P. The equivalent of 5 oocytes (plasma membranes, **A**) and 0.5 oocytes (oocyte lysate, **B**) injected were loaded into each lane: **lane 1** (NodS), **lane 2** (L230W), and **lane 3** (Y229P). Immunodetection was achieved with affinity purified anti-nodulin 26 antibodies (Zhang & Roberts, 1995).

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they show defective targeting to the plasma membrane (**Fig. 11A**). L230W shows no detectable signal in the plasma membrane, and Y229P shows only a slight signal compared to nodulin 26, explaining the low activity of these oocytes. Interestingly, similar mutations have little effect on the targeting of AQPcic to the plasma membrane (Lagree et al , 1999)

Because of these difficulties and unpredictabilities in folding and targeting, further mutagenesis of the nodulin 26 protein was not pursued and attention was focused on studies of the gating and regulation of the wild type protein.

EFFECTS OF pH ON THE PERMEABILITY PROPERTIES OF NODULIN 26

From biophysical measurements it is clear that nodulin 26 shows water transport, however the single channel rate was found to be extremely low compared to AQP1 (Zeidel et al., 1992; Rivers et al., 1997, Dean et al., 1999; Van Hoek & Verkman, 1992) and various other aquaporins (Yang & Verkman, 1997; reviewed in Verkman & Mitra, 2000). Thus, the possibility that nodulin 26 undergoes regulation (e.g. stimulation) by external signals was entertained.

Regulation by pH has been observed in at least three mammalian aquaporins: AQP0, AQP3, AQP6 (reviewed in Engel et al., 2000), suggesting that structural pH sensors may exist in these proteins. In the case of AQP0, another aquaporin with an extremely low single channel rate, it was observed that lowering pH results in a 3-4-fold increase in water permeability in expressed oocytes (Nemeth-Cahalan & Hall, 2000).

These studies have led us to investigate whether the permeability properties of Nod26 were also affected by changes in pH. As shown in **Figure 12**, the relatively low water permeability (P_f) observed at the standard recording pH (7.6) is enhanced by reducing the pH. By background subtracting the water control and standardizing to the P_f at the standard recording pH a relative P_f was determined (Nemeth-Cahalan & Hall, 2000). At pH 6.5 and pH 5.5 the P_f is elevated approximately 2-fold and 3-fold higher respectively (**Fig. 12).** Measuring over a wide range, it is clear that the optimum pH for the osmotic water permeability of nodulin 26 is 5.5 (**Fig. 13**). These data show that, similar to AQP0, pH is capable of gating water flow through nodulin 26.

EFFECTS OF CALCIUM ON THE PERMEABILITY PROPERTIES OF NODULIN 26

Besides pH, a potential role for calcium in regulating MIP function has been proposed by Nemeth-Cahalan & Hall (2000), Girsch & Peracchia (1991), and Louis et al. (1985). The standard concentration of calcium in frog Ringer's solution is 0.6 mM. To address the potential role of calcium in the modulation of nodulin 26 function, the effects of high and low calcium incubations on P_f was assessed (**Fig. 14**). Simply removing calcium from the frog Ringer's solution results in a significant increase in permeability, with a relative P_f that is two-fold higher than that observed under standard

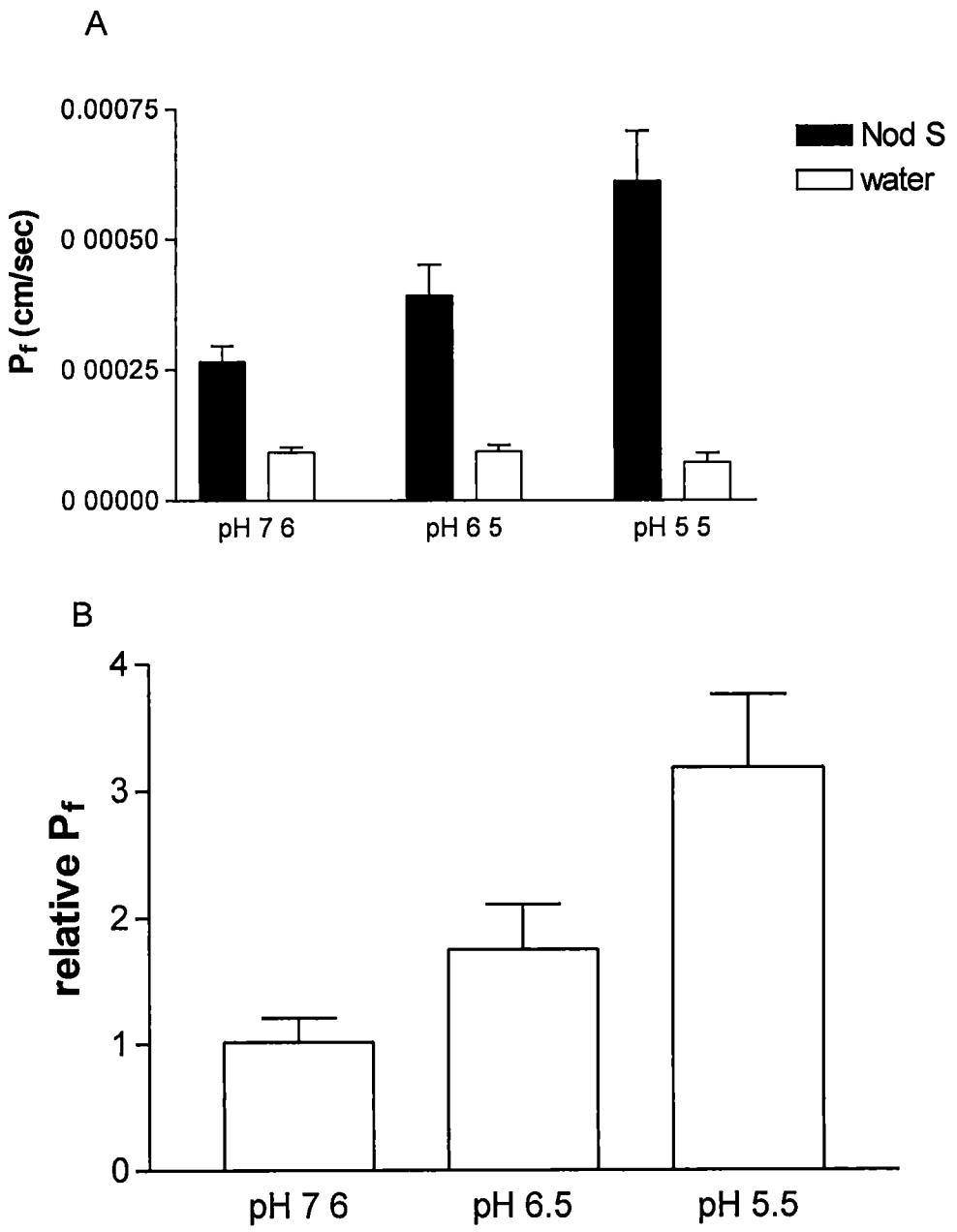


Figure 12. Effect of pH on the water permeability of nodulin 26. Oocytes were injected with 46 nL of 1 μg/μL NodS cRNA or DEPC-treated water (control). Water permeability assays were performed using Ringer's varying in pH: pH 7.6 (standard) (n=6), pH 6.5 (n=6), pH 5.5 (n=6). **A.** Osmotic water permeability of oocytes. Error bars represent SE. **B.** Relative water permeability of oocytes.

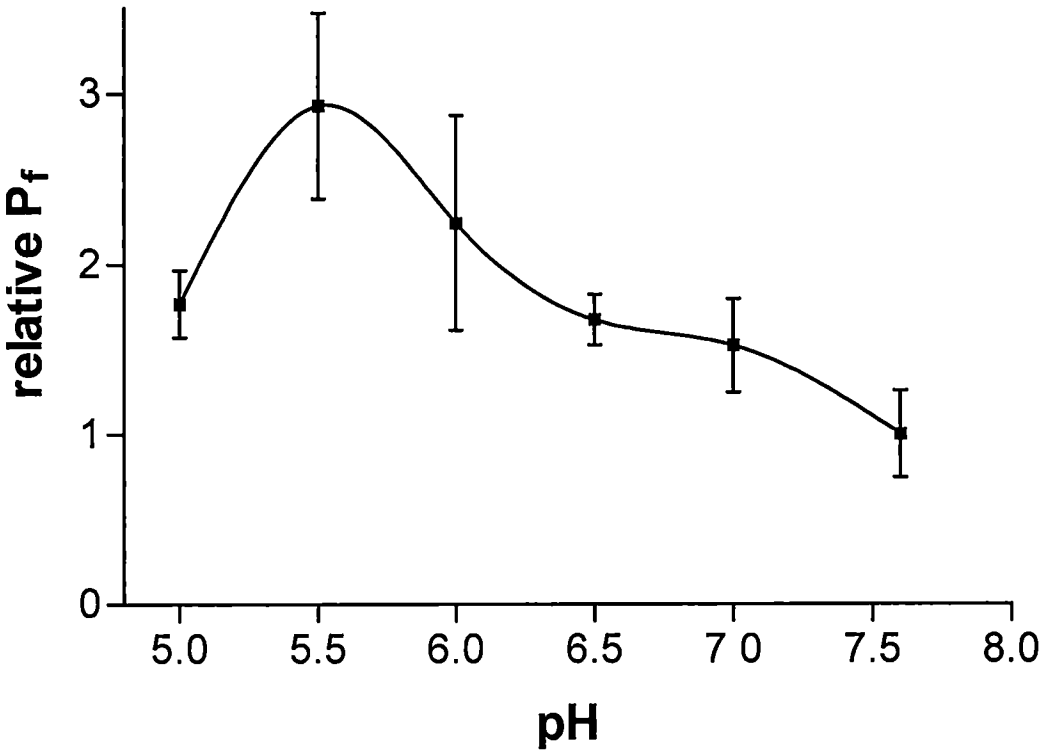


Figure 13. Effect of varying pH (5.0 to 7.6) on the water permeability of nodulin 26. Titration curve of the relative permeabilities of nodulin 26-injected oocytes from pH 5.0 to pH 7.6· pH5.0 (n=6), pH 5.5 (n=6), pH 6.0 (n=5), pH 6.5 (n=6), pH 7.0 (n=6), pH 7.6 (n=6).

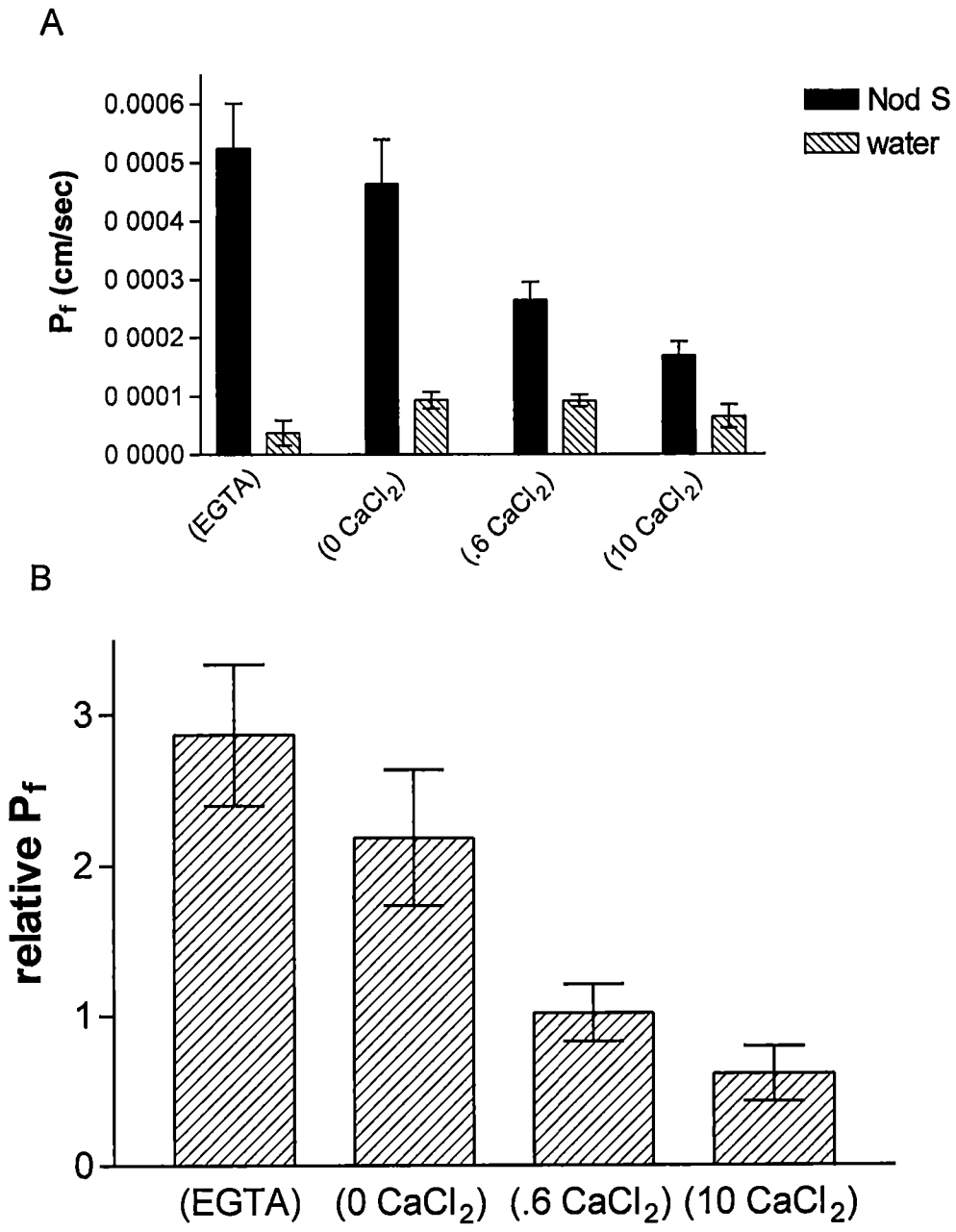


Figure 14. Effect of calcium on the water permeability of nodulin 26. Oocytes were injected with 46 nL of 1 μg/μL NodS cRNA or DEPC-treated water (control). Water permeability assays were performed using Ringer's solution with varying calcium concentration: 1 mM EGTA (n=6), none added (n=6), 0.6 mM (standard) (n=6), and 10 mM (n=7). **A.** Osmotic water permeability of oocytes. Error bars represent SE. **B.** Relative water permeability of oocytes.

conditions. Furthermore, inclusion of the calcium chelator EGTA further elevated P_f to a value that is 3-fold higher than the standard value. Conversely, raising the external calcium to 10 mM exerts the opposite effect, with the relative permeability reduced two-fold (**Fig. 14**).

While finding that modulation of calcium levels outside of the cell suggests that calcium is an inhibitor of water transport through nodulin 26, the site of action of calcium is not clear. Raising or lowering calcium levels outside the oocyte could potentially influence the protein from the external space but also could indirectly affect the internal concentration of calcium within the oocyte. This later possibility makes more sense from a regulatory perspective since calcium signal transduction takes place within the cytosolic compartment. To test whether the site of calcium sensing is internal, we used the calcium chelator BAPTA. As show in **Figure 15**, microinjection of BAPTA results in a four-fold enhanced water permeability suggesting that the clamping of internal calcium to low levels enhances the rate of water flow through nodulin 26. Thus, the effects of elevating calcium in the oocyte bath could effect nodulin 26 by altering the intracellular calcium concentration In this model, a calcium flux into the cell would be a negative regulator of water permeability.

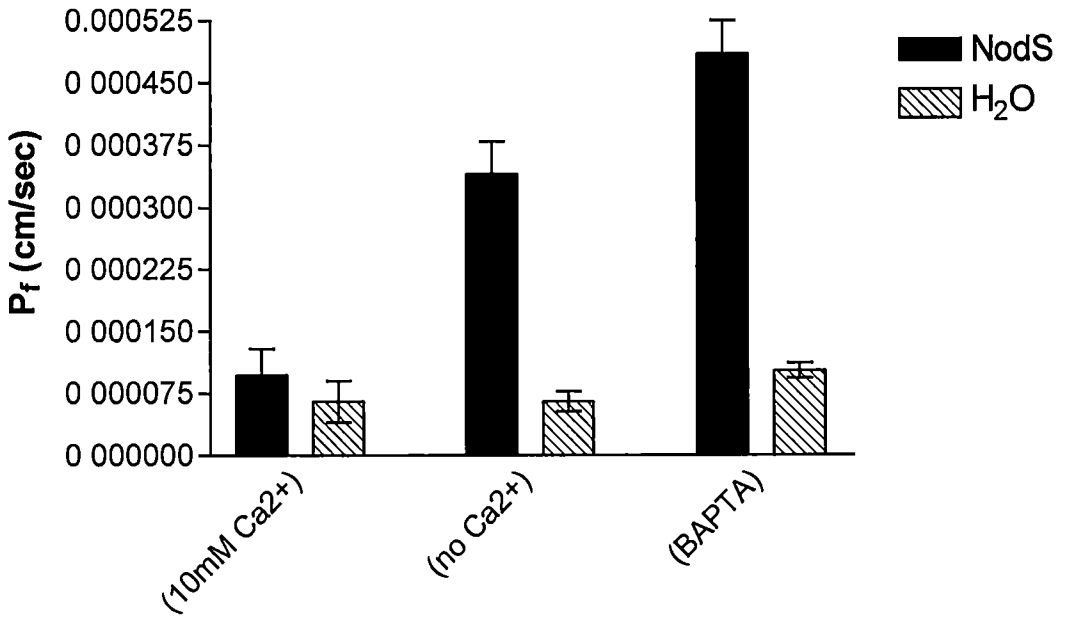


Figure 15. Effect of internal calcium on nodulin 26 water permeability. Osmotic water permeability of *Xenopus* oocytes initially injected with either 46 nL of DEPC-treated water (H_2O) or 1 μg/μL of nodulin 26 cRNA (NodS) and subsequently incubated in Ringer's containing 10 mM calcium (n=6), no calcium (n=6), or were microinjected with 46 nL of 2 mM BAPTA (n=7) and assayed under standard conditions. Permeability analyses were done as described in the Materials and Methods.

EFFECTS OF PHOSPHORYLATION ON THE PERMEABILITY PROPERTIES OF NODULIN 26

As previously discussed, several members of the MIP family, including nodulin 26, have been observed to be phosphorylated. Phosphorylation has varied effects on their activity and targeting (Maurel et al., 1995; Johansson et al., 1998; Han et al., 1998; Marples et al., 1995; Fushimi et al., 1997). The effects of phosphorylation of nodulin 26 in planar lipid bilayers has been investigated previously (Lee et al., 1995), but its role in the regulation of aquaporin activity has not been addressed. We wanted to investigate the effects of phosphorylation on the ability of oocytes expressing nodulin 26 to flux water.

Nodulin 26 mutants were generated that possessed substitutions at the unique site of phosphorylation, serine 262: one that contains a nonphosphorylatable alanine (NodA) and one that possesses a negative charged aspartate (NodD). Analysis of these mutants in Xenopus shows that they are isofunctional with wild type NodS, showing identical swelling rates and P_f values (**Fig. 4**). Further, Western analysis indicates that these mutations at the 262 position of nodulin 26 did not affect the stability of the protein or its targeting to the plasma membrane. The proteins were present in both the oocyte and the plasma membrane in similar quantities (**Fig. 16**), indicating that these mutations did not affect the intrinsic water fluxing property of the protein. Thus, the simple substitution of a neutral residue, or negatively charged residue that might mimic phosphoserine, has no apparent effect on P_f.

A. Immunoblot of plasma membrane

B. Immunoblot of oocyte lysate

Lanes: 1 2 3 1 2 3

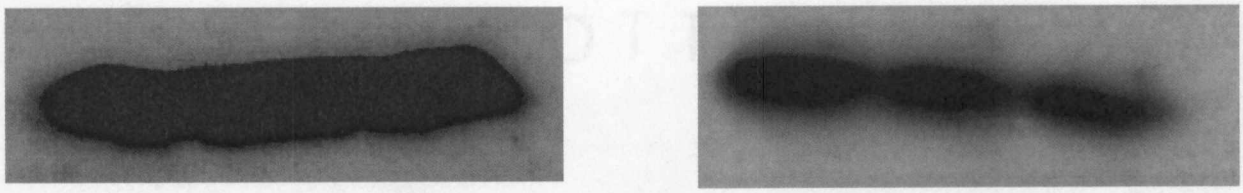


Figure 16. Western blot analysis for NodS, NodA, and NodD. The equivalent of 5 oocytes (total oocyte membranes, **A**) and 0.5 oocytes (oocyte lysate, **B**) were loaded into each lane: **lane 1** (NodS), **lane 2** (NodA), and **lane 3** (NodD). Immunodetection was achieved with affinity purified anti-nodulin 26 antibodies (Zhang & Roberts, 1995).

To address the effects of phosphorylation on nodulin 26, we used a constitutive recombinant form of CDPK (Harper et al., 1994). The strategy involved injection of this CDPK into oocytes two days after initial injection of cRNAs, followed by an additional day of incubation, and then oocyte swelling assay. Three independent experiments revealed that nodulin 26 oocytes injected with CDPK 24- hours prior to the assay showed a reduction in the rate of osmotic driven water flux. A representative result is shown in **figure 17**. As a control, to determine whether this effect is due to phosphorylation of nodulin 26 at serine 262, we used the serine to alanine mutant (NodA) as a control. As shown in **figure 18**, the NodA mutant did not show the same reduction in water permeability that was observed with wild type (NodS), suggesting that the reduction in P_f requires the presence of serine 262 and presumably is due to phosphorylation by CDPK. Glycerol transport properties were also made to determine if phosphorylation by CDPK affects the glycerol permeability of nodulin 26. Nodulin 26 (NodS) oocytes injected with CDPK showed a reduction in glycerol permeability compared to the nodulin 26 oocytes not injected with CDPK. Again, the NodA did not show any significant reduction in glycerol permeability upon CDPK injection (**Fig. 19**).

The initial interpretation of the water and glycerol permeability data led us to propose that the phosphorylation of nodulin 26 could exert a negative effect on its intrinsic transport activities. However, to ensure that the injection of CDPK did not alter the expression or trafficking of the protein, Western blot analysis was done. As shown in **figure 20**, the injection of CDPK into oocytes results in an overall decrease in the level of nodulin 26 in oocytes and virtually no nodulin 26 on isolated plasma membranes. Thus,

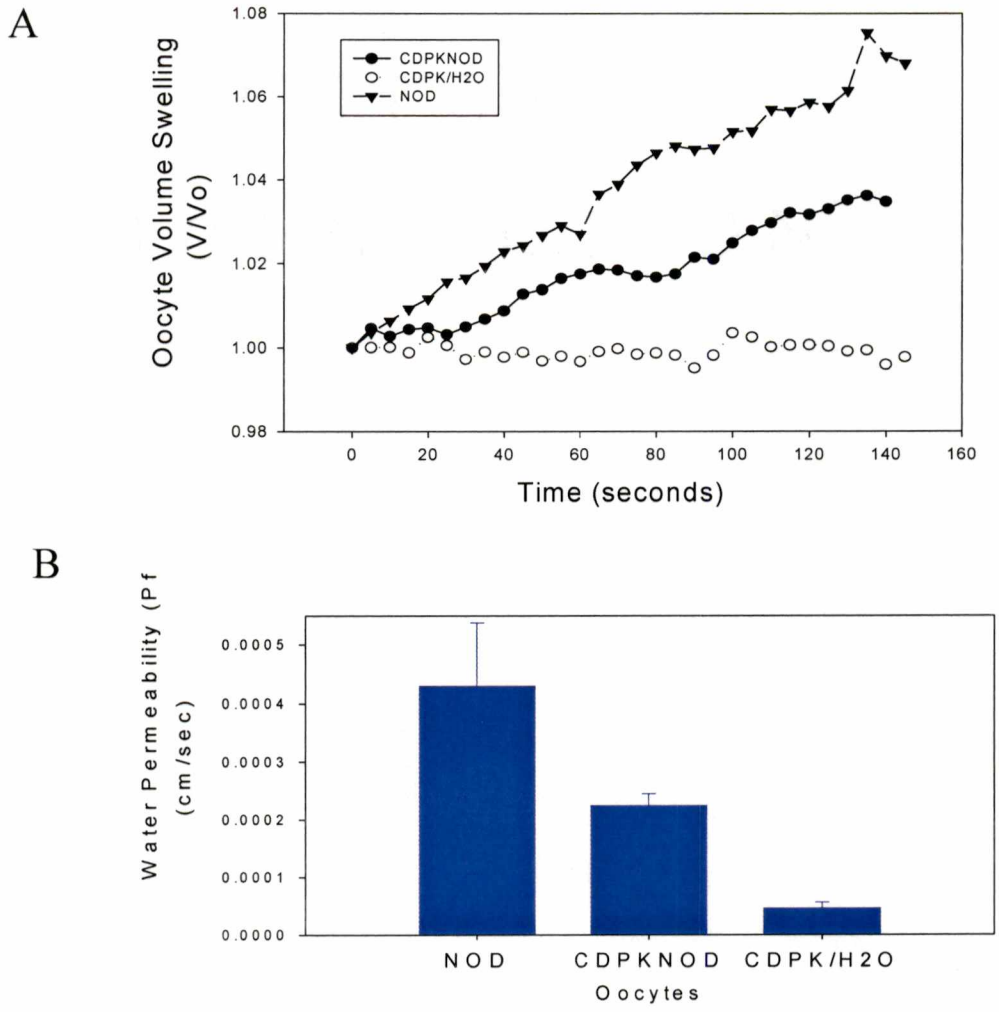


Figure 17. Effect of CDPK microinjection on Nodulin 26 Water Permeability. A. The increase of the relative oocyte swelling (V/Vo) of individual oocytes was measured as a function of time after placing the oocytes in hypoosmotic frog Ringer's solution. Depicted are the relative oocyte volume swelling for single oocytes of three samples: CDPK injected nodulin 26 oocytes (**CDPKNOD**), CDPK injected water control oocytes (**CDPK/H2O**), and nodulin 26-injected oocytes (**NOD**). **B.** Histogram showing the calculated P_f derived from measurement of swelling rates (average of three oocytes with error bars showing SD).

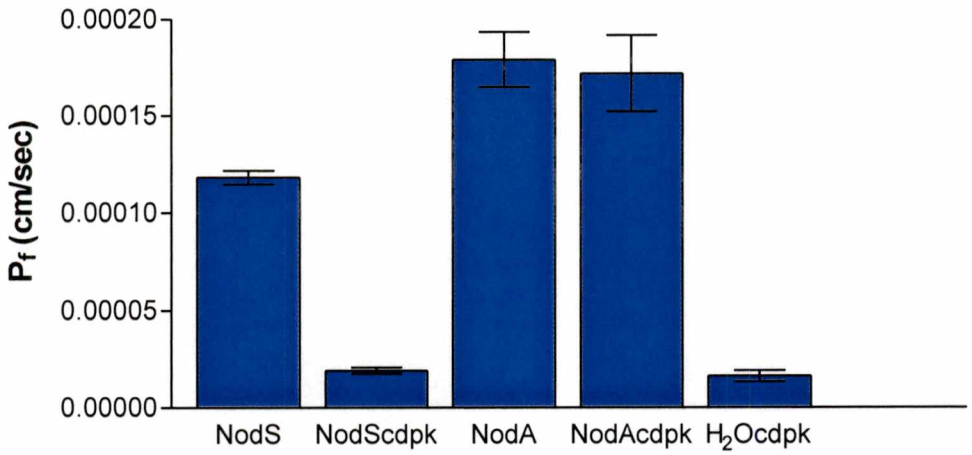


Figure 18. Comparison of the effects of CDPK microinjection on NodS and NodA. Oocytes were injected with 46 nL of 1μg/μL cRNA or DEPC-treated water (control) and CDPK: **H_2O** (n=8); **NodS** (n=8); **NodScdpk** (n=9); **NodA** (n=8); **NodAcdpk** (n=9), and **H_2Ocdpk** (n=7). The data for each sample were corrected by subtracting the rate of water permeability of uninjected control oocytes. Error bars represent SE

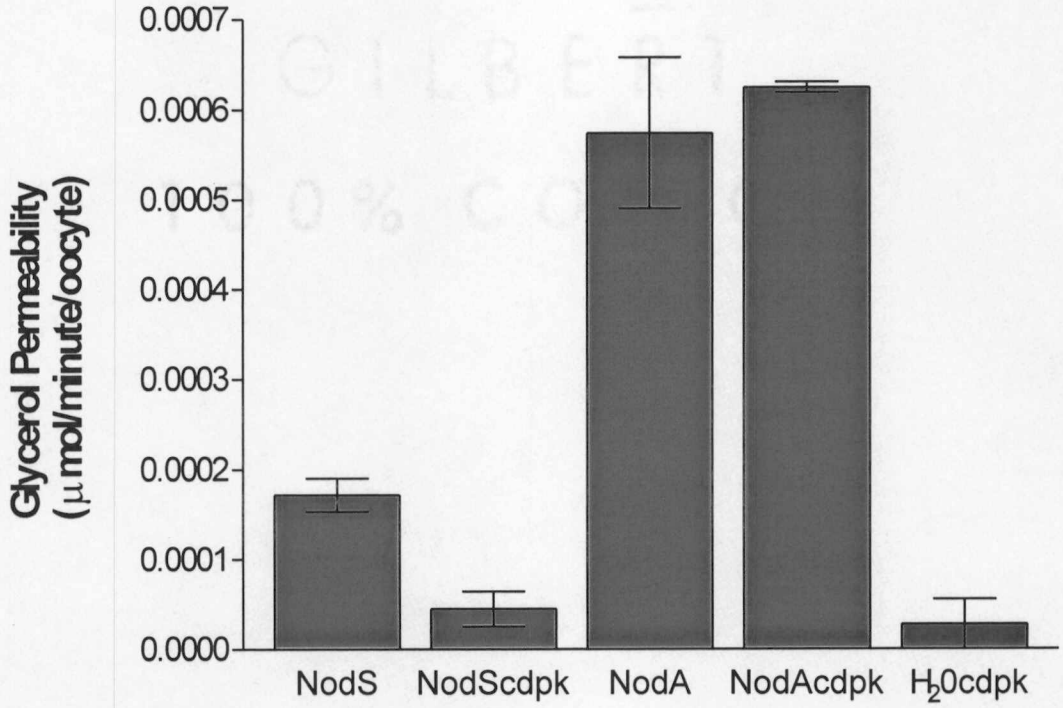


Figure 19. Comparison of the effects of CDPK microinjection on NodS and NodA glycerol permeability. Glycerol uptake of oocytes incubated in 150 mM glycerol concentrations (isoosmotic conditions). Oocytes were injected with 46 nL of 1μg/μL cRNA or DEPC-treated water (control) and 46 nL of CDPK: **NodS** (n=7); **NodScdpk** oocytes (n=7); **H_2Ocdpk** (n=7), **NodA** (n=7); and **NodAcdpk** (n=9). The data for each sample was corrected by subtracting the basal rate glycerol uptake into uninjected control oocytes. Error bars represent SD.

A. Immunoblot of oocyte lysate

B. Immunoblot of Plasma Membrane



Figure 20. Western blot analysis of NodS in CDPK microinjected oocytes. The equivalent of 0.5 oocytes for the (**A**) oocyte lysate and 2 oocytes for the (**B**) plasma membrane was loaded into each lane: **lane 1** (NodS) and **lane 2** (NodS with CDPK). Immunodetection was achieved with affinity purified anti-nodulin 26 antibodies (Zhang & Roberts, 1995)

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the decrease in nodulin 26 observed in CDPK-injected oocytes appears to be the result of decreased stability or targeting of nodulin 26 rather than due to a change in the intrinsic activity of the protein.

To address further the issue of the effects of phosphorylation of nodulin 26 on the activity of the protein, we wished to do the experiments in a shortened time frame to prevent long term effects on the synthesis and trafficking of the protein. In previous studies, the effects of phosphorylation within oocytes have been addressed by adding pharmacological agents that stimulate existing protein kinases within the oocytes (Maurel et al., 1995, Johansson et al., 1998; Han et al., 1998; Fushimi et al., 1997).

Since *Xenopus* does not possess CDPK, which is a plant enzyme, we decided to investigate the use of protein kinase C The reason for this is as follows: 1) The region of phosphorylation recognized by protein kinase C (basic residues surrounding a serine or threonine) (reviewed in Newton, 1997) is similar to the sequence in the carboxyl terminal lobe of nodulin 26, which is phosphorylated by CDPK (**Fig. 21**); 2) AQP0 is readily phosphorylated by protein kinase C on an identically placed serine residue within its carboxyl terminus (Lampe & Johnson, 1989); and 3) oocytes readily respond to protein kinase C agonists such as TPA (Han et al., 1998). To test whether protein kinase C (α isoform) will recognize the phosphorylation motif in nodulin 26, we performed a protein kinase assay with CK-15 which possesses the 14 carboxyl terminal residues in nodulin 26 including serine 262 (Weaver et al., 1991). As shown in **figure 22**, the CK-15 peptide functioned as a suitable substrate for protein kinase C phosphorylation. Histone III (S) is a commonly used substrate in protein kinase C assays to verify the activity of the kinase.

Nodulin 26 sequence: CTKSAS*FLKGRAASK

Protein Kinase C peptide: FKKSFKL-NH_2

Figure 21. Peptide substrates for protein kinase C. Shown is the sequence of the CK-15 peptide which contains the 14 carboxyl-terminal residues found in nodulin 26 (Weaver et al., 1991) including serine 262 (asterisked). Basic residues are underlined. For comparison, a synthetic peptide substrate commonly used for protein kinase C is shown (Chakravarthy et al., 1991).

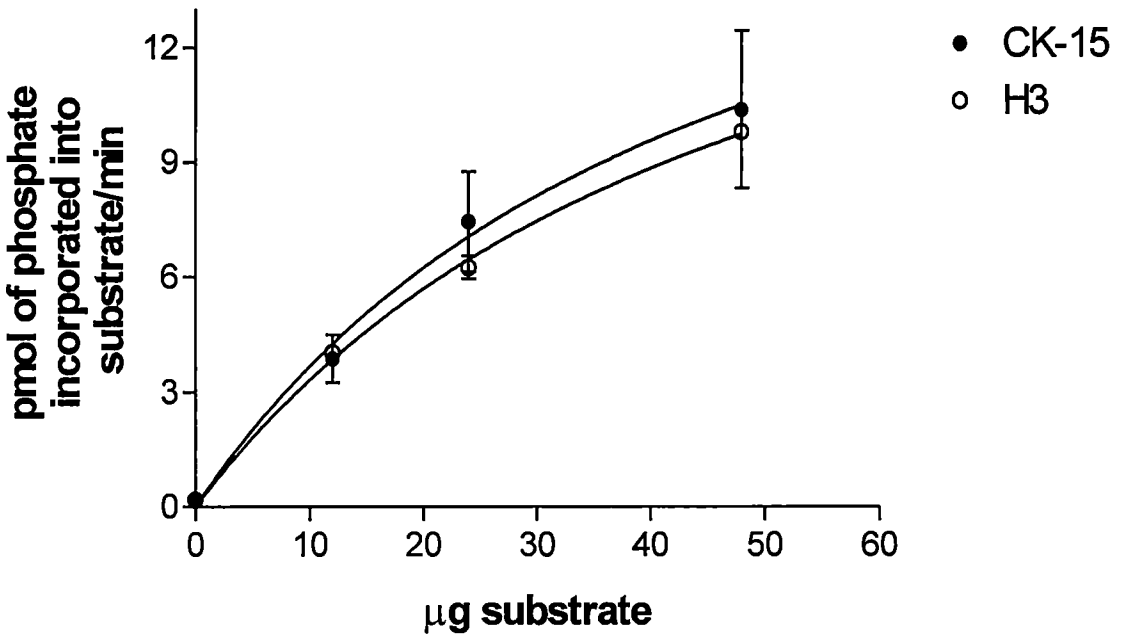


Figure 22. Protein kinase C assay with CK-15 peptide from nodulin 26. Protein kinase assay with protein kinase C (α) and either CK-15 (●) or Histone III (S) (○) as substrates in the presence of 20 μg/mL L-α-1,2 dioleoylglycerol, 100 μg/mL phosphatidylserine.

The similarity in the level of activity obtained using histone III (S) and CK-15 indicates that CK-15 functions as effectively in its role as a substrate for protein kinase C as histone III (S) (**Fig. 22**). CK-15 is phosphorylated by protein kinase C (α) with a K_m (520 μM ± 154 μM) that is comparable to that observed with CDPK (142 μM; Lee et al , 1995), the endogenous protein kinase that phosphorylates nodulin 26 *in vivo* (Weaver et al., 1991).

To test the effects of phosphorylation on nodulin 26 activity, two approaches were taken. First, the protein kinase C agonist TPA was used to stimulate protein kinase C in oocytes, secondly the protein phosphatase 1 and 2A inhibitor okadaic acid was used to enhance the basal phosphorylation state of the protein. As shown in **figure 23**, both reagents stimulated the P_f of water flux through nodulin 26 two-fold. To determine whether the observed effects were due to the phosphorylation of serine 262 on nodulin 26, we used the two nodulin 26 mutants nod A and nod D as controls. Unlike the wild type nod S, neither showed any sensitivity to okadaic acid or to TPA (**Fig. 23**). The data strongly suggest that phosphorylation of nodulin 26 at serine 262 stimulates the rate of water permeability.

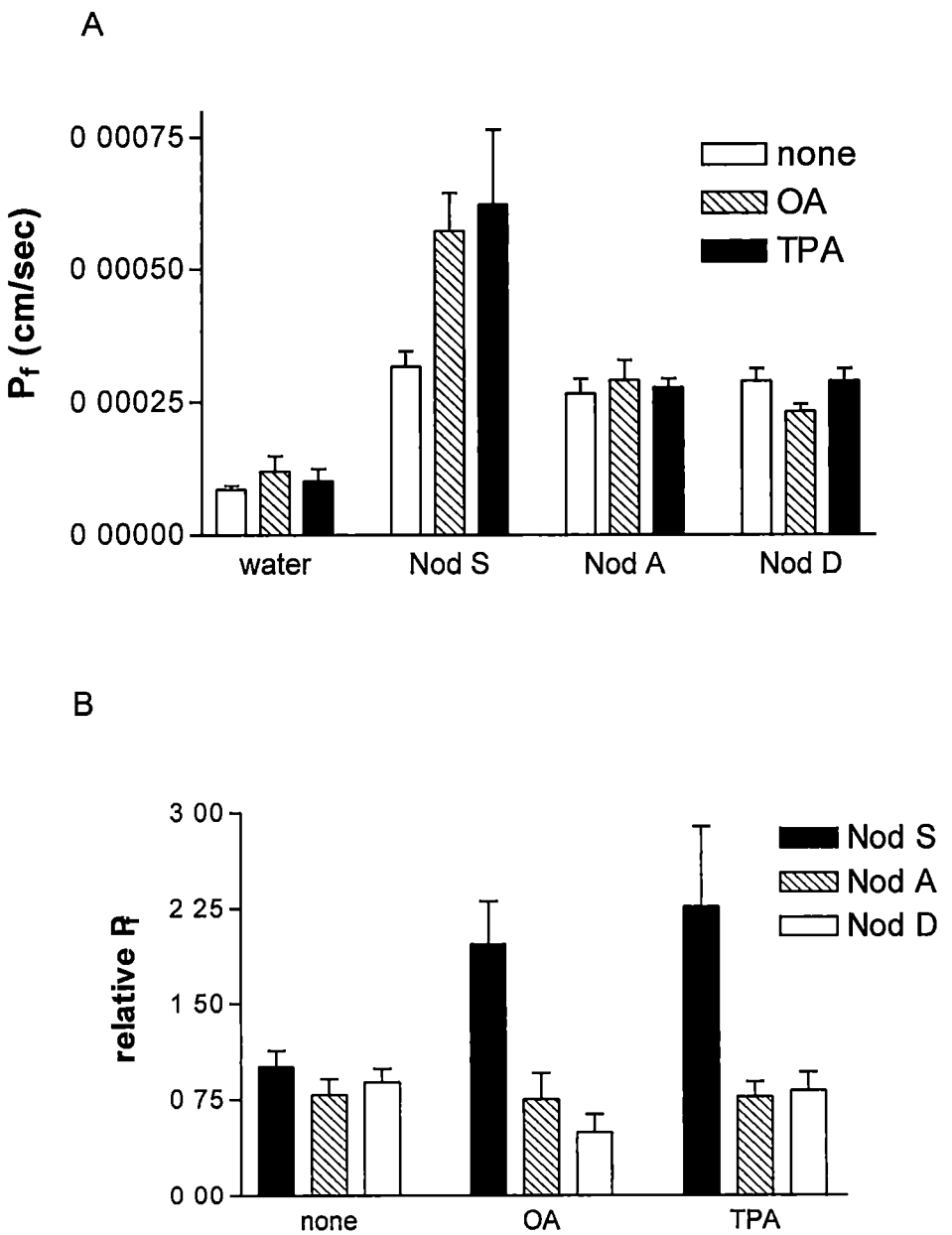


Figure 23. Effects of TPA and okadaic acid on NodS, NodA, and NodD water permeability. Oocytes were injected with 46 nL of 1μg/μL cRNA or DEPC-treated water (control) Water permeability assays were performed in the frog Ringer's solution supplemented with either 5 μM okadaic acid or 10 nM TPA as described in 'Materials and Methods'. **A.** Osmotic water permeability. Error bars represent SE (n=6-8). **B.** Relative water permeability. Error bars represent SE

CHAPTER IV

DISCUSSION

RESIDUES IMPORTANT FOR WATER PERMEABILITY AND SELECTIVITY

The highly conserved structure shared by members of the MIP family suggests that understanding the selectivity of the proteins must be examined in terms of subtle differences in the chemistry of the channel lumen (reviewed in Heymann et al., 1998). The selectivities in the MIP family that have been demonstrated are (1) the distinction between water and glycerol and (2) against ions (reviewed in Heymann et al., 1998). Three explanations that have been presented to explain selectivity include: size-exclusion effects, subtle arrangements of hydrogen-bonding partners in the channel entrances, and the possibility of multiple pathways for water and uncharged solutes (reviewed in Heymann et al., 1998).

Size exclusion may be an important factor for establishing the specificity of aquaporins. Only certain uncharged solutes are able to permeate aquaglyceroporins and glyceroporins . For example, the mammalian aquaglyceroporin AQP3 was demonstrated to permeate glycerol (with van der Waals dimensions of 4.8Å X 5.1Å X7.8Å) but not the smaller molecule urea (with van der Waals dimensions of 3.6Å X 5.2Å X 5.4Å) (Meinild

et al., 1998). Interestingly a similar selectivity was also found for nodulin 26 (Rivers et al., 1997). From these analyses, pore size and van der Waal dimensions of the solutes are not sufficient to explain the specificity of these aquaglyceroporins for one solute (glycerol) while excluding another smaller solute (urea). It was concluded that another factor determines the specificity of an aquaporin, and possibly directs solute-pore interactions. For instance, $-NH_2$ and –OH moieties of the solutes can form hydrogen bonds with oxygens facing the wall of the pore. In addition to size-exclusion, residues near the pore may facilitate the movement of water across the channel pore by establishing a water hydrogen-bonding network as well as interacting with bound water chains through their sidechains (reviewed in Heymann et al., 1998).

The third explanation for the selectivity of aquaporins is that water and uncharged solutes may travel through the channel via different pathways. According to this view, some MIP family proteins (aquaporins) form one type of pore that is selective for water transport, whereas other MIP family proteins (glyceroporins) form a pore that is permeable only to some uncharged solutes. Aquaglyceroporins, such as AQP3 and nodulin 26, may have components of both groups and therefore form multiple permeation pathways Therefore, water could travel through one pathway, glycerol and other uncharged solutes could travel through another pathway. In support of this theory, the water permeability of AQP3 expressed in *Xenopus* oocytes was diminished upon the addition of the sulfhydryl reagent p-chloromercuriphenylsulfonate, whereas the glycerol permeability was unaffected suggesting that water and uncharged solutes do not share the same pathway (Echevarria et al., 1996). Also, Niemietz and Tyerman (2000) have shown

that NH_3 flux, presumably facilitated by nodulin 26, shows a different mercurial sensitivity compared to water flux.

In this study we were interested initially in addressing two unique facets of nodulin 26 structure and function 1 The reason for its low intrinsic water permeability and 2. Its multifunctional aquaglyceroporin activity. To address the structural reason for these properties, and perhaps to shed light on which model for selectivity is relevant, we used site-directed mutagenesis in an attempt to identify residues essential for transport.

We used the structural model for the aqueous pathway through AQP1 (Heymann et al., 1998) as a starting point for a mutagenesis study to investigate residues involved in water permeability. As discussed in the introduction, this model proposes an aqueous pathway through the AQP1 monomer based on: 1) The hourglass model and highest resolution crystal structure, and 2) location of internal water molecules and water binding residues in other membrane protein structures; and 3) positions of highly conserved AQP residues (summary of these proposed residues and their positions are summarized in Table 3).

Four residues were investigated, three of which are conserved in AQP1 and other aquaporins with a high water transport (Reizer et al., 1993; Froger et al., 1998) and the fourth, constituting one of the two conserved cysteines in nodulin 26, a possible site for mercury inhibition of transport. The three residues chosen were: Q114 which is found near the putative cytosolic face of helix 3 (Fig. 3) and which is usually a conserved arginine in other aquaporins; V197 which is located in helix 5 near the putative extracellular face adjacent to the NPA loop E and which is usually a histidine in other

aquaporins; and I226 which is found in the putative helix 6/loop E hinge and which is a highly conserved histidine in most other aquaporins. In the Heymann et al. (1998) model, the sidechains of these residues contribute hydrogen bond contacts with water molecules helping to maintain an aqueous conduit through the aquaporin monomer.

While the substitution of AQP1-like sequences here might be expected to enhance water permeability, and possibly selectivity, this was not the case. Based on comparisons of water and glycerol permeabilities, Q114R is identical to nodulin 26 suggesting that this substitution does not confer a higher water permeability characteristic of aquaporin 1. This result suggests that the glutamine for arginine substitution characteristic of the nodulin 26 subfamily, does not contribute to its distinct transport properties. However, we cannot exclude the possibility that this residue lies along the aqueous pathway proposed in Heymann et al. (1998). Although, arginine is a highly hydrophilic and charged residue, glutamine residues have been observed to form contacts with waters within the pore/interiors of other membrane proteins, such as cytochrome F (Martinez et al., 1996).

The remaining three mutants showed lower water permeabilities than wild-type nodulin 26 and Q114R, although the reasons for this are distinct. The I226H mutant shows an enhanced water and glycerol permeability compared to water-injected control oocytes but a reduced level compared to nodulin 26 and Q114R. Interestingly, Western blot analyses of lysates and plasma membranes from oocytes injected with I226H show normal expression and membrane targeting. Thus, the simple substitution of a

hydrophilic residue that is characteristic of a high transport aquaporin, and which is proposed to lie along the water channel, is not adequate to restore high water transport.

The remaining two substitutions, C172F and V197H, gave oocytes that were indistinguishable from water-injected controls. Western blot analysis showed that neither mutant was successfully targeted to the plasma membrane. This may be due to improper expression, folding, or trafficking of the protein. In the Western analysis of the lysate and plasma membrane of oocytes injected with C172F a lower molecular weight band was observed instead of the normal nodulin 26 band at 28,000 kDa (data not shown), suggesting a potential breakdown product. Apparently these substitutions result in a disruption of nodulin 26 structure, resulting in misfolded proteins being retained in the endoplasmic reticulum. This is discussed further below.

In addition to investigating the low intrinsic water permeability of nodulin 26, we also attempted to address the underlying reasons for its aquaglyceroporin activity. The basis for this study came from the sequence analysis performed by Froger et al. (1998). As described previously, five discriminant residues were proposed for water-selective aquaporins as well as glyceroporins (**Table 1**). Discriminant residues P_2 and P_3 are located in loop E containing the second NPA motif. P_4, P_5 and possibly P_1 are very close to this NPA motif and could interact with P_2 and P_3 to aid in determining the selectivity of the pore (Froger et al., 1998). Recently, Lagree et al. (1999) substituted two discriminant residues (P_4 and P_5) that were glyceroporin-like for aquaporin-like residues in the sixth helix of AQPcic (Y222P and W223L). These substitutions resulted in switching the selectivity of AQPcic from a water-selective aquaporin to a glyceroporin.

This study supports the contention by Froger et al. (1998) that the discriminant residues may determine the selectivity of a channel protein.

In lieu of this study, we wanted to investigate these corresponding residues in nodulin 26 to determine if they also determined its selectivity. An examination of the nodulin 26 sequence shows that it contains a hybrid sequence between the aquaporin-like and glyceroporin-like signature sequences with P_1 and P_5 showing glyceroporin-like properties and P_2-P_4 showing aquaporin-like properties (**Table 1**). We attempted both mutations, the substitution of a proline for a tyrosine at position 229 (glyceroporin-like) and a tryptophan for a leucine at position 230 (aquaporin-like).

Both substitutions gave oocytes that were indistinguishable from water-injected controls with respect to water and glycerol transport properties. Western blot analysis indicates that neither mutant was expressed normally and showed defects in targeting to the plasma membrane.

These results were surprising since the studies of Lagree et al (1999) suggested that these types of substitutions in AQPcic were tolerated and the proteins were properly folded and targeted. These results underscore the fact that mutagenesis studies of aquaporins, especially in *Xenopus*, are unpredictable and one cannot anticipate what mutations will be tolerated and which will lead to internal retention of misfolded or dysfunctional proteins These results are common and have been reported for many aquaporins. For example, while attempting to determine which cysteine residue is important for mercury inhibition of AQPcic using the *Xenopus* expression system, Lagree et al. (1998) demonstrated that a substitution of serine for the cysteine at position 134 in

loop C resulted in the protein not being correctly targeted to the plasma membrane. In a similar experiment, Mulders et al. (1997a) examined whether the cysteine located at position 181 of AQP2 was associated with its mercury-sensitivity by expressing mutant versions of AQP2 (AQP2-C181S and AQP2-C181A) in *Xenopus*. In this study, both mutations resulted in AQP2 proteins that were not correctly targeted to the plasma membrane. Interestingly, a similar mutation in AQP1 was functional and allowed the determination of the mercury-sensitive site (Preston et al., 1993; Zhang et al., 1993). While attempting to determine the possibility that loops B and E are located near the narrowing of the water channel aperture, Jung et al. (1994a) made conservative substitutions of slightly greater mass for residues located within and around the "NPA" motifs of loops B and E These substitutions also resulted in proteins that were unsuccessfully localized to the plasma membrane. Naturally occurring mutations in AQP2 have also been identified in which the mutation affects the folding or targeting of the water channel (Kuwahara, 1998; Mulders et al., 1997b; Deen et al., 1995). These mutations were associated with Nephragenic Diabetes Insipidus (NDI) which is characterized by the inability of the kidney to concentrate urine in response to vasopressin.

In addition to point mutations, domain exchanges between different aquaporins have also been performed and demonstrated to have mixed results (Mulders et al , 1998, Kuwahara et al., 1999). Kuwahara et al. (1999) investigated the structural reasons for the differences in levels of water permeability between AQP0 and AQP2, which are highly homologous (58%) proteins. In their study, several parts of AQP0 were replaced with the

corresponding parts of AQP2 and then functionally studied using *Xenopus* oocytes. Among the many exchanges performed, only certain exchanges of loops D and E and helix 5 resulted in proteins that remained targeted to the plasma membrane (Kuwahara et al., 1999). Mulders et al., (1998) also performed domain exchanges between AQP0, AQP2, and AQP3. Analyses of their expression in *Xenopus* oocytes revealed that six out of the nine chimeric proteins were observed to be non-functional due to misrouting (Mulders et al., 1998).

The difficulties with improper expression, folding or trafficking to the plasma membrane has made it difficult for us to make any conclusions about the influence of these residues in the selectivity or single channel rate of nodulin 26. Because of the unpredictability of this approach, it is not clear how much useful information might be gained by further mutagenesis of the protein One possible alternative is to use other expression systems. For example, expression in yeast systems has been successful for recombinant aquaporin mutants that are not expressed or are mistargeted in *Xenopus* (Lagree et al , 1998; Coury et al., 1998; Laize et al., 1995). These proteins can then be functionally analyzed in reconstituted proteoliposomes (Lagree et al., 1998).

From the limited data that we have obtained, the simple single substitutions of residues within the putative aqueous pore do not significantly affect the transport rate of nodulin 26. Thus, the structural basis of the differences of the single channel rate between nodulin 26 and AQP1 may be more complex involving the contribution of multiple residues along the channel pore. Further, as discussed below, nodulin 26 also differs from the AQP1 prototype in that its activity can be up and down regulated by

multiple factors. Thus, the protein may also undergo a conformational transition between more active and less active states. To obtain more detailed structural information about nodulin 26, which may aid in providing insight into the functional differences from AQP1, electron diffraction crystallography is currently being pursued.

REGULATION OF NODULIN 26 BY pH

In the present study three separate factors were found to modulate nodulin 26 activity: pH, calcium, and phosphorylation. Reduction of the pH of the external bathing medium raised the P_f of the oolemma of the *Xenopus* oocytes expressing nodulin 26, with the highest activity observed at pH 5.5. These observations suggest that the differences in pH found in the symbiosome space may control the rate of water flux through nodulin 26 on the SM. The topology of nodulin 26 (Miao et al., 1992) suggests that the protein is inserted into the membrane with the hydrophilic amino and carboxyl terminal domains exposed to the cytosol, similar to other aquaporins and MIPs (reviewed in Agre et al., 1998; Engel et al., 2000). Thus, in *Xenopus* the portion of the protein that is exposed to the extracellular space is likely to be loops A, C, and E (**Fig. 3**) and this is likely to be the site of action for the pH effect *In vivo,* this region of the protein would be facing the symbiosome space (**Fig. 3**). Previous work shows that the SM possesses an electrogenic, H^+-pumping ATPase (Blumwald et al., 1985; Bassarab et al., 1986; Udvardi & Day, 1989; Udvardi et al., 1991; reviewed in Udvardi & Day, 1997) which transports H^+ into

the lumen of the symbiosome generating a pH and electrical gradient. This gradient drives the uptake of anions and dicarboxylates (Udvardi et al., 1991; Ou Yang et al., 1990; Day et al., 1995; Tyerman et al., 1995; Corzo et al., 1997; reviewed in Udvardi & Day, 1997). Based on our work, modulation of H^+-ATPase could also serve to regulate nodulin 26 activities by acidification of the symbiosome space. Furthermore, the uptake and respiration of the dicarboxylic acids and the excretion of ammonia during times of peak metabolism could also serve to further decrease the pH of the symbiosome space (reviewed in Udvardi & Day, 1997). One interesting possibility is that the establishment of the pH gradient could signal increased metabolic flux and a need for increased water flow through nodulin 26 that serves an osmoregulatory function. Understanding the factors that control the proton pump *in vivo* could shed more light on the role of pH in controlling nodulin 26.

Nodulin 26 represents the fourth aquaporin that has been reported to be modulated by pH with AQP0 (Nemeth-Cahalan & Hall, 2000), AQP6 (Yasui et al., 1999), and AQP3 (Zeuthen & Klaerke, 1999) representing the other three. Interestingly, other aquaporins (e.g AQP1, AQP2, AQP4, and AQP5, Zeuthen & Klaerke, 1999) showed little or no sensitivity to pH, further underscoring the concept that while the MIP/aquaporin family has a common structural framework, details of their regulation are often unique. This is further shown by the fact that the four pH-sensitive aquaporins show considerable differences in the manner in which they respond to pH. Similar to nodulin 26, AQP0 (Mulders et al., 1995; Yang & Verkman, 1997) and AQP6 (Ma et al., 1993; Yasui et al., 1999) transports water at low intrinsic rate at neutral pH. However, a

reduction in pH was observed to increase the permeation of water through AQP0 (Nemeth-Cahalan, 2000) and AQP6 (Yasui et al., 1999). However in each case the effect was different.

In the case of AQP0 (Nemeth-Cahalan & Hall, 2000), a pH dependent rise in permeability was observed with an optimum (3-fold elevation) observed at pH 6.5, suggesting the involvement of a histidine residue. This hypothesis was supported by several observations. First, pretreatment with the histidine-selective reagent, diethylpyrocarbonate, increased the P_f 4-fold and abolished pH sensitivity (Nemeth-Calahan & Hall, 2000). Site-directed mutagenesis further identified histidine 40 in loop A as the residue involved in the sensitivity of the wild-type AQP0 to pH (Nemeth-Cahalan, 2000). The sensitivity of AQP0 to pH permits the protein to switch between high and low permeability states, and it was proposed as a mechanism for modulating the permeability state of the lens. This may help to increase the circulation of water when metabolic activity is high (Nemeth-Cahalan & Hall, 2000). Also, as discussed below, similar to nodulin 26, it appears as if calcium may also be involved in gating the AQP0 channel (Nemeth-Cahalan, 2000).

The AQP6 case is unique among aquaporins. Upon expression in *Xenopus*, AQP6 exhibits low water permeability, but upon treatment with known water channel inhibitors (Hg^{2+}) its water permeability increases by 10-fold and is accompanied by ion conductance. Mutational analysis indicate the involvement of two cysteine residues in the mercury activation of AQP6: C155 and C190. In this same study, AQP6 was demonstrated to colocalize with H^+-ATPase in intracellular vesicles of acid-secreting α-

intercalated cells of the renal collecting duct suggesting that low pH may naturally activate AQP6 A reduction in pH was observed to increase the water permeability of AQP6-injected oocytes by over 2-fold and to enhance ion conductance (Yasui et al., 1999). Analysis of the current demonstrated that it has a greater selectivity for anions than cations (Yasui et al., 1999). Combining information from the hourglass model for aquaporins (Jung et al., 1994b) with the observation that selectivity of ion channels are often determined by specific charged residues (Fahlke et al., 1997; Friedrich et al., 1999; Blachly-Dyson et al., 1990), lysine at position 72 of loop B at the cytoplasmic mouth of the AQP6 pore was proposed to be the residue important in establishing the channel's selectivity. Substitution of a glutamic acid for this residue resulted in a change in the anion/cation selectivity of AQP6 at pH 4.0. The observations that AQP6 exhibits anion selectivity and its localization to intracellular vesicles of acid-secreting cells of the renal collecting duct may provide a clue to the role of AQP6 in biological processes. Since the intracellular organelles are acidified by H^+-ATPase and the vesicles have a pH of 5.0 or lower, (Schwartz & Al-Awqati, 1986), this suggests that anion conductance may be needed to maintain electroneutrality The intracellular chloride channel ClC-5, which colocalizes with H^+-ATPase in the kidney (Gunther et al., 1998) is inhibited at pH less than 6 5 (Friedrich et al , 1999) Therefore, since AQP6 conductance is activated at low pH it may function at a later stage in acidification than ClC-5 The activation of the water permeability of AQP6 may contribute to vesicle swelling and membrane fusion during exocytosis or other cellular processes (Jena et al., 1997).

Unlike AQP0, AQP6 and nodulin 26, the influence of pH on AQP3 is negative. At neutral pH, AQP3 shows a high single channel rate for water flux (Yang & Verkman, 1997) as well as the ability to transport various uncharged solutes (Ishibashi et al., 1994; Echevarria et al , 1996; Ishibashi et al., 1997b). However, Zeuthen and Klaerke (1999) showed that the rate of both water (pK_a = 6.4) and glycerol transport (pK_a = 6.1) through AQP3 is inhibited by pH. The dependence of the omsotic water permeability of AQP3 exhibited a Hill coefficient of 3, suggesting that at least three cooperating titratable sites determine the osmotic water permeability. According to this model, these titratable sites are located on the aqueous pathway and determine the energy barriers for water permeability. Titration of these sites would abolish their capacity for forming hydrogen bonds and render them hydrophobic. One model for the regulation of the water permeability of AQP3 by pH suggests that under anaerobic conditions, AQP3 is localized in membranes to prevent excessive cellular swelling by reducing the permeability of water. Furthermore, unlike water permeability, the glycerol permeability of AQP3 did not begin to decrease until pH 6.25, showing a steeper decrease with a pK_a of 6 1 and a Hill coefficient of 6 Zeuthen and Klaerke (1999) proposed that glycerol also permeates the channel by forming six successive hydrogen bonds (Zeuthen & Klaerke, 1999. The lower pK for glycerol suggests that the cells endeavor to maintain their ability to take up glycerol under acidosis (Zeuthen & Klaerke, 1999), and also supports a model in which the water and glycerol permeation pathways are distinct.

The site of the pH effect for nodulin 26 remains undetermined. Unlike AQP0, there is no histidine at a comparable position in loop A. However, there are multiple ionizable

groups on the extracellular side of the molecule including a glutamate residue at a comparable location (loop B) as histidine 40 in AQP0, as well as other groups in loops C and E (**Fig. 2**). Future work with substitutions at these positions, similar to the approach of Nemeth-Cahalan and Hall (2000) might help identify which residues are important in pH gating. Additionally, it has been observed that nodulin 26 exhibits an ion conductance under certain conditions in planar lipid bilayers (Weaver et al., 1994; Lee et al., 1995). Although experiments in *Xenopus* failed to demonstrate an ion conductance associated with nodulin 26, it may be interesting to test whether pH affects ion conductance in light of the data obtained with AQP6 (Yasui et al., 1999).

REGULATION OF NODULIN 26 BY Ca^{2+}

Besides pH, it is clear that nodulin 26 is also sensitive to calcium. However, unlike pH, calcium ion appears to be a negative regulator of nodulin 26 in oocytes. By using the calcium buffer, BAPTA, we showed that the site of calcium action appears to be intracellular. Thus, the inhibition of water transport that is observed upon raising extracellular calcium is likely due to an indirect effect on intracellular calcium homeostasis. This effect is similar to that observed with AQP0 (Nemeth-Cahalan & Hall, 2000). They observed that reduction of external calcium concentration enhances the water permeability of AQP0 expressed in oocytes by almost 4-fold and similar to our findings, they found that the effect of calcium appears to be cytosolic/intracellular.

Several questions remain regarding the regulation of nodulin 26 by calcium. First, what is the calcium sensor for calcium regulation of the activity? Secondly, is there coordinated regulation of nodulin 26 by calcium, phosphorylation and pH? The latter point is particularly complex since the phosphorylation of nodulin 26 is also regulated by calcium, but in this case the activity is up-regulated instead of inhibited (see discussion below). Studies with AQP0 show that simultaneous reduction of both external pH and calcium affects AQP0 water permeability more than reducing pH or calcium alome, although the effects are not additive (Nemeth-Cahalan & Hall, 2000). Futhermore, the histidine 40 mutants that lack pH sensitivity are also insensitive to calcium, suggesting that calcium regulation requires the critical histidine 40 residue (Nemeth-Cahalan & Hall, 2000).

The calcium sensor, or site of calcium action of inhibition of nodulin 26 remains unknown. Based on previous studies with AQP0, the mechanism of calcium regulation may be through the activation of calmodulin. Calmodulin, a 17 kDa member of the EF hand family, serves as a ubiquitous calcium sensor in eukaryotes which is activated by cytosolic calcium fluxes (reviewed in van Eldik & Watterson, 1998; Nelson & Chazin, 1998; Klee & Vanaman, 1982). Nemeth-Cahalan & Hall (2000) showed that inhibition of AQP0 with calcium is reversed by trifluoperazine, calmidazolium, and N-(6aminohexyl)-5chloro-1-naphthalenesulfonamide (W-7). These compounds are well known antagonists of calcium-calmodulin (reviewed in Nelson & Chazin, 1998), and it was proposed that the calcium sensor leading to inhibition of AQP0 is calmodulin. How does calmodulin interact with AQP0? Early work with AQP0 showed that calcium-

calmodulin interacts with the carboxyl terminal, cytosolic domain of AQP0 (Girsh & Peracchia, 1991; Louis et al., 1990) One possible scenario is that the calcium activation of calmodulin leads to an interaction with the carboxyl terminus of AQP0, which may then block the passage of water and/or solutes across the channel pore. It is not yet clear whether nodulin 26 interacts with and is regulated by calmodulin. Previous work shows that calmodulin prefers sequences that form basic amphipathic α-helices (reviewed in Clore et al., 1993; Crivici & Ikura, 1995; Rhoads & Friedberg, 1997). An examination of the carboxyl terminal domain of nodulin 26 (**Fig. 3**) shows that it is basic and amphipathic, similar to AQP0, and thus may be able to interact with calmodulin. This model requires further investigation.

PHOSPHORYLATION OF NODULIN 26

As discussed previously, several members of the MIP family undergo phosphorylation resulting in changes in activity and/or membrane targeting (Maurel et al., 1995; Johansson et al., 1998; Han et al., 1998; Marples et al , 1995; Fushimi et al., 1997, Johnson & Chrispeels, 1992, Lampe & Johnson, 1989) Previous studies demonstrated that nodulin 26 is phosphorylated at serine 262 of the carboxyl terminus by a CDPK that is colocalized to the SM (Weaver et al., 1991; Weaver et al., 1992). In an attempt to address the effects of CDPK phosphorylation, purified recombinant KJM-CDPK was coinjected into nodulin 26-expressing oocytes 24-hours prior to the assay.

Both water and glycerol transport properties of nodulin 26 decreased upon injection of CDPK However, Western blot analysis indicates that CDPK injection affects the stability or targeting of nodulin 26 instead of its intrinsic transport properties. Interestingly, this effect was not observed for nodulin 26 S262A, suggesting that a serine at position 262 is necessary for this effect and that phosphorylation by CDPK is necessary. One possibility is that phosphorylation of nodulin 26 is accompanied by a change in structure and function that alters its stability and/or trafficking. Previous work with AQP2 shows that phosphorylation can trigger a change in membrane trafficking (Fushimi et al., 1997). Whether this phenomenon for nodulin 26 is observed *in vivo* in nodules merits further study. One possible scenario is that a long-term outcome of nodulin 26 phosphorylation may be down regulation of its activity by increased degradation or altered membrane localization

Because of the difficulties observed with CDPK injection studies, we adopted a different tact for analysis of the effects of nodulin 26 phosphorylation. An examination of the nodulin 26 sequence reveals that serine 262 is found within a consensus site for CDPK phosphorylation including hydrophobic-X-basic-X-X-serine (X = any amino acid) (Roberts & Harmon, 1992). Animal protein kinase C on the other hand is less strict and recognizes a serine flanked by basic residues (reviewed in Newton, 1997). By using the CK-15 peptide we have shown (**Fig. 22**) that protein kinase C (α) is capable of recognizing and readily phosphorylating this region of nodulin 26, similar to its effects on AQP0 (Lampe et al., 1986; Lampe et al., 1989) Since protein kinase C is already present in *Xenopus* oocytes, we used a selective agonist of this activity (the phorbol ester, TPA)

as well as okadaic acid (a phosphatase inhibitor) to address the short term effects of phosphorylation of nodulin 26. Unlike CDPK co-injection, short term treatment with these reagents (on a time scale that should not affect the levels of nodulin 26 protein on the plasma membrane) enhanced the rate of water flux through wild-type nodulin 26, but not through mutants with substitutions at position 262. This finding suggests that phosphorylation by CDPK enhances the rate of water flow through nodulin 26, similar to its proposed effects on α-TIP (Maurel et al., 1995) and PM28A (Johansson et al., 1998).

SUMMARY OF FACTORS MODULATING NODULIN 26 ACTIVITY

In **figure 24**, we present a model for the current understanding of nodulin 26 regulation. According to this model, calcium may be involved in a dual regulation of nodulin 26. First, a calcium flux would result in the activation of a SM-associated CDPK, which would phosphorylate and up regulate nodulin 26 activity However, in addition, calcium could exert a second effect, possibly through calmodulin, in which interaction with nodulin 26 results in a decreased rate of water flux. These apparently contradictory effects could serve to attenuate the response Such opposing influences in regulation have been observed in other systems For example, calmodulin stimulates contraction by smooth muscle MLCK activation, but a second calmodulin target, CaM kinase II, attenuates this response by phosphorylating MLCK and blocking its interaction with calmodulin (Allen & Walsh, 1994). By analogy, a localized calcium flux would be

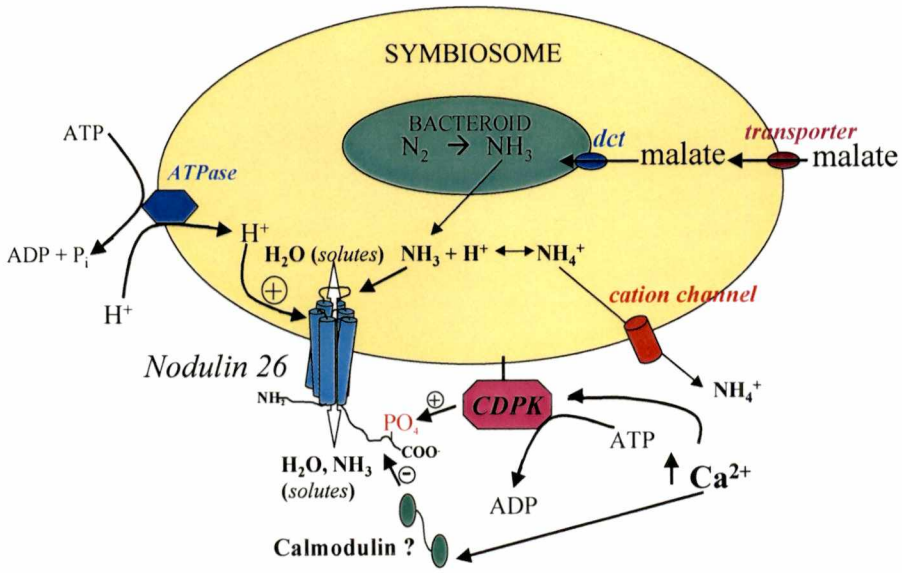


Figure 24. Model for the regulation of nodulin 26. Illustrated are the metabolic pathways and other activities associated with the symbiosome membrane. Fixed nitrogen is effluxed in two potential forms, NH_4^+ which is transported to the plant cell cytosol through cation channels, NH_3 which is potentially fluxed through the nodulin 26 channel. Malate is transported into the symbiosome space by a specific SM transporter and is taken up through the dct protein and metabolized by the bacteroid. The SM-ATPas acidifies the symbiosome space by pumping H^+ into the symbiosome. The proposed pathway for the regulation of nodulin 26 by pH, calcium, and CDPK phosphorylation is also shown. The acidity of the symbiosome space may regulate the transport of H_2O and solutes through the nodulin 26 channel. A rise in the calcium level in the plant cell cytosol may also regulate the transport property of nodulin 26 through the activation of CDPK (calmodulin-like domain protein kinase) and also by inhibition, possibly by calmodulin.

expected to trigger the rapid phosphorylation of nodulin 26 by CDPK because of the proximity effect (both proteins colocalized to the symbiosome membrane). A sustained calcium flux would lead to the activation of calmodulin, its diffusion to the symbiosome and potential interaction with nodulin 26. To test this model, we need to: 1) determine whether calmodulin is the calcium sensor that leads to calcium-dependent inhibition, 2) determine the site of interaction of calmodulin binding. This latter point is particularly important since there is evidence that the site of potential interaction may overlap the phosphorylation site at the carboxyl terminal end, raising the possibility that these two factors may compete for their effects on nodulin 26. In other words, the phosphorylation of nodulin 26 by CDPK may reduce the affinity of calmodulin for the carboxyl terminus of nodulin 26, and conversely, the binding of calmodulin to nodulin 26 may prevent the phosphorylation by CDPK. In addition, long term effects of phosphorylation of nodulin 26 on its stability or targeting need to be addressed in the nodule.

The regulation of nodulin 26 by pH and the ATPase also needs to be addressed. As shown in **figure 24**, activation of the ATPase would result in the acidification of the symbiosome space. Presently, factors that control the activity of the ATPase and acidity of the symbiosome space remain to be established. However, as pointed out for AQP0, pH regulation may be triggered by the metabolic state of the cell, and the need for a higher water permeability and osmoregulation. One last point, the effects of pH and calcium are exerted on the opposite sides of the membrane (pH from the symbiosome space and calcium from the cytosolic space) The possibility has recently been proposed that water and solute flux through nodulin 26 might be rectified (Niemietz & Tyerman,

unpublished observations) Since the sites of interactions of these factors are asymmetric, these factors may not affect the flux of water and solute in both directions equally. This may contribute to uneven flow of water and solutes, and rectification.

The regulation of nodulin 26 by pH, calcium, and phosphorylation may contribute to the functioning of nodulin 26 in the SM. The regulation of nodulin 26 by these factors would permit the protein to shift between an activated, high permeability state to a less active, low permeability state to allow osmoregulation and possibly adaptation to environmental factors. In other plant cells, the vacuole, which occupies most of the cell interior, has been implicated in osmoregulation (Maurel et al., 1995; reviewed in Maurel, 1997). In a similar manner, an abundance of symbiosomes occupies most of the intracellular space of infected cells (Roth et al., 1988; reviewed in Udvardi & Day, 1997), and it seems likely that they might also function in osmoregulation. Reversible water flux through nodulin 26 may function to maintain osmotic equilibrium and turgor as the osmotic gradient changes due to the movement of ions and/or metabolites across the SM. In addition, nodulin 26 may also function in adapting to osmotic stresses. Several other plant aquaporins have been implicated in osmotic stresses such as drought and salinity (Weig et al., 1997; Maurel et al., 1995; reviewed in Maurel, 1997) where they may be involved in the ability of the plant to change the membrane permeability to adapt to changes in water potential. In support of this, nodules were observed to be extremely sensitive and responsive to osmotic stresses (Hunt & Layzell, 1993)

As a final note, considering the evidence that the solute pore is different than the water pore in aquaglyceroporins, the investigation of pH, phosphoryalation, and calcium on solute flux needs to be addressed

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